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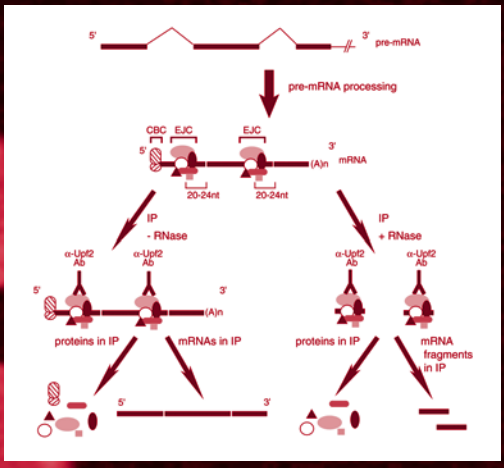

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Using Chromatin Immunoprecipitation to Map Cotranscriptional mRNA Processing in *Saccharomyces cerevisiae*

Michael-Christopher Keogh and Stephen Buratowski

Summary

The chromatin immunoprecipitation (ChIP) technique has been used to determine where and under what conditions DNA binding proteins associate with specific DNA sequences. Proteins are crosslinked in vivo with formaldehyde, and chromatin is then isolated and sheared. The protein of interest is then immunoprecipitated and the associated DNA sequences identified via PCR. Although this technique was originally designed to assay DNA binding proteins, it can also be used to monitor mRNA processing factors associated with transcription complexes.

Key Words

Chromatin immunoprecipitation; epitope tagging; polymerase chain reaction; tandem affinity purification (TAP) tag.

1. Introduction

Synthesis of mRNA by RNA polymerase II (RNApII) is a complex process involving the transient association of large protein complexes with DNA ***(1,2)***. Much work in the field has concentrated on in vitro reconstitution, examining the role of individual proteins or complexes at different steps of the transcription cycle. However, study of this process in its natural chromosomal environment is required for more complete understanding.

This chapter describes chromatin immunoprecipitation (ChIP), a method used to determine where and when a particular protein is located near specific DNA sequences ***(3–6)***. Chromatin immunoprecipitation has been used extensively in the budding yeast *Saccharomyces cerevisiae*, but the technique has

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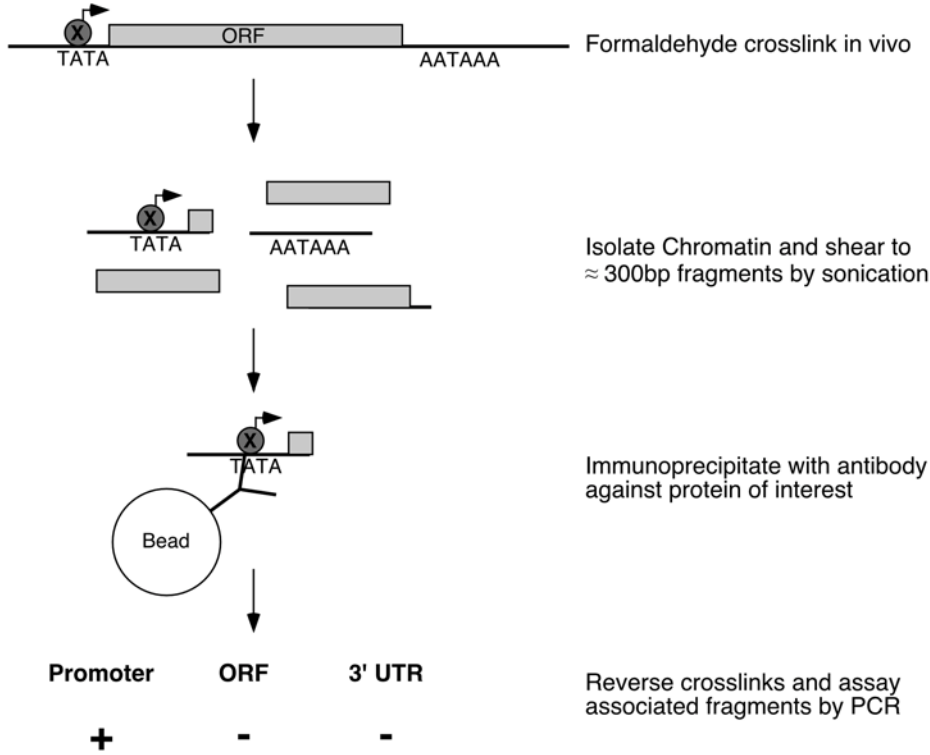

Fig. 1. Chromatin immunoprecipitation schematic. Protein X is localized in the region of the promoter (TATA) during transcription, but not throughout the open reading frame (ORF) or at the 3' UTR (AATAAA). Following formaldehyde crosslinking, the cells are lysed and the chromatin isolated and sheared to smaller fragments by sonication. Protein X remains crosslinked and associated with the promoter-region chromatin throughout these manipulations. Protein X is further purified by immunoprecipitation, the crosslinks reversed, and the associated chromatin isolated. The specific DNA sequences bound to protein X can be assayed by PCR with specific primers.

been adapted successfully to many species ***(3,4,7–10)***. Simply put, the protein of interest is crosslinked in vivo to chromatin, which is then isolated and sheared to the desired size. The protein is then immunoprecipitated, along with any associated DNA. The chromatin is decrosslinked and specific DNA sequences are assayed using the polymerase chain reaction (PCR) (**Fig. 1**). The exquisite sensitivity of PCR and the availability of complete genomic sequences have made this technique very powerful.

Formaldehyde is the crosslinking agent of choice for these experiments (**Subheading 3.2.**). It is easy to handle, water-soluble, and active over a wide

range of concentrations. Most importantly, it readily traverses biological membranes, allowing crosslinking to be performed on intact cells ***(3,11)***. Formaldehyde crosslinks primary amino groups such as those on lysines and the bases adenine, guanine and cytosine. Protein–protein and protein–DNA crosslinks are formed between groups within distances of approx 2 Å. These modifications are reversible: extended incubation at 65°C breaks the protein-DNA bonds, while the protein–protein crosslinks can be reversed by boiling ***(3)***.

After crosslinking, the yeast cells are mechanically lysed (**Subheading 3.3.**). However, DNA fragments in these lysates are too long to determine the precise genomic location of chromatin-associated proteins. Sonication is a rapid and straightforward way to shear the chromatin fragments and generate the smaller-sized fragments desired. By controlling the sonication time and strength, it is possible to generate relatively uniformly sized populations and increase the resolution of the technique ***(12)***. In our experience, the maximum resolution achievable is approx 200 bp.

Once the technique is established, the main variable encountered is the immunoprecipitation step (**Subheading 3.4.**). Not all primary antibodies are amenable to the relatively stringent conditions employed. This variable can be avoided by epitope-tagging the protein of interest ***(13)***, although it must be shown that the tag does not interfere with the function of the protein. Epitope tagging of genomic loci in *S. cerevisiae* is a relatively straightforward process ***(14,15)***, which greatly increases the utility of the technique in this species.

In our experience, the HA (human influenza virus hemagglutinin) epitope and protein A tags work well in chromatin immunoprecipitation. The small HA-epitope (YPYDVPDYA) is recognized by the commercially available 12CA5 monoclonal antibody, which binds with equal efficiency to protein A or protein G Sepharose ***(16)***. The HA-epitope works well in most locations within the tagged protein. However, it is best to use three or more copies of the epitope for maximum efficiency. Although the protein A module is larger, it has some advantages. For immunoprecipitation, relatively inexpensive IgG agarose is used. Also, the popular tandem affinity purification (TAP) tag ***(17)*** contains one copy of the protein A module, and many TAP-tagged strains are already available. Although the TAP tag was originally designed for purification of tagged proteins, it also works well in chromatin immunoprecipitation.

After reversal of crosslinking, the PCR step (**Subheading 3.5.**) enables investigation of whether specific DNA sequences are bound to the protein under study. Each reaction contains two or more primer pairs. It is highly advisable to include a control primer pair that amplifies a nontranscribed region (i.e., no open reading frame, marked with an asterisk in **Figs. 2** and **3**). This serves as an internal negative control for background and PCR efficiency, and this signal can be used to normalize separate ChIP experiments. In addition, the reaction can contain

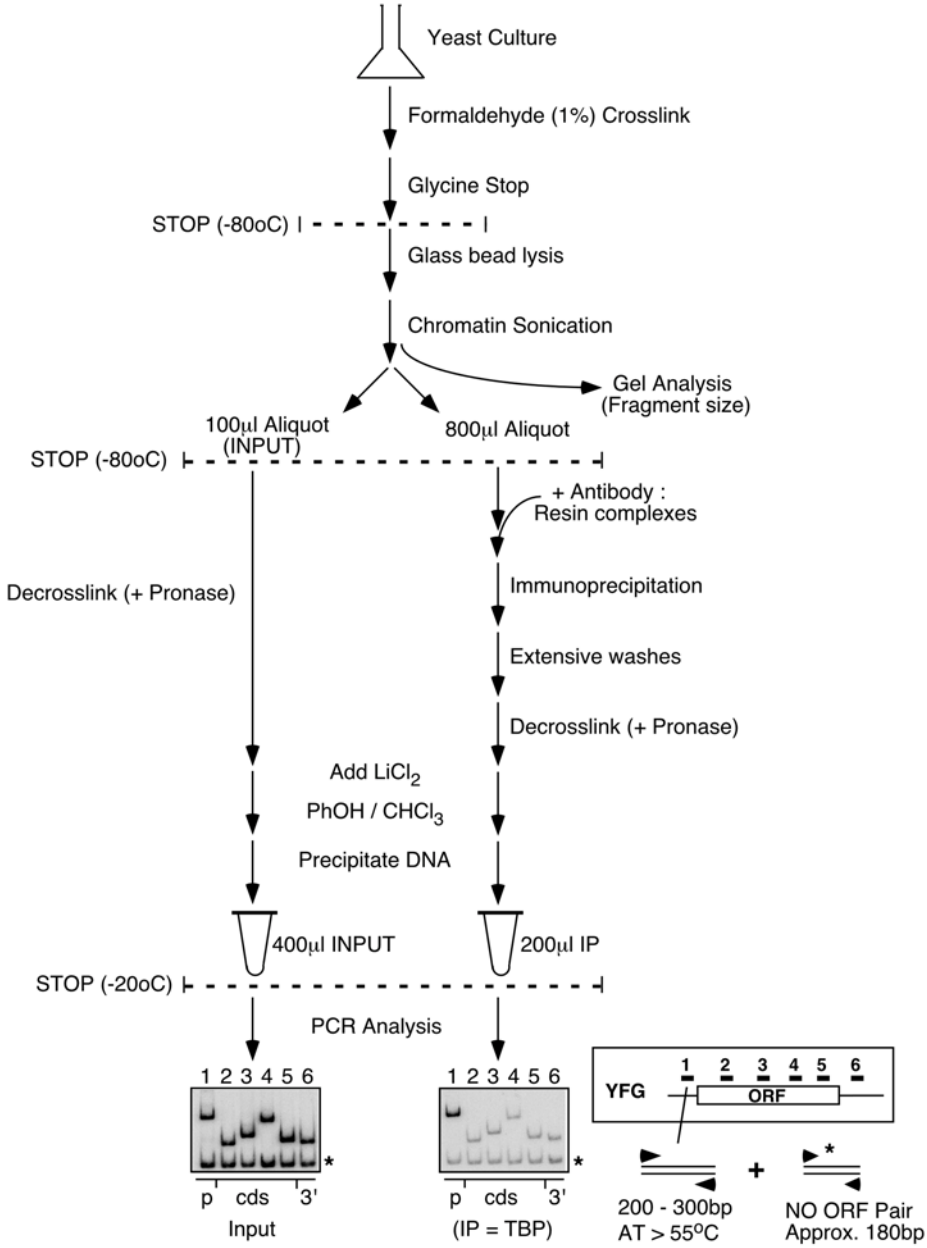

Fig. 2. Chromatin immunoprecipitation protocol. An overview of the steps in the technique is shown. The points at which the protocol can be safely interrupted and samples stored are indicated. The panels at bottom show a representative PCR analysis of an input sample and immunoprecipitation, which, in this case, were performed

one or more primer pairs that amplify a specific region of interest (**Figs. 2** and **3**). Primers are designed primarily on the basis of location, but are typically 24–30-mers with an annealing temperature of approx 55°C. A BLAST search of the primer sequences against the entire genome is recommended to assure that hybridization is specific to the desired region. It is also worthwhile to use one of the many available computer programs that tests primer sequences for internal hairpins, primer-dimers, and so on.

Polymerase chain reaction products are easily resolved on a nondenaturing polyacrylamide or agarose gel. Of course, if multiple primer pairs are used in the same reaction, the amplified products must be of different sizes. The inclusion of radiolabeled nucleotide in the reactions allows quantitation of two or more products (the negative control and specific sequences) in each tube. If a protein crosslinks to a specific DNA sequence, there should be an increase in the relative abundance of that PCR product compared to the control standard (**Fig. 3**). For accurate quantitation, the PCR reactions must be assayed while still in the exponential phase.

A schematic of the protocol is shown in **Fig. 2**, which also indicates the points at which the procedure can be safely interrupted. A typical ChIP experiment (assuming the current availability of all strains and materials) takes 4–5 d. Up to the point that PCR-ready samples are prepared, we generally deal with no more than 12 crosslinked samples at once, a bottleneck imposed in our case by the ultra-centrifugation steps on day two (*see* **Subheadings 3.3.3.** and **3.3.5.**). The PCR throughput is determined by the capacity of the thermocycler(s).

Although the length of the protocol can be daunting, it is relatively simple to master if each step is well controlled. For the worker learning the technique, it is useful to initially perform the analysis with previously characterized factors. As a transcription lab, we generally use the crosslinking of TBP and Rpb3 as controls. The former should crosslink specifically to promoters, the latter at promoters and throughout coding regions ***(6,18–19)***. These positive controls can verify the quality of the chromatin and the proper execution of the protocol. These patterns serve as points of comparison for crosslinking of new fac-

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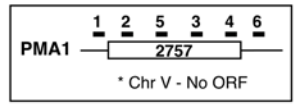
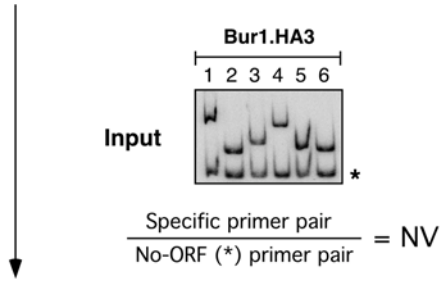
Fig. 2. *(continued)* with the promoter-localized TATA-binding protein (TBP). Six specific primer pairs throughout your favorite gene (YFG) are depicted (upper band in each case). Each tube also contains a second primer pair (*) specific to a smaller nontranscribed region of DNA, which acts as an internal standard and negative control. The increased intensity of the primer pair 1 band, corresponding to the promoter in the IP panel, indicates specific occupancy of TBP at this location.



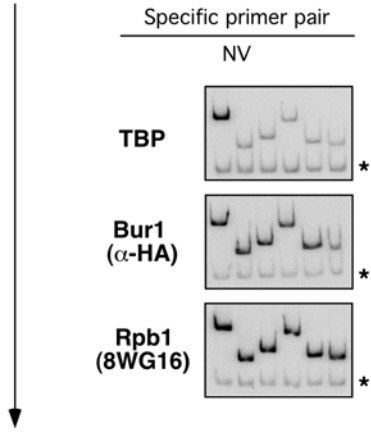
Step 1 - Use Input to derive Normalization Value (NV)



Specific NV

Primer	Bur1
1	0.84
2	1.03
3	0.99
4	1.26
5	0.71
6	0.84

Step 2 - Use each NV to derive the Corrected Value (CV)



Calculated OV for each IP

Primer	TBP	Bur1	Rpb1
1	4.0	6.5	6.9
2	1.0	5.7	6.0
3	1.0	4.5	5.0
4	1.0	5.0	5.9
5	1.0	4.7	5.5
6	1.0	1.9	5.0

Step 3 - Use CV to determine Occupancy Value (OV)

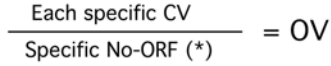


Fig. 3. Quantitating occupancy by ChIP. After PAGE, PCR products are quantitated by phosphoimager (we use a Fujix BAS 2040 PhosphoImager and the allied Fuji *ImageGauge* software). The experiment depicted utilizes six primer pairs that amplify different regions of the PMA1 gene. A graphical location of each primer is shown in the top panel and the specific sequence of each given in **Table 1**. The Input sample is used to calculate the normalization value (NV) between each specific primer pair (numbered 1–6) and the control "no-ORF" primer pair (*). This ratio compensates for any variation in PCR efficiency and label content by converting the signal from different

tors. It is important to analyze occupancy at multiple genes (*see* **Note 1**) before any specific observations can be generalized.

Chromatin immunoprecipitation has been used for mapping various factors involved in DNA-related processes, including replication, chromatin modifications, and transcription. However, other factors associated with transcription complexes but not directly associated with DNA, such as the mRNA capping enzyme and other mRNA processing factors, can also generate a signal in ChIP experiments. Such crosslinking is strongly indicative of cotranscriptional mRNA processing.

2. Materials

2.1. Growth of Yeast Cells

1. Appropriate growth media.
2. Incubator shaker.

2.2. Formaldehyde Crosslinking and Chromatin Preparation

2.2.1. Equipment

1. Preparative centrifuge (Sorvall RC5B+ or equivalent).
2. Ultracentrifuge (Beckman Coulter Optima LE-80K or equivalent).
3. Beckman Ti50 rotor.
4. Ultracentrifuge tubes (10.4 mL polycarbonate, Beckman, cat. no. 335603 or equivalent).
5. Microcentrifuge (Eppendorf 5415C or equivalent).
6. Centrifuge flasks/tubes (preparative).
7. 14-mL Round-bottom Falcon tube (Falcon, cat. no. 2059 or equivalent).
8. Acid-washed glass beads, 425–600 μ (Sigma, G-8772).
9. Glass Pasteur pipets (VWR 14672-380 or equivalent).
10. 2-mL Vials (Corning, cat. no. 430289 or equivalent).
11. Probe sonicator with microprobe tip (MSE 2/76 Mk2 or equivalent).

Fig. 3. (*continued*) primer pairs into normalized units of the control primer pair. This operation generates the corrected value (CV) for each specific primer pair in each immunoprecipitation as shown. Finally, each CV is divided by the no-ORF (*) signal from each immunoprecipitation to give the occupancy value (OV).

In the experiment shown, three different proteins are localized along the constitutively transcribed PMA1 gene. Immunoprecipitation of TBP and the large RNA polymerase II subunit Rpb1 demonstrates that TBP is localized at the promoter and Rpb1 throughout the gene, as expected. We can see that the factor Bur1 is recruited in the region of the promoter and present thoughout the coding sequence, but shows displacement in the region of the 3' UTR ***(19)***.

2.2.2. Reagents

1. 37% Formaldehyde (HCHO): molecular biology grade; VWR, cat no. EM-FX0415-5.
2. Glycine stop solution: 3 *M* glycine, 20 m*M* Tris base; do not adjust the pH.
3. Diluent, pH 7.5: 150 m*M* NaCl, 1.5 m*M* EDTA, 70 m*M* HEPES; adjust pH with KOH.
4. TBS: 20 m*M* Tris, pH 7.5, 150 m*M* NaCl.
5. 2X FA lysis buffer: 100 m*M* HEPES: KOH pH 7.5, 300 m*M* NaCl, 2 m*M* EDTA, 2% Triton X-100, 0.2% sodium deoxycholate.
6. 1X FA lysis buffer/0.1% SDS.
7. 1X FA lysis buffer/0.5% SDS.
8. 5 *M* NaCl.
9. Protein A Sepharose CL-4B (Amersham Pharmacia Biotech, cat. no. 17-0780-01).
10. Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, cat. no. 17-0618-01).
11. Rabbit IgG agarose (Sigma, cat. no. A-2709).

2.3. Immunoprecipitation and Decrosslinking

2.3.1. Reagents

1. TBS: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
2. 2X FA lysis buffer (*see* **Subheading 2.2.2.**).
3. 5 *M* NaCl.
4. Wash 1: 1X FA lysis buffer/0.1% SDS/275 m*M* NaCl.
5. Wash 2: 1X FA lysis buffer/0.1% SDS/500 m*M* NaCl.
6. Wash 3: 10 m*M* Tris, pH 8.0, 1 m*M* EDTA, 0.25 *M* LiCl, 0.5% NP40, 0.5% sodium deoxycholate.
7. Wash 4: TE, pH 8.0 (10 m*M* Tris, pH 8.0, 1 m*M* EDTA).
8. Elution buffer: 50 m*M* Tris, pH 7.5, 10 m*M* EDTA, 1% SDS.
9. 20 mg/mL Pronase (Roche, 165 921).
10. 4 *M* LiCl.
11. PCI: phenol/chloroform/isoamylalcohol, 25:24:1.
12. 10 mg/mL Glycogen (Roche, 901 393).
13. 100% EtOH.

2.4. PCR Analysis

2.4.1. Equipment

1. 0.5-mL Thin-walled PCR tubes.
2. PCR machine with heated lid (MJ Research PTC-100 or equivalent).
3. Vertical polyacrylamide electrophoresis system.
4. Detection and quantitation system, e.g., Phosphorimager plates and analysis system or autoradiography film (Kodak X-OMAT AR or equivalent) and developing system.

2.4.2. Reagents

1. dNTP mix: 2.5 m*M* dATP, dTTP, dCTP, dGTP.
2. Platinum *Taq* (Invitrogen, cat. no. 10966-034, 5 U/μL or equivalent, *see* **Note 2**).
3. 10X Platinum *Taq* reaction buffer.
4. 50 m*M* $MgCl_2$.
5. 10 μ*M* primer mixes.
6. α-[^{32}P]dATP (specific activity 3000 Ci/mmol, 10 mCi/mL).
7. 6X Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.

3. Methods

A schematic of the protocol is presented in **Fig. 2**. The protocol can be stopped for long term storage at the indicated points; if performed without stopping, it takes 4 d. The protocol as described supplies sufficient chromatin for an Input sample and 10 immunoprecipitations (IP). Each immunoprecipitation can be used for approx 50 PCR amplifications. If multiple analyses of a single preparation are not required, the protocol can be scaled back accordingly, although as a rule we keep all volumes as indicated until **Subheading 3.3.5.**

3.1. Growth of Yeast Cells

Grow a starter culture overnight to late log phase/saturation. At the beginning of Day 1, dilute the overnight culture to an $OD_{\lambda 600}$ of approx 0.15 and grow under the appropriate conditions to $OD_{\lambda 600}$ of approx 0.65–0.8 (*see* **Note 3**). For a wild-type strain at the optimal growth temperature (30°C) in yeast extract-peptone-dextrose (YPD) media with 2% glucose as the carbon source, the process will take approx 5 h. Cells are in the exponential growth phase during crosslinking.

3.2. Formaldehyde Crosslinking

1. To a 250-mL culture, add 25 mL 11% HCHO (freshly made from commercial 37% solution and diluent) such that the final formaldehyde concentration is 1%. Incubate 20 min at room temperature with gentle mixing.
2. Add 37.5 mL glycine stop solution and incubate for a further 5 min with gentle mixing. Although the stop solution can be made in advance, it often precipitates; redissolve crystals by heating to >50°C with stirring before use. Alternatively, the solution can be freshly made before each experiment.
3. Pellet cells by centrifugation at 1500*g* in a Sorvall SLA-3000 rotor or the equivalent. All steps from this point on are performed on ice with precooled solutions unless indicated otherwise. The cell pellet should be washed by resuspending and repelleting twice with 100 mL TBS and once with 10 mL FA lysis buffer/0.1% SDS. Transfer to a 14-mL round-bottom Falcon tube, pellet cells and aspirate buffer. Pellets can be stored at this point at –80°C.

3.3. Preparation of Chromatin

1. Resuspend the cell pellet in 1 mL FA lysis buffer/0.5% SDS and an equal volume of acid washed glass beads. Vortex vigorously in 10 cycles of 30 s mixing, 30 s on ice. A large amount of foaming occurs; therefore, it is important to ensure that the beads do not settle during vortexing.
2. Add 6.5 mL FA lysis buffer/0.1% SDS and puncture the tube bottom and cap with a 22G needle. Place the Falcon tube into a 30-mL Nalgene Oakridge tube and centrifuge in the SLA-3000 rotor at 170*g* for 4 min. Collect the lysate in the Oakridge tube and discard the Falcon tube containing the glass beads.
3. Resuspend the loose pellet and transfer the entire lysate to an ultracentrifuge tube. Centrifuge at 155,000*g* in a Ti50 rotor for 10 min at 4°C. Discard the supernatant and resuspend the pellet in 8 mL FA lysis buffer/0.1% SDS (*see* **Note 4**). Centrifuge the resulting suspension at 155,000*g*, 4°C, for 20 min. Discard the supernatant, resuspend the pellet in 1.5 mL FA lysis buffer/0.1% SDS, and transfer to a 2-mL microcentrifuge tube.
4. Sonicate the suspension to break the chromatin into fragments of approx 200–500 bp (*see* **Note 5**). For this step, the conditions used need to be determined empirically for each sonicator. For an MSE Sonifier (2/76 Mk2) fitted with a microprobe tip, five or six 20-s pulses of constant output (amplitude 12) are sufficient. Keep the sample tubes on ice throughout sonication and allow 20-s breaks between pulses to prevent excessive warming. Ensure that the sonicator tip is fully immersed in the solution to reduce foaming.
5. Transfer to an ultracentrifuge tube, add 6.5 mL FA lysis buffer/0.1% SDS, and centrifuge at 45,000 rpm, 4°C, for 20 min. Carefully remove the supernatant containing the sheared chromatin and freeze in 800-µL aliquots at –80°C. Also set aside one 100-µL aliquot to be used as an Input sample (*see* **Subheading 3.4.**, **step 6**).

3.4. Immunoprecipitation and Decrosslinking

1. Prepare the appropriate resin:antibody complexes (*see* **Note 6**). As an example, for each immunoprecipitation of an HA-epitope tagged protein, prebind 10 µL of pre-equilibrated protein A-Sepharose beads (TE, pH 8.0) and 5 µL 12CA5 ascites in 100 µL TE pH 8.0 for 30 min at 25°C with gentle mixing. Collect the complexes by centrifugation at low speed in a microcentrifuge, discard the supernatant, and wash the resin with 100 µL TE pH 8.0. Resuspend the antibody-bound resin in 20 µL TE pH 8.0. This prebinding step can be scaled up for the desired number of immunoprecipitation reactions.
2. Thaw chromatin aliquots for immunoprecipitation on ice. Add 5 *M* NaCl to a final concentration of 275 m*M* (20 µL for 800-µL aliquot from **Subheading 3.3.**, **step 5**) (*see* **Note 7**). Add the resin:antibody complex from above and bind overnight with gentle rolling at 4°C.
3. Collect the beads by centrifugation at low speed in a microcentrifuge and discard the supernatant. Add 1.4 mL of wash buffer 1 and place on a rotator 4 min at 25°C. Collect the beads by centrifugation, discard the supernatant and wash

sequentially with wash buffers 2, 3, and 4. Discard the supernatant following the last wash.

4. Add 250 µL elution buffer to the washed beads and elute at 65°C for 10 min. Collect the beads by centrifugation and transfer the supernatant to a new microcentrifuge tube. Wash the beads with 250 µL TE, pH 8.0, collect the beads by centrifugation, and combine this wash with the previous eluate. This sample is the IP (**Fig. 2**).
5. To reverse the crosslinks, add 20 µL of 20 mg/mL Pronase and incubate for 1 h at 42°C, then 4 h at 65°C.
6. To prepare the Input sample, add 400 µL TE, pH 8.0, to 100 µL of chromatin from **Subheading 3.3.**, **step 5**. Add 20 µL of 20 mg/mL Pronase and incubate for 1 h at 42°C and 4 h at 65°C. This sample is then processed through all the following steps in parallel with the IP sample.
7. Add 50 µL 4 *M* LiCl per tube and vortex. Sequentially extract with 400 µL PCI and 300 µL chloroform. At each step, mix by vortexing, separate the phases by centrifugation, and collect the upper aqueous layer. To precipitate DNA, add 1 µL of 10 mg/mL glycogen and 2.5 vol 100% EtOH per tube. Incubate for 1 h to overnight at –80°C. Centrifuge at 4°C to collect the DNA and wash precipitate with 1 mL 100% EtOH. Remove the liquid, and dry the pellet at 25°C for 10 min. Resuspend the IP in 200 µL TE, pH 8.0, and the Input in 400 µL TE, pH 8.0. Store at –20°C. The precipitated DNA samples are stable for at least 6 mo.

3.5. PCR Analysis

1 Each primer pair is generally selected to be 250–300 bp apart and designed to have similar melting temperatures (~55°C) (*see* **Note 1**). As an internal control, each reaction also contains another primer pair (the intergenic primer pair in **Fig. 1**) that amplifies an approx 180 bp product from a nontranscribed region.
2. Thaw reaction components on ice and vortex well before use. Each 10-µL PCR reaction contains 4 µL DNA (IP or Input) from **Subheading 3.4.**, **step 7** and 6 µL PCR mix. With these small sample volumes, it is preferable to use a heated lid PCR machine rather than a mineral oil overlay.
3. Prepare a PCR master mix on ice: 5.75 µL per reaction, consisting of 0.25 µL 10 µ*M* intergenic primer mix (internal standard), 0.4 µL 2.5 m*M* dNTP mix, 1 µL 10X platinum *Taq* buffer, 0.3 µL 50 m*M* $MgCl_2$, 0.1 µL Platinum *Taq* (5 U/µL) (*see* **Note 2**), 0.03 µL α-[^{32}P]dATP, 3.67 µL H_2O. To each individual reaction, add 0.25 µL of the specific primer pair (10 µ*M*) and 4 µL of the chromatin sample to be tested. Centrifuge briefly in a microcentrifuge to collect the mix at the bottom of the tube.
4. Each sample is PCR-amplified by the following protocol: one cycle at 94°C for 90 s, followed by 26 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Reactions are completed by an additional incubation at 72°C for 10 min (*see* **Note 8**).
5. When PCR is complete, add 2 µL 6X gel loading buffer to each tube and briefly centrifuge to mix. The PCR products are separated on an 8% vertical polyacryla-

mide gel with 1X TBE buffer at 180 V for 3 h. The authors generally use 0.8- or 1.5-mm thickness gels, although thicker gels are easier to handle. Polymerase chain reaction products of 180–300 bp migrate near the xylene cyanol FF dye. The unincorporated NTPs (including the radioactive dATP) will run near the bottom of the gel; this strip can be cut off to reduce background and contamination. The gel is dried onto Whatman 3MM paper and exposed to phosphorimager plates or autoradiography film.

6. PCR products can be quantitated using a phosphorimager. To determine whether a specific protein is associated with a particular sequence, the relative abundance of the PCR product from the region in question is compared to a reference (**Fig. 3**). The efficiency of amplification and labeling for each primer pair relative to the internal negative control pair (no-ORF control in **Fig. 3**) is calculated from their ratio in the Input sample. This ratio is termed the normalization value (NV). The intensity of each specific primer pair band from each IP is then divided by the relevant NV to give the corrected value (CV). The CV is then divided by the no-ORF signal from each IP to give the occupancy value (OV). An OV greater than 1 theoretically indicates the presence of the immunoprecipitated protein at the relevant region or DNA. However, the authors generally feel most confident when the OV is more than 2. For a strongly expressed gene, the typical OV seen for TBP is between 4 and 6. Some variability is seen between experiments.

4. Notes

1. The authors have found that the best signals come from genes transcribed at higher levels (>30 mRNA copies/h). If the researcher is interested in determining occupancy at a specific yeast locus, the Young laboratory of the Whitehead institute provides a useful resource (http://web.wi.mit.edu/young/expression). This database indicates the transcription frequency of most yeast genes. Some primers used to determine occupancy at a number of strongly expressed genes (including the PMA1 gene shown in **Figs. 2** and **3**) are listed in **Table 1**.
2. The authors have found that not all thermostable polymerases work well for ChIPs. Platinum *Taq* (Invitrogen) is used in the authors' laboratory, although other labs have successfully used other "hot-start" formulations.
3. The appropriate growth conditions will depend on the experiment. Published conditions exist for the analysis of samples following temperature shift to induce heat shock genes, inactivate temperature sensitive alleles, induce genes on alternative carbon sources, and other desired results. For rapid temperature shift, grow 250 mL of cells in a 1-L flask to an OD of approx 0.5, then add an appropriate volume of prewarmed medium to immediately bring the culture to the desired temperature. Flasks are then incubated at this temperature for the duration of the experiment. For the induction of GAL genes, grow cells in medium containing 2% raffinose/0.5% glucose to $OD_{\lambda 600}$ approx 0.3. Add galactose to 2% to induce and incubate for a further 2–4 h prior to crosslinking and analysis.
4. The chromatin/debris pellet is resuspended with a Pasteur pipet that has been flame-sealed and bent at the end. It is not possible to completely resuspend this material, so the pellet should be disrupted as much as possible.

Table 1
Selection of Primers Used for ChIP on the Constitutively Expressed PMA1 and ADH1 and the Galactose-Inducible GAL1 Gene

Name/location*	Oligo sequence
ADH1 p, –235	TTCCTTCCTTCATTCACGCACACT
ADH1 p, –18	GTTGATTGTATGCTTGGTATAGCTTG
ADH1 cds1, +146	ACGCTTGGCACGGTGACTG
ADH1 cds1, +372	ACCGTCGTGGGTGTAACCAGA
ADH1 cds2, +844	TTCAACCAAGTCGTCAAGTCCATCTCTA
ADH1 cds2, +1018	ATTTGACCCTTTTCCATCTTTTCGTAA
ADH1 3'UTR top	ACCGGCATGCCGAGCAAATGCCTG
ADH1 3'UTR bottom	CCCAACTGAAGGCTAGGCTGTGG
PMA1 p, –370	GGTACCGCTTATGCTCCCCTCCAT
PMA1 p, –70	ATTTTTTTTCTTTCTTTTGAATGTGTG
PMA1 cds1, +168	CGACGACGAAGACAGTGATAACG
PMA1 cds1, +376	ATTGAATTGGACCGACGAAAAACATAAC
PMA1 cds2, +1010	GTTTGCCAGCTGTCGTTACCACCAC
PMA1 cds2, +1235	GCAGCCAAACAAGCAGTCAACATCAAG
PMA1 cds3, +2018	CTATTATTGATGCTTTGAAGACCTCCAG
PMA1 cds3, +2290	TGCCCAAAATAATAGACATACCCCATAA
PMA1 cds4, +584	AAGTCGTCCCAGGTGATATTTTGCA
PMA1 cds4, +807	AACGAAAGTGTTGTCACCGGTAGC
PMA1 3'UTR top	GAAAATATTTGGTATCTTTGCAAGATG
PMA1 3'UTR bottom	GTAAATTTGTATACGTTCATGTAAGTG
GAL1 p, –190	GGTAATTAATCAGCGAAGCGATG
GAL1 p, +54	TGCGCTAGAATTGAACTCAGGTAC
GAL1 cds1 +427	CCGGAAAGGTTTGCCAGTGCTC
GAL1 cds1 +726	CGGAGTAGCCTTCAACTGCGGTTTG
GAL1 cds2 +1039	GAAGAGTCTCTCGCCAATAAGAAACAGG
GAL1 cds2 +1331	GAACATTCGTAAAGTTTATCGCAAG
GAL1 3'UTR +1764	CCACAAACTTTAAAACACAGGGAC
GAL1 3'UTR +2079	CCTCCTCGCGCTTGTCTACTAAAATC
Chr V no-ORF up	GGCTGTCAGAATATGGGGCCGTAGTA
Chr V no-ORF down	CACCCCGAAGCTGCTTTCACAATAC

*Primers are used in pairs. Numbers are relative to the first nucleotide of the open reading frame initiation codon. P, promoter; CDS, coding sequence; UTR, untranslated region; ORF, open reading frame.

5. The efficiency of sonication determines the resolution of the technique, so efficiency at this point in the experiment is extremely important. The sonication conditions must be determined empirically for each sonicator. Gel electrophoresis can be used to determine the average fragment size after sonication. Samples should be heat-treated to reverse crosslinks and extracted with phenol/$CHCl_3$ to remove protein prior to gel analysis. To demonstrate efficient chromatin sonication, it is useful to examine the chromatin on an agarose gel after reversal of crosslinking. Another useful control is immunoprecipitation with a well-characterized antibody specific for a given region. As an example, the authors use anti-TBP (TATA binding protein), because TBP is recruited specifically to the promoter. We have designed primers to overlapping regions near the promoter of ADH1 (**Table 1**), moving sequentially 3' into the coding sequence. If anti-TBP appears to crosslink to coding sequence products removed from the TATA box by more than 300 bp, it is an indication that sonication conditions are less than optimal.
6. The resin:antibody complexes used for each immunoprecipitation are obviously dependent on the protein under study. We routinely use protein A or protein G Sepharose, depending on the antibody isotype. IgG-Agarose resin works well for protein A (TAP)-tagged proteins. The amount of antibody in each experiment is determined empirically. For HA-tagged proteins, 5 μL 12CA5 ascites fluid is used for each immunoprecipitation.
7. The immunoprecipitation and wash conditions depend on the antibody used. The conditions described are standard, although the authors have found that lower-affinity interactions, such as those with IgM antibodies, are not always stable under these stringent treatments. In these cases, less stringent conditions can be used. The NaCl (**Subheading 3.4., step 2**) can be omitted and wash buffers 1–3 can be replaced with 1X FA lysis buffer/0.1% SDS.
8. For accurate quantitation, it is essential that PCR products be analyzed when the amplification is still in the exponential phase. The conditions described here are used for single-copy genes. For the study of multiple-copy loci (such as ribosomal genes), the number of PCR cycles can be reduced accordingly. It also recommended that the PCR signals be shown to increase linearly with the amount of input by assaying several dilutions of the Input sample before a set of experiments.

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2

Identifying PhosphoCTD-Associating Proteins

Hemali P. Phatnani and Arno L. Greenleaf

Summary

The C-terminal repeat domain (CTD) of the largest subunit of RNA polymerase II is hyperphosphorylated during transcription elongation. The phosphoCTD is known to bind to a subset of RNA processing factors and to several other nuclear proteins, thereby positioning them to efficiently carry out their elongation-linked functions. The authors propose that additional phosphoCTD-associating proteins (PCAPs) exist and describe a systematic biochemical approach for identifying such proteins. A binding probe is generated by using yeast CTD kinase I to exhaustively phosphorylate a CTD fusion protein. This phosphoCTD is used to probe fractionated yeast or mammalian extracts in a Far Western protein interaction assay. Putative PCAPs are further purified and identified by mass spectrometry.

Key Words

RNA polymerase II; CTD; CTD kinase I; PCAPs; phosphoCTD; transcription elongation; RNA processing; nuclear organization; hyperphosphorylation; Far Western; protein interaction blot; protein–protein interaction.

1. Introduction

The C-terminal repeat domain (CTD) of elongating RNA polymerase II (RNAP II) is highly phosphorylated, principally on Ser2 and Ser5 of the consensus repeats YSPTSPS. It is thought that the kinase activity of TFIIH adds phosphates onto Ser5 positions in conjunction with the initiation process and that subsequently an "elongation" CTD kinase (yeast CTDK-I or mammalian P-TEFb) subsequently adds multiple phosphates onto Ser2 positions in association with the commitment to effective elongation ***(1)***. It has been found that phosphorylation of the CTD leads to the binding of factors involved in RNA processing and in other nuclear events ***(2–4)***. These findings suggest that one significant role of the phosphoCTD is to spatially and functionally organize nuclear components associated with transcription. The phosphoCTD is well

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suited to this role, as it probably exists in a largely extended state; also in yeast and mammals it potentially extends from the polymerase more than 600 Å and 1200 Å, respectively ***(4–6)***.

The first few phosphoCTD-associating proteins (PCAPs) identified were uncovered using a yeast two-hybrid approach ***(7)***. After the CTD was shown to be required for pre-mRNA processing, the next several PCAPs were discovered among known pre-mRNA processing factors ***(8–11)***. After these discoveries, the authors felt that only a fraction of the PCAPs actually present in nuclei had been discovered and that the surface of the PCAP-ome had barely been scratched. Proposing that many more PCAPs remained to be found, the authors developed a biochemical approach to identify proteins likely to be associated with elongating RNAP II. The authors used CTDK-I ***(12–14)***, the yeast CTD kinase known to be involved in phosphorylating elongating polymerase ***(15)***, to generate a fully phosphorylated recombinant CTD fusion protein that was then employed as a binding probe to identify PCAPs. Using this approach, the authors have identified a number of new PCAPs in both yeast and mammalian cells, most of which appear to be involved with elongation-coupled events ***(4,6,16–18)***. However, the authors predict that still more elongation-related PCAPs remain to be found and propose that finding and characterizing them will lead to new insights into nuclear functional organization. It is also of note that there are three other known CTD kinases in yeast, with functions and specificities different from those of CTDK-I ***(1)***. It is expected that use of the other kinases to prepare a phosphoCTD probe will lead to the discovery of distinct sets of PCAPs.

Here, the authors' approach for identifying phosphoCTD-associating proteins is presented.

2. Materials

2.1. Preparation of Mammalian Extracts

1. Wash buffer: 20 m*M* HEPES-KOH, pH 7.5, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 0.2 *M* sucrose. The following are added just before use: 0.5 m*M* DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
2. Hypotonic buffer: Follow instructions for wash buffer, but omit sucrose.
3. Extraction buffer: 50 m*M* HEPES-KOH, pH 7.5, 10% sucrose, plus the following added just before use: 0.5 m*M* DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
4. HGE(0.5): 25 m*M* HEPES, pH 7.6, 15% glycerol, 0.1 m*M* EDTA, 0.5 *M* NaCl. The following are added just before use: 0.5 m*M* DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.

2.2. Preparation of Yeast Extracts

1. Commercial yeast blocks: Eagle brand, moist cakes, used for baking.
2. Extract buffer: 25 m*M* Tris-HCl, pH 7.6, 25 m*M* KCl, 1 m*M* EDTA. The following are added just before use: 1 m*M* DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
3. Polyethylenimine (Polymin P): 10% (v/v) solution adjusted to pH 7.9.
4. HGE: 25 m*M* HEPES, pH 7.6, 15% glycerol, 0.1 m*M* EDTA. The following are added just before use: 1 m*M* DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.

2.3. Detecting PCAPs by Far Western Blotting

1. Electroblotting buffer: Tris/Glycine, 20% methanol ***(19)***.
2. BRB, blocking/renaturation buffer: PBS containing 3% nonfat dry milk, 0.2% Tween 20, 0.1% PMSF, 5 m*M* NaF.
3. PBS-Tw: PBS (10 m*M* Na-PO_4, pH 7.2, 150 m*M* NaCl) containing 0.2% Tween 20.
4. HEPES dialysis buffer: 10 m*M* HEPES-KOH, pH 7.6, 50 m*M* NaCl, 0.1 m*M* PMSF.

2.4. Preparation of Hyperphosphorylated CTD

1. 10X Reaction buffer: 250 m*M* HEPES-KOH, pH 7.6, 100 m*M* $MgCl_2$, 50 m*M* NaF.
2. 2% (v/v) Tween 20: store at 4°C, but check carefully for contamination before use.
3. γ-[^{32}P]ATP, 10 mCi/mL, specific activity 4500 Ci/mmol.
4. 3 m*M* ATP.
5. Quick Spin Protein Column (Roche Molecular Biochemicals).

2.5. Purifying CTDK-I

1. HGE(X): HGE containing X *M* KCl.
2. P11 phosphocellulose.
3. DE52 DEAE-cellulose.
4. MonoS column (FPLC, Amersham, Piscataway, NJ).

3. Methods

The ideal starting material contains a high percentage of the PCAPs present in the cells being studied. Thus, one goal of initial extract preparation is to retain PCAPs while discarding other constituents. The method described here for preparing mammalian extracts appears to meet this goal well; the initial steps enrich for elongationally engaged RNAP II0 under conditions that should not disrupt most PCAP-phosphoCTD associations. In contrast, the yeast extract used to date in the authors' laboratory was actually prepared for the purpose of purifying CTDK-I, and the early steps used in its preparation may have resulted in loss of some PCAPs (especially polymin-P precipitation). The authors are in the process of developing an alternative method for preparation of yeast extracts.

3.1. Preparation of Mammalian Cell Extracts

1. Grow cells by standard techniques appropriate for the cell line to the desired density (for HeLa, 4–7 × 10^5/mL), then harvest by centrifugation (8 min at 3000*g* for HeLa cells).
2. Resuspend cells in wash buffer (20 mL/L medium), distribute into centrifuge bottles, and pellet at 3300*g* for 5 min.
3. Resuspend the cells and swell in hypotonic buffer on ice for 10 min (3 mL per gram of cell pellet weight ***[20]***).
4. Homogenize on ice using a Dounce homogenizer with a B pestle to 80–100% lysis and pellet nuclei at 4°C (2000*g*) for 5 min.
5. Decant the supernatant and resuspend the nuclear pellet in cold extraction buffer (1.5 mL per gram of cell pellet weight). Measure the volume of the resuspension and add 0.031 vol 5 *M* NaCl stock solution (final NaCl concentration approx 150 m*M*).
6. Rock or rotate the mixture at 4° for 1 h, then centrifuge at 4°C (14,500*g*) for 20 min.
7. Decant the supernatant (low-salt nuclear extract) and save if desired. Estimate the volume of the pellet (low-salt extracted nuclei) and add 8 vol of buffer HGE(0.5). Gently but thoroughly homogenize the pellet on ice (Dounce), then centrifuge the homogenate at 150,000*g* for 60 min at 4°C.
8. Decant and save the supernatant (0.5 *M* NaCl extract); it serves as the source of PCAPs. Residual pellet can be extracted with higher salt if desired (*see* **Fig. 1** in ***[4]***).

3.2. Preparation of Yeast Cell Extracts

Commercial baker's yeast is a convenient and inexpensive starting material if a specific genetically defined strain is not initially required. Baker's yeast moist cakes are crumbled into liquid nitrogen and stored in convenient aliquots at –80°C. Similarly, frozen batches of defined strains can also be used. The following methods are described in refs. ***12*** and ***14***. All steps are carried out at 4°C.

1. Place approx 300 g of crumbled, frozen yeast in a stainless steel Waring blender (approx 3.8-L capacity) and add liquid nitrogen to cover yeast. Pulse on medium power in 30-s bursts. Monitor breakage by measuring soluble protein (Bradford assay, e.g., Bio-Rad), and continue pulverizing until soluble protein begins to plateau (usually 4–5 min).
2. After liquid nitrogen evaporates, scrape the yeast powder into a beaker and suspend it in 1 L extract buffer.
3. Centrifuge at 15,000*g* for 30 min and retain the supernatant.
4. With stirring, add a solution of 10% polyethylenimine, pH 7.9 to a final concentration of 0.3% to the supernatant, stir for an additional 30 min.
5. Centrifuge at 10,000*g* for 15 min and retain the pellet.
6. Resuspend the pellet in 200 mL extract buffer using Dounce homogenization and centrifuge again. Retain the pellet.

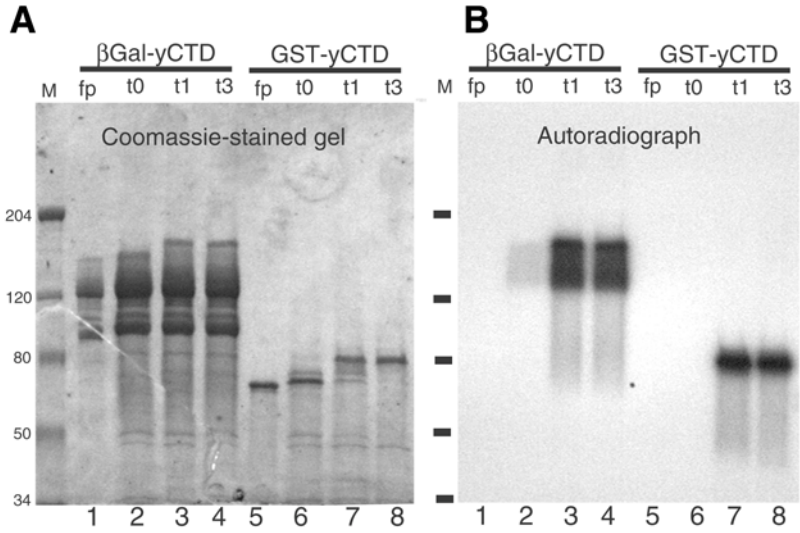

Fig. 1. Phosphorylation of yeast CTD fusion proteins by yeast CTDK-I. CTD fusion proteins (GST-yCTD and, separately, βGal-yCTD) were incubated with CTDK-I as described in the text. Time points were taken at 0, 1, and 3 h (aliquots representing about 1/15 of the reaction = t0, t1, t3), heated in SDS sample buffer, and subjected to SDS-PAGE. (**A**) Coomassie blue-stained gel with marker proteins in lane *M* (kDa on left). Lanes 1 and 5 contain untreated fusion proteins. Note that intact βGal-yCTD fusion protein is the top band in lanes 1–4, whereas faster-migrating bands between the intact form and approx 120 kDa are proteolyzed forms. (**B**) Autoradiograph of gel in (**A**). The t0 time points were taken a few seconds after adding unlabeled ATP to start the reaction. For the βGal-yCTD reaction, radiolabeled ATP was added just before unlabeled ATP, whereas for the GST-yCTD reaction, unlabeled ATP was added first.

7. Extract kinase activity by homogenizing the pellet in 200 mL 0.4 *M* KCl in extract buffer using a Dounce homogenizer. Centrifuge homogenate at 10,000*g* for 15 min and retain the supernatant.
8. Stirring slowly, add solid ammonium sulfate to the supernatant to 45% saturation, stir 30 min, and centrifuge at 15,000*g* for 30 min. Resuspend the pellet in HE; dialyze against HGE for the minimum amount of time needed for the conductivity to reach that of HGE containing 0.15 *M* KCl. Centrifuge the dialysate at 15,000*g* for 10 min and retain the supernatant.
9. Divide the extract into aliquots and store at –80°C.

The authors are in the process of developing alternative methods for preparation of yeast extracts. One approach that has succeeded demonstrably in identifying proteins associated with elongating RNAP II ***(21–23)*** is that of Woontner and colleagues ***(24)***. This type of extract should also serve as a starting material for CTDK-I purification.

3.3. Detecting PCAPs by Far Western Blotting

1. Heat aliquots of the protein mixtures to be probed (e.g., 0.5 *M* NaCl extract or a column fraction) in SDS sample buffer according to standard procedures (e.g., 90°C for 5 min).
2. Subject the protein samples to SDS-PAGE (e.g., 4–15% gradient gels, Bio-Rad) along with prestained marker proteins (Bio-Rad).
3. Electroblot the proteins from the gel to supported nitrocellulose (Hybond-C Extra, Amersham Biosciences). Typically, the authors transfer for 2 h at 0.75 amp at 4°C using a Hoeffer TE50X apparatus.
4. Incubate the nitrocellulose overnight in BRB (above) plus 2 m*M* dithiothreitol (this and subsequent steps at 4°C).
5. Probe nitrocellulose with hyperphosphorylated CTD.
   a. For [^{32}P]-labeled phosphoCTD, use at least 2.5 μg GST-phosphoCTD fusion protein (≥300,000 cpm; *see* **Note 4**) in 5 mL fresh BRB. Rock for 1–4 h at 4°C. Rinse the nitrocellulose membrane in PBS-Tw, 4 × 8 min, ≥50 mL per rinse, and monitor the washes until no radioactivity is detected. Blot dry by placing nitrocellulose on thick filter paper for a few seconds, protein side up. Cover with plastic wrap and expose to film or to a storage phosphor screen.
   b. For nonradioactive phosphoCTD, use at least 2.5 μg probe (GST-phosphoCTD) in 5 mL fresh BRB. Rock and rinse as **step 5a**, above. Incubate with the primary antibody (e.g., rabbit anti-GST IgG) in fresh BRB for 30 min. Wash 3 × 10 min in PBS-Tw and incubate with the secondary antibody (e.g., peroxidase-coupled donkey anti-rabbit IgG) in fresh BRB for 30 min. Wash 4 × 10 min in PBS-Tw and develop. Antibody dilutions should be determined in trial experiments.

3.4. Preparation of the Hyperphosphorylated CTD Probe

3.4.1. Preparing [^{32}P]-Labeled PhosphoCTD

1. Express GST-CTD fusion protein in bacteria and purify using an affinity matrix ***(25)***; check the purity and amount of intact protein using SDS-PAGE (*see* **Note 1**). Dialyze the fusion protein into HEPES dialysis buffer. Store frozen in aliquots at –80°C.
2. Set up the kinase reaction by adding the following to the microfuge tube:

   10 μL 10X reaction buffer
   x μL GST-CTD fusion protein (approx 25 μg)
   5 μL 2% Tween-20 solution
   y μL CTD kinase (approx 200 ng enzyme)
   Add deionized H_2O to make 100 μL [72.5 – (*x* + *y*) μL]; this can also be added first.
   10 μL (100 μCi) g-[^{32}P]ATP
   2.5 μL 3m*M* ATP (final concentration = 75 μ*M*).
3. Mix gently by micropipetting.
4. Immediately remove 2–5 μL, pipet into SDS sample buffer, and heat for 5 min at 90°C to generate the time zero point.

5. Incubate the kinase reaction at 30°C for 1 h.
6. Add 7.5 µL 3 m*M* stock nonradioactive ATP (final = 300 µ*M*) (*see* **Note 3**).
7. Incubate at 30°C for an additional 60 min.
8. Remove 2–5 µL and pipet into SDS sample buffer; heat for 5 min at 90°C to generate the 2-h time point. Place the remainder of the kinase reaction at –80°C.
9. Check the extent of CTD phosphorylation by analyzing the t = 0 and t = 2 h samples, along with untreated fusion protein and prestained marker proteins, using SDS-PAGE (e.g., 4–12% or 4–15% acrylamide gradient gel). Electrophorese the samples until prestained standards of approximately the same size as the CTD fusion protein migrate two-thirds of the distance to the bottom of gel. Stain with Coomassie blue. Destain and monitor the mobility shift of fusion protein in the t = 2 h sample as compared to the untreated and t = 0 samples. *See* **Fig. 1** for examples of the time course of phosphorylation.
10. If all of the (intact) fusion protein is not shifted to slower mobility, it will be necessary to further kinase the sample. Thaw the remainder of the kinase reaction by gently agitating the tube in room temperature water until just thawed. Add another aliquot of CTD kinase (e.g., *y* µL or 0.5*y* µL). Incubate at 30°C for an additional 1–2 h. Remove 2–5 µL and analyze by SDS-PAGE as before. To determine the extent of radioactivity that has been incorporated, the gels can be analyzed by autoradiography or PhosphorImager analysis (*see* **Fig. 1** for an example).
11. Following the kinase reaction, [^{32}P]-ATP is removed and the buffer is exchanged into PBS or other desired buffer using a 1-mL quick-spin protein column following the manufacturer's instructions. This procedure yields a final volume of about 400 µL. The authors assume a spin column yield of approx 80%. The phosphorylated CTD fusion protein is stored at –80°C. This procedure should yield sufficient probe for 4–8 Far Western blots.

3.4.2. Preparation of Nonradioactive PhosphoCTD

To prepare phosphoCTD without a radioactive label, proceed as above, but increase the initial concentration of nonradioactive ATP to 300 µ*M* and omit radioactive ATP.

3.5. Purifying CTD Kinase

CTD kinases differ in the positions on the CTD to which they add phosphate groups, although a rigorous determination of this site specificity has yet to be carried out for any CTD kinase ***(1)***. Likewise, PCAPs display binding preferences for CTDs carrying phosphates in particular patterns. Thus, the set of PCAPs detected will depend on the CTD kinase used to prepare the phosphorylated CTD. The authors have used yeast CTDK-I exclusively in studies and expect that use of the yeast Kin28, Bur1, and Srb10 CTD kinases will result in the identification of different sets of proteins. It will also be interesting to note whether the use of a mammalian homolog of CTDK-I (e.g., P-TEFb) might result in the discovery of additional PCAPs.

3.5.1. Purification of CTDK-I From Yeast (**refs. 12,14**; see also **Subheading 3.2.**)

1. Apply extract (**Subheading 3.2.**) to phosphocellulose (P11) column (50-mL bed volume) equilibrated in HGE(0.15).
2. Wash the column extensively with the same buffer, then elute with a 250-mL linear gradient of 0.2–0.8 *M* KCl in HGE.
3. Assay fractions for CTDK-I activity (*see* **Subheading 3.5.2.**) and pool peak fractions (enzyme usually elutes at approx 0.32 *M* KCl).
4. Dialyze sample (vs HGE) to 0.12 *M* KCl and apply to a DE52 column (8-mL bed volume) equilibrated in the same buffer. The kinase is recovered in the flow-through fraction.
5. Apply the flow-through fraction directly to a 1-mL MonoS column and elute with a 0.15–0.45 *M* KCl gradient. CTDK-I elutes at approx 0.35 *M* KCl. Assay fractions for CTDK-I activity and pool fractions containing the peak of enzymatic activity.

3.5.2. Assay for CTD Kinase I (CTDK-I) Activity

1. For a 20-μL reaction, add 2 μL 10X reaction buffer (**Subheading 2.4.**), yeast CTD fusion protein to a final concentration of 0.15 mg/mL intact protein, 2–10 μL of each column fraction, ATP to 300 μ*M*, γ-[^{32}P]ATP (1–3 μCi), and water to 20 μL.
2. Incubate the mixture for 15–60 min at 30°C.
3. Terminate the reaction by adding SDS sample buffer and heating at 90°C for 5 min.
4. Analyze the products by SDS-PAGE followed by autoradiography (*see* **Fig. 1**).

3.6. Fractionation and Purification of PCAPs

The overall approach is four-pronged: (1) fractionate the extract, (2) test resulting fractions for presence of PCAPs, (3) fractionate/purify PCAPs further if necessary, and (4) identify the PCAPs.

1. Extracts are fractionated by conventional ion-exchange chromatography. A high-capacity cation-exchange resin that will handle large amounts of material and to which most PCAPs are expected to bind is a good choice. The authors have successfully used Macro-Prep High S, Macro-Prep CM (Bio-Rad, Hercules, CA), and HiTrap S (Amersham, Piscataway, NJ) supports ***(4,18)***. After application of the sample at low salt (e.g., 0.15 *M* KCl), the column is developed with a gradient of increasing salt concentration (e.g., 0.15–1.0 *M* KCl).
2. Identification of PCAPs in column fractions: An aliquot of each fraction is applied to duplicate SDS-PAGE gels. One gel is stained with Coomassie blue; the other is analyzed by Far Western blotting using a PCTD probe (*see* above). If a PCAP corresponds to a single protein band, it is excised from the stained gel and identified by mass spectrometry (*see* **Subheading 3.7.**). Fractions containing less pure PCAPs are fractionated further as described below.
3. Additional purification of PCAPs: PCAP-containing fractions are pooled, diluted to 0.15 *M* KCl, and applied to a second column. Good candidates for this column

are P-Serine-agarose (Sigma-Aldrich, St. Louis, MO) ***(4)***, or phosphoCTD-agarose ***(18)***. The second column is eluted with steps of increasing solvent concentration (e.g., phosphoserine, salt), and recovered fractions are analyzed on parallel SDS-PAGE gels as previously mentioned. Many PCAPs should now correspond to single protein bands and can be identified by mass spectrometry. Others may require one more fractionation step (e.g., MacroPrep High Q [Bio-Rad], ***[4]***).

3.7. Identifying PCAPs by Mass Spectrometry

PCAPs are identified by excising the band from the Coomassie blue-stained gel corresponding to PCTD-binding activity (Far Western blot) and subjecting it to mass spectrometry analysis. The authors have submitted numerous samples to the Laboratory for Protein Microsequencing and Proteomic Mass Spectrometry, University of Massachusetts Medical School, Worcester Foundation Campus, Shrewsbury, MA (http://www.umassmed.edu/proteomic/) for in-gel trypsinization and MALDI mass spectrometry analysis. Note that mass spectrometry technology is rapidly evolving; check with experts for the current best approach.

3.8. Confirming PCAP Identity

The mass spectrometry-based identification of a PCAP needs to be checked by testing *bona fide* protein for its ability to bind the phosphoCTD. The most convincing approach is to demonstrate that purified, recombinant putative PCAP binds the phosphoCTD. If recombinant protein is not available conventionally-purified putative PCAP can be used.

3.8.1. Far Western Analysis With phosphoCTD as Probe

1. Obtain (express and purify) recombinant protein to be tested.
2. Separate the test protein on three individual SDS PAGE gels and blot all three gels onto nitrocellulose for Far Western analysis. Include positive (known PCAP) and negative (known non-PCAP) control proteins in the SDS gels.
3. One blot is probed with a phosphoCTD fusion protein (e.g., GST-phosphoCTD) as described above. A second blot is probed with the fusion partner (e.g., GST). Binding of the test protein to phosphoCTD fusion protein but not to the fusion partner confirms that the test protein identification was correct and that it is actually a PCAP. The third blot is probed with the nonphosphorylated CTD fusion protein (e.g., GST-CTD). The binding of the putative PCAP to the phosphoCTD fusion protein but not to the nonphosphorylated CTD fusion protein indicates that binding depends on CTD phosphorylation.

3.8.2. Far Western Analysis of PhosphoCTD Binding Using the Putative PCAP as Probe (Reverse Far Western, see ***Note 5***)

1. To adjacent lanes of an SDS-PAGE gel, apply phosphoCTD fusion protein (e.g., GST-phosphoCTD), nonphosphorylated CTD fusion protein, and the fusion part-

ner (e.g., GST) together with prestained marker proteins (*see* **Note 6**). Multiple sets should be applied to a single gel.
2. Electrophorese and blot onto nitrocellulose membrane as described previously.
3. Cut the nitrocellulose membrane to separate each set of three lanes.
4. Probe one set of lanes with the recombinant putative PCAP (e.g., MBP-PCAP). A *bona fide* PCAP will bind to the phosphoCTD fusion protein (i.e., show a signal in lane 1) but not to the non-phosphoCTD or the fusion partner (i.e., no signal in lanes 2 and 3). For an example, *see* Phatnani and Greenleaf, 2004 ***(18)***.

4. Notes

1. A CTD fusion protein with affinity tags at both ends (e.g., GST-yCTD-His_6) affords the opportunity to use sequential affinity purifications to generate only full-length protein ***(25)***. However, a single affinity purification step frequently yields a preparation with sufficient full-length protein for many uses. Of course, the CTD can be fused to purification tags other than GST (*see* **Fig. 1**). Interestingly, in the authors' experience, the efficiency of phosphorylation of the CTD varies depending on its fusion partner; this phenomenon has not been thoroughly investigated.
   The authors have observed that the nonfused CTD (i.e., free CTD, not attached to a protein fusion partner) displays some unusual properties. It is not stained well by either Coomassie blue or silver, and it is not easily detected after SDS-PAGE and Western blotting. On the other hand, because each CTD repeat contains a tyrosine, its absorbance at 280 nm can be easily measured.
2. If fully shifted, exhaustively phosphorylated fusion protein is required, small-scale trial experiments should be first performed to determine how much CTD kinase to use. This practice will help conserve kinase.
3. CTDK-I has an apparent Km for ATP of approx 30 μ*M* ***(12)***; incubation with total ATP concentration much below this level (e.g., using carrier-free radiolabeled ATP) results in very little incorporation. The authors attempt to maximize the specific radioactivity of the final product by incubating the kinase reaction for an hour with [ATP] somewhat above the Km, and then for an additional 1 h or more at "saturating" [ATP].
4. To estimate the amount of [^{32}P]phosphoCTD-fusion protein, place 2 μL of the material recovered from the spin column in **Subheading 3.4.5.** at the bottom of a 1.5-mL microfuge tube. Place this against the detector of a Geiger counter and determine the counts per second. This figure is then converted to cpm/μL. This method gives estimated cpm that are about six- to eightfold lower than counts determined by scintillation counter (using scintillation fluid).
5. In the authors' experience, segments of certain PCAPs subjected to SDS-PAGE and electroblotting to nitrocellulose sometimes fail to bind phosphoCTD fusion protein probe, presumably because the phosphoCTD-interacting domain does not renature under the conditions used. In such cases, a reverse Far Western approach can be used. The advantage is that the putative PCAP is used in its native (not denatured and renatured) state to probe the phosphoCTD fusion protein that has

been subjected to SDS-PAGE and electroblotting to nitrocellulose. The phosphoCTD is thought to be normally largely unstructured in solution ***(5)*** and therefore does not suffer irreversible denaturation during this procedure.

6. Exhaustively phosphorylated (i.e., fully shifted) CTD fusion protein is not absolutely necessary for the reverse Far Western approach. To conserve kinase, a small-scale phosphorylation reaction can be carried out on 1–2 μg fusion protein for 1 h. If some nonshifted (hypophosphorylated) fusion protein remains, it can serve as an internal negative control for the dependence of binding on phosphorylation (e.g., lane 7 in **Fig. 1**).

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3

Imaging Alternative Splicing in Living Cells

Eric J. Wagner, Andrea Baines, Todd Albrecht, Robert M. Brazas, and Mariano A. Garcia-Blanco

Summary

We have developed an in vivo reporter of alternative splicing decisions that allows for the determination of FGF-R2 splicing patterns without the destruction of cells. This method has broad applications, including the study of other alternatively spliced genes in tissue culture and in whole animals, and may be useful in creating imaging markers for the study of tumor progression and metastasis. In this chapter, the authors present one example of this method using fluorescence reporters. As with any new assay, a series of experiments were performed to validate the method. This chapter documents some of these experiments.

Key Words

Alternative splicing; in vivo imaging; fluorescence; intronic splicing silencers; green fluorescent protein; fibroblast growth factor receptor.

1. Introduction

1.1. Alternative Splicing

Versatility, or "multitasking," by a relatively small number of genes may be a requirement to establish the development and function of metazoans ***(1,2)***. Complex genes in metazoans can each encode multiple protein products by differentially selecting which exons will be included in a mature transcript. This process is known as alternative splicing ***(3)***, likely to be the most important engine driving the proteome, the diverse array of proteins observed in any one cell. Alternative RNA synthesis and processing yields different mRNAs from one gene by altering one or all of the following: (1) the transcription initiation site, thus modifying the 5' end of the RNA; (2) the site of cleavage and polyadenylation, thus altering the 3' end of the transcript; and (3) the definition of exons, providing for the different assortment of these packets of coding

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information. Alternative splicing is observed among pre-mRNAs from over two-thirds of protein-coding genes in humans ***(4,5)***. Alternative splicing can be divided into four general categories: the choice to remove or not to remove an intron, the alternative use of 5' splice sites, or the alternative use of 3' splice sites that will change the length of an exon, and the choice between exon inclusion and exon skipping. This last form of alternative splicing involves one or more alternatively used exons between two exons that are constitutively included.

1.2. Alternative Splicing of FGF-R2 Transcripts

Even though the methodology described is generally applicable to many examples of alternative splicing (*see* **Note 1**), this section focuses on the mutually exclusive choice made between two exons FGF-R2IIIb (IIIb) or FGF-R2IIIc (IIIc) ***(6–14)***. Although exons IIIb and IIIc encode homologous subdomains, the differences between them are sufficient to dramatically alter the ligand-binding specificity of the FGF-R2 isoforms. The choice between IIIb and IIIc is tissue specific. Epithelial cells and well-differentiated prostate tumors of epithelial origin (e.g., DT3 rat prostate tumors) include only IIIb, whereas mesenchymal cells or dedifferentiated prostate carcinomas (e.g., AT3 rat prostate tumors) include only IIIc ***(9)***. The IIIb/IIIc choice must be dictated by differences in the splicing machinery in different cells (i.e., DT3 vs AT3) and by unique *cis*-acting elements in FGF-R2 transcripts. The identities of these *trans*-acting factors and *cis*-elements have led to a model for the tissue-specific regulation of IIIb/IIIc choice ***(6–9,11–14***; **Fig. 1**). The choice of FGF-R2 isoforms critically depends on weak splice sites bordering the IIIb exon, contributing to poor exon definition ***(6,14)***. An hnRNP A1 binding site in exon IIIb mediates poor recognition of this exon ***(8)***. Intronic splicing silencers (ISS) flank exon IIIb and regulate profound repression of IIIb inclusion ***(11,16)***. This silencing is mediated by the polypyrimidine tract binding protein (PTB) ***(11)***. In fibroblasts, these repressors of exon IIIb dictate exon choice, whereas in epithelial cells, these negative *cis*-elements are counteracted to activate exon IIIb and repress exon IIIc. An intronic splicing activator and repressor (ISAR) and an intronic activating sequence (IAS2) are located in the intron between IIIb and IIIc and are absolutely required for cell-type specific activation of IIIb ***(7,9)***. These elements are also required for IIIc repression (Wagner et al., unpublished results).

1.3. Methods to Study Alternative Splicing of FGF-R2 Transcripts

Most methods currently used to study alternative splicing in mammals employ one of two general approaches. Biochemical analysis involves the use of cellular or subcellular extracts capable of recreating splicing reactions in vitro. This approach has the advantage of facilitating the study of individual proteins using

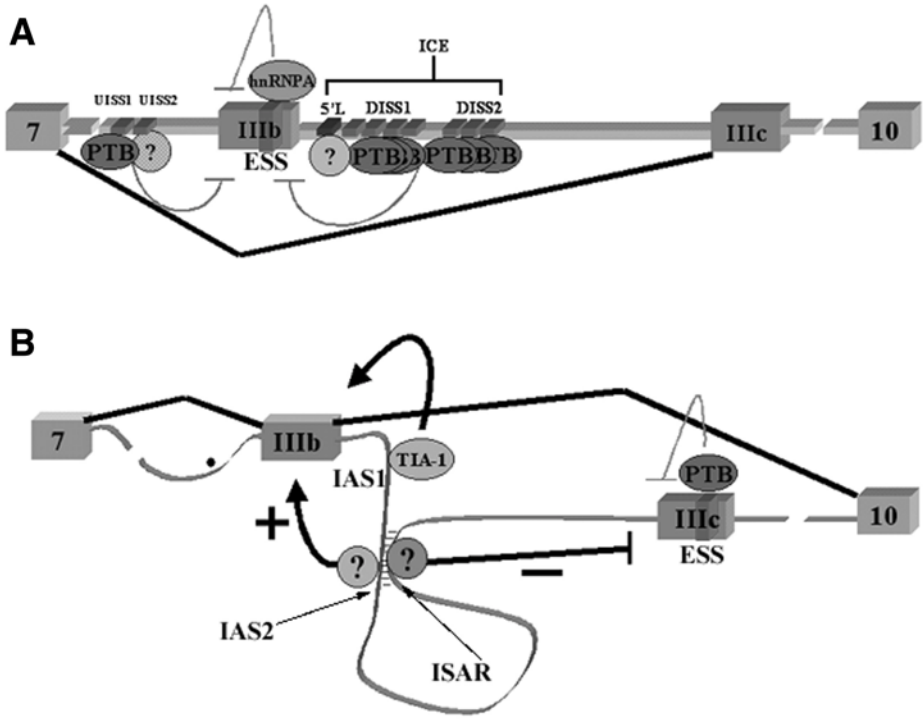


Fig. 1. A model for the regulation of FGF-R2 alternative splicing. Several factors have been implicated in mediating IIIb silencing; the polypyrimidine tract binding protein (PTB) binds the ISS elements that flank IIIb and silences this exon ***(11)***. HnRNP A1 has also been implicated in IIIb silencing. Artificial recruitment of hnRNP A1 protein to IIIb leads to decreased IIIb inclusion ***(18)***. IIIb inclusion is likely mediated by an epithelial specific factor, which we posit interacts with and stabilizes a secondary structure predicted to be formed by ISAR-IAS2 base pairing ***(7,9)***. Given the overlap between IAS2 and the downstream silencers within ICE, it is likely that the putative ISAR-IAS2 secondary structure perturbs silencer function.

classical biochemistry. The disadvantages are that, to date, many cell types have not produced splicing-competent nuclear extracts, and most available extracts can only partially recreate splicing decisions made in vivo. A molecular genetics approach involves the use of minigenes, in which genomic sequences from alternatively spliced genes are inserted into mammalian expression vectors and then transfected into cells in culture that regulate the alternative splicing event of interest. The advantage to this approach is that it facilitates the discovery of *cis*-elements and is experimentally facile. This approach, combined with the use of RNA interference, can lead to the identification of *trans*-acting factors ***(11)***. Additionally, the use of knockout mice has led to an understanding of the

roles of a few alternative splicing factors (e.g., Nova-1) ***(15)***. The details of the RNA transactions in these cases were obtained by lysing the cells and analyzing the isolated RNA. In some cases alternative decisions have been observed in fixed tissues using *in situ* hybridization. All of these approaches, although quite useful, do not allow for observation of splicing in real time.

The authors have developed an in vivo reporter of alternative splicing decisions (*see* **Note 2**) that allows for the determination of FGF-R2 splicing patterns without the destruction of cells. This method has broad applications, including the study of other alternatively spliced genes in tissue culture and in whole animals, and may be useful in creating imaging markers for the study of tumor progression and metastasis. The following sections present one example of this method using fluorescence reporters (*see* **Note 3**). As with any new assay, a series of experiments, some of which are documented in this chapter, were performed to validate the method. The creation of these fluorescent splicing reporters is described, and the correlation between the alternative splicing of the reporter and the fluorescence seen in the cells is demonstrated.

2. Materials

2.1. Plasmids

1. Plasmid pEGFPN1:pEGFPN1 (Clontech, Inc.) has a multicloning site (MCS) between the immediate early promoter of CMV (PCMV IE) and EGFP coding sequences, as well as an SV40 polyadenylation signal downstream of the EGFP open reading frame (ORF) to direct proper processing of the 3' end of the mRNA. A neomycin-resistance cassette (neo^r) allows stably transfected eukaryotic cells to be selected using geneticin (also known as G418) and a kanamycin resistance (kan^r) marker is used for selection of kan^r in *E. coli*. A complete description and sequence of this vector can be found at the Clontech website: http://www.clontech.com/techinfo/vectors/vectorsE/pEGFP-N1.shtml (*see* **Note 4**).
2. Plasmid pGInt: Plasmid pGInt was constructed by introducing an intron from pI–12 ***(11)*** into sequences encoding the EFGP ORF in pEGFPN1 (*see* **Note 5**).
3. Plasmid pGIIIb: The rat FGF-R2 exon IIIb and upstream and downstream intronic sequences flanking the exon, including UISS and ICE, were subcloned into pGInt (*see* **Fig. 2**).
4. Plasmids pGΔUISS, pGΔICE and pGΔΔ: Plasmids pGΔUISS, pGΔICE, and pGΔΔ are identical to pGIIIb except that the UISS, ICE, or both were deleted, respectively (**Fig. 2**).
5. Plasmid pcDNA5/FRT/TO (Invitrogen, Inc.). This plasmid is similar to standard cytomegalovirus (CMV) immediate early promoter driven mammalian expression vectors except for the presence of three important features: First, there is the FRT site, which is a recognition/target sequence for the yeast 2 μ plasmid site-specific recombinase, Flp. The presence of this site allows for this plasmid to be integrated into a second specific FRT site located within a host cell chromosome

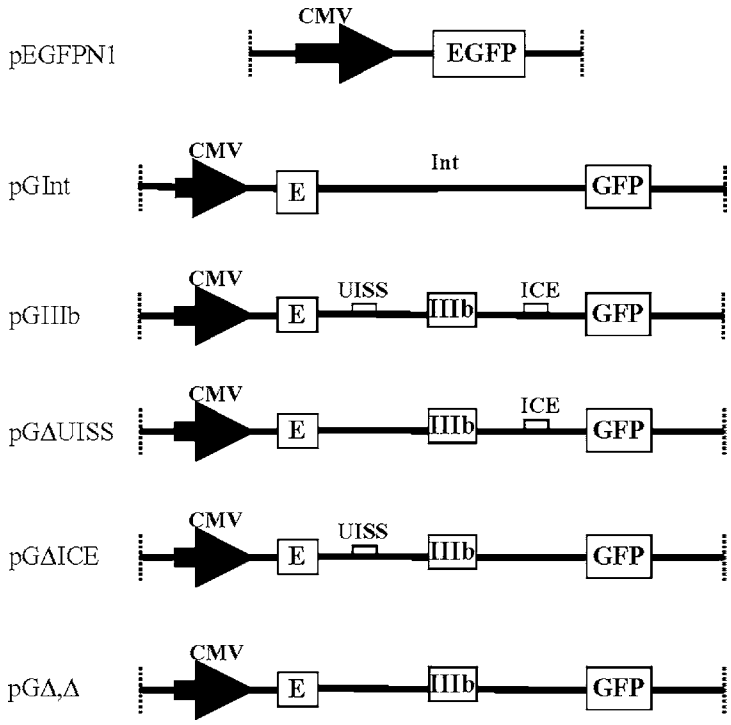


Fig. 2. Schematics of EGFP reporters used to image alternative splicing. All constructs represented have the backbone sequence of pEGFPN1 (Clontech, Inc.). The pGInt reporter contains an intron derived from pI-12 inserted within the EGFP open reading frame (*see* **Materials** and **Methods**). The pGIIIb reporter is identical to pGInt with the exception of having FGF-R2 exon IIIb as well as the flanking intronic elements, UISS and ICE, inserted between the *Bam*HI and *Apa*I sites of the intron (*see* **Subheadings 2.** and **3.**). The pGΔUISS, pGΔICE, and pGΔ,Δ reporters are identical to pGIIIb with the exception of having either the UISS or the ICE (or both) intronic elements deleted.

by Flp-dependent, site-specific recombination (*see* **Fig. 3**). Second, the CMV promoter has two tetracycline (tet) repressor operators within it, making it sensitive to the presence of tet repressor. In the absence of tetracycline, the tet repressor will bind the operator and repress the CMV promoter; thus, there will be no expression of the gene of interest. Upon addition of tetracycline, expression of the gene of interest will be induced strongly (Tet-ON), similar to the levels seen using the standard CMV promoter (*see* **Note 6**). Third, this plasmid backbone contains a hygromycin resistance gene just downstream of the FRT site that lacks a mammalian promoter. The promoter for the hygromycin resistance gene is

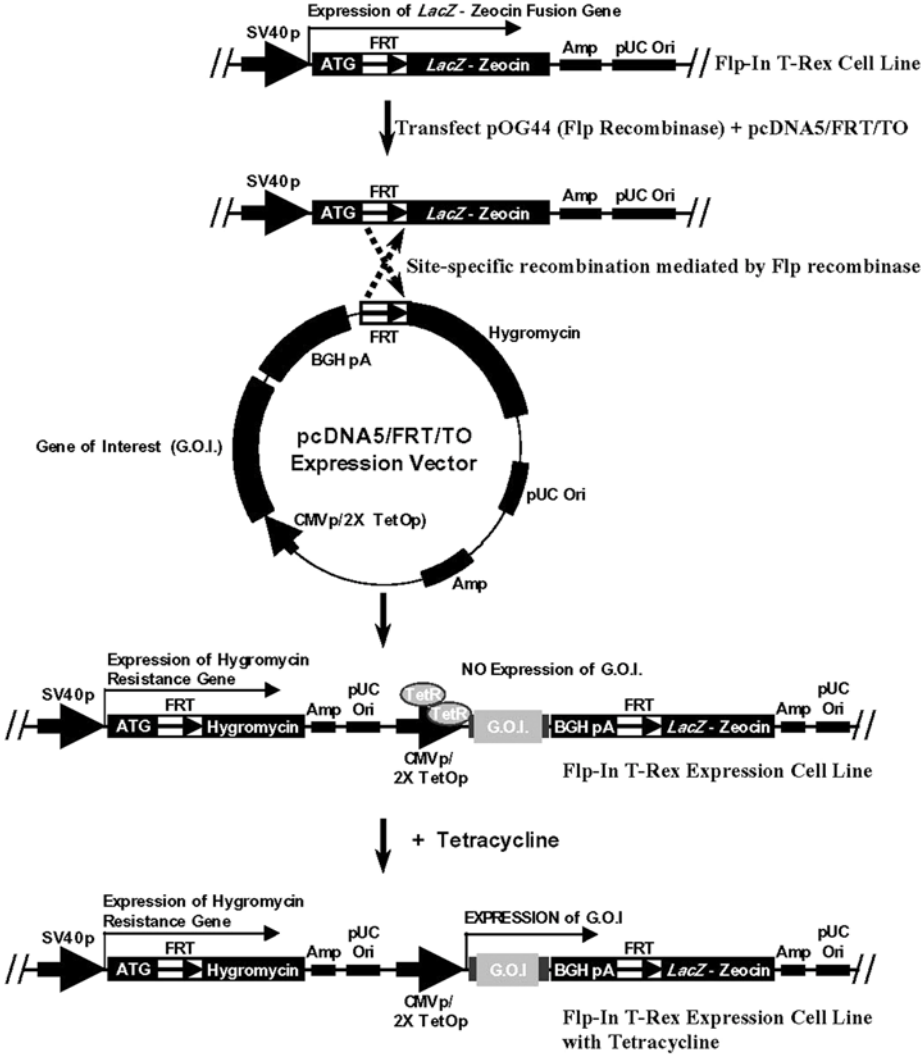


Fig. 3. The Flp-In T-Rex system (Invitrogen, Inc.) for creating tetracycline-inducible stable cell lines. A cell line is created that contains the pFRT/lacZeo vector integrated at a single chromosomal location and multiple copies of the pcDNA6/TR tet repressor expression vector. This cell line is then transfected with the pcDNA5/FRT/TO expression vector containing the gene of interest (G.O.I.) and the pOG44 Flp recombinase expression vector. The Flp recombinase catalyzes the recombination of the pcDNA5/FRT/TO vector (through its FRT site) and the chromosomal FRT site, which is part of the integrated pFRT/lacZeo. The integration of the expression vector disrupts the lacZeo fusion gene expression and induces the expression of the hygromycin resistance gene making the stably transfected cells hygromycin resistant.

acquired upon integration into the chromosomal FRT site, which prevents spurious integration events from being selected (*see* **Fig. 3**, *see* **Note 7**).

6. Plasmids pcDNA5/FRT/TO–Gint, pcDNA5/FRT/TO–GIIIb, pcDNA5/FRT/TO–GΔUISS, pcDNA5/FRT/TO–GΔ,Δ. Each of these plasmids has the EGFP region, including the intron and IIIb sequences from pGInt, pGIIIb, pGΔUISS, and pGΔ,Δ, cloned into the polylinker region of pcDNA5/FRT/TO downstream of the CMV immediate early promoter. These plasmids can be integrated into a chromosomal FRT site using Flp recombinase (*see* **Subheading 3.2.1.** for details).
7. Plasmid pOG44. This vector expresses a temperature-sensitive mutant of the yeast 2 μ plasmid-derived Flp recombinase under the control of the CMV immediate early promoter. It is used as a source of Flp recombinase during Flp-mediated recombination into the chromosomal FRT site. *See* Invitrogen's website for additional details (http://www.invitrogen.com).

2.2. Cell Culture and Transfections

1. DT3 and AT3 cell lines are derivatives of the rat Dunning prostate tumor and were kindly given to the laboratory by Dr. Wallace McKeehan (Texas A & M University Health Sciences Center, Houston, TX). These cells were maintained in DMEM-L (media described in the following steps).
2. Flp-In T-Rex 293 cell line (Invitrogen, Inc.). This cell line is derived from standard human embryonic kidney 293 cells (ATCC, CRL-1573). Flp-In T-Rex 293 cells have a single copy of an FRT site integrated by stable transfection of the pFRT/lacZeo plasmid. Invitrogen has demonstrated high level expression from pcDNA5/FRT/TO-derived vectors integrated at this chromosomal FRT location. The pcDNA6/TR tet repressor expression vector (Invitrogen, Inc.) has also been stably integrated, yielding high-level constitutive expression of tet repressor. Therefore, in the absence of tetracycline, there will be no expression from pcDNA5/FRT/TO-integrated plasmids, while there will be strong expression in the presence of tetracycline. These cells were maintained in DMEM–High Glucose (Invitrogen) with tetracycline-free 10% fetal bovine serum (FBS) (*see* **Note 8**), L-glutamine, penicillin/streptomycin, 100 μg/mL Zeocin, and 15 μg/mL blasticidin.
3. Dulbecco's modified eagle medium with low glucose, L-glutamine, 110 mg/L sodium pyruvate, and pyridoxine hydrochloride (DMEM-L). (AT3 and DT3 cell lines). Dulbecco's modified eagle medium with high glucose, L-glutamine, 110 mg/L sodium pyruvate and pyridoxine hydrochloride (DMEM-H) (Flp-In T-Rex 293 cell line). Unless otherwise indicated, tissue culture supplies were obtained from Invitrogen, Inc.
4. FBS was heat inactivated at 56°C for 30 min (Hyclone, Inc.).
5. Penicillin-streptomycin (100X–10,000 U/mL).
6. Trypsin–EDTA (0.05% trypsin, 0.53 m*M* EDTA–4Na).

Fig. 3. *(continued)* After the stable cells have been allowed to grow as a polyclonal population in the absence of tetracycline, the expression of the G.O.I. can be induced by the addition of tetracycline.

7. Dulbecco's phosphate-buffered saline (PBS).
8. OPTI-MEM reduced serum medium with 1X HEPES buffer and L-glutamine and without phenol red was used for transfection media (Invitrogen, Inc.). Fluorescence detection was done in DMEM maintenance media.
9. Lipofectamine (Invitrogen, Inc.).
10. FuGENE 6 (Roche Molecular Biochemicals).
11. Geneticin (G418): stock solution is 50 mg/mL (Invitrogen, Inc.).
12. Zeocin: stock solution is 100 mg/mL (Invitrogen, Inc.).
13. Blasticidin: stock solution is 10 mg/mL (Invitrogen, Inc.).
14. Qiaquick PCR purification kit (Qiagen) for purification of DNA fragments for transfection.
15. Hygromycin: stock solution is 50 mg/mL (Invitrogen, Inc.).

2.3. Fluorescent Microscopy

1. Leica DMRA fluorescent microscope.
2. Chroma FITC filter set no. 41001.
3. SPOT digital camera (Diagnostic Instruments).
4. SPOT RT Software for image acquisition (Diagnostic Instruments).
5. Dell Dimension 4100 computer.
6. Glass slides, glass coverslips, and all other conventional microscopic supplies are used.
7. Crystal/mount aqueous/dry mounting medium (Biomeda).

2.4. Fluorescence-Activated Cell Sorter

1. FACS caliber flow cytometer with 488-nm argon laser (Becton Dickinson). The instrument is part of the Duke University Comprehensive Cancer Center fluorescence-activated cell sorter shared resource facility (Dr. J. Michael Cook, director).
2. Standard 530-nm FITC bandpass filter.
3. CELLQuest software was used for fluorescence analysis.

3. Methods

3.1. Cell Culture Conditions

1. All cell lines are maintained in T75 vented tissue culture flasks (Corning) at 37°C with 5% carbon dioxide in vented cap. Transfections are performed in six-well plates (Falcon) (*see* **Note 9**).
2. Cell lines are replaced every 3 mo with frozen stocks from liquid nitrogen. Cells are thawed by placing a 1-mL frozen stock in 37°C water bath. The entire 1-mL aliquot is then placed in a T25 tissue culture flask with 5 mL maintenance media and placed at 37C with 5% carbon dioxide.
3. Cell lines are prepared for liquid nitrogen storage by treating a T75 tissue culture flask with 1.5 mL of trypsin at 37°C until all cells have detached. Trypsinized cells are resuspended with 8.5 mL of DMEM growth medium placed in a sterile 15-mL conical tube, and centrifuged at 2000*g* for 1 min in a tabletop clinical centrifuge (International Equipment Company) with a fixed-angle rotor. The supernatant

fraction is removed and the cell pellet is resuspended in 3 mL of maintenance media with 10% (v/v) DMSO. 1-mL Aliquots are distributed into 1.5-mL cryogenic tubes (Nalgene, Inc.). Cells are placed in a freezing chamber (Nalgene, Inc.) containing isopropanol at –80°C for 24 h and then moved to liquid nitrogen.

4. Once the cell monolayer is confluent, cells are transferred to new flask as follows. Cells in a T75 flask are treated with trypsin as described previously. Resuspend by adding 8.5 mL of maintenance media in addition to the 1.5 mL of trypsin. Add 1 mL of the resuspended cells to a T75 flask already containing 9 mL of maintenance media.

3.2. Transfections With Conventional Vectors

1. Plate cells in six-well plates the day before transfection (approx 20 h before transfection) at a dilution of 3×10^5 cells/well.
2. Linearize plasmids pGInt and derivatives (but not the plasmids containing FRT sites) by digestion with Apa LI at 37°C (*see* **Note 10**).
3. For transfection, add 5 µL lipofectamine reagent to 95 µL OPTI-MEM in a 1.5-mL microcentrifuge tube and incubate at room temperature for 2 min.
4. In a separate 1.5-mL microcentrifuge tube bring 1 µg linearized plasmid DNA to a total volume of 100 µL with OPTI-MEM.
5. Mix the 100 µL plasmid DNA with the 100 µL diluted lipofectamine reagent (both in OPTI-MEM) and incubate at room temperature for 20 min.
6. Add 800 µL OPTI-MEM to the 200 µL plasmid DNA and lipofectamine solution.
7. Wash one well of a six-well plate twice with 2 mL OPTI-MEM, then replace media with 1 mL lipofectamine reagent and plasmid DNA solution.
8. Incubate for 4 h at 37°C with 5% CO_2.
9. Replace media with 2 mL maintenance media and allow cells to recover for 24 h at 37°C with 5% CO_2.
10. Replace 2 mL maintenance media with 2 mL of this media containing a 1:100 dilution of the stock geneticin antibiotic (final concentration 500 µg/mL).

3.2.1. Frt/flp Vectors

Figure 3 illustrates the overall approach used to integrate the pcDNA5/FRT/TO expression vectors into a single chromosomal FRT site in each cell using Flp recombinase. The following steps were used to integrate the pcDNA5/FRT/TO–EGFP series of vectors:

1. The Flp-In T-Rex cells are seeded in T75 tissue culture flasks 1 d prior to transfection so that 24 h later (the day of transfection), they will be approx 75% confluent (*see* **Note 11**). The total amount of media in the flask is 10 mL (*see* **Note 12**).
2. For each T75 flask to be transfected, the following transfection mix is made in this order:
   a. Place 800 µL OPTI-MEM in a sterile polystyrene tube.
   b. Add 48 µL FuGENE 6 to the OPTI-MEM below the surface.
   c. Flick the tube five times to mix the FuGENE 6 and OPTI-MEM.

d. Add a mixture of 14.4 μg pOG44 and 1.6 μg pcDNA5/FRT/TO (each expression vector should be done separately) to the tube and flick five more times.
e. Allow this mixture to sit for 25 min at room temperature.
f. Add the transfection mixture to the flask of Flp-In T-Rex 293 cells, mix thoroughly (*see* **Note 13**), and place back in the incubator at 37°C at 5% CO_2 for 48 h.

3. After allowing the cells to grow for 2 d, trypsinize each flask and replate into media supplemented with 15 μg/mL blasticidin and 150 μg/mL hygromycin.
4. Allow the cells to grow in this selective media until large colonies of stable transfectants form in the flask. Change the media every 3 d during this incubation.
5. Trypsinize the flask, then replate into a T75 flask in the same selective media (*see* **Note 14**).
6. After sufficient numbers of blasticidin/hygromycin resistant cells have been produced, the cells are ready for flow cytometry analysis.

3.3. Fluorescent Microscopy

1. Cells are plated at 3×10^5/well in a six-well plate, where each well contains a sterile glass coverslip.
2. The following day, remove the media and wash the cells once with 2 mL PBS.
3. In a chemical fume hood, remove the PBS, then add 2 mL PBS-fix (PBS with 3.7% formaldehyde, made freshly) to the wells. Incubate at room temperature for 15 min.
4. Remove the PBS-fix and add 2 mL of 20 m*M* NH_4Cl to quench the formaldehyde.
5. Wash the cells once more with PBS, then place the coverslip onto a conventional glass microscope slide containing one drop of antifade/sealant (Biomeda).
6. Observe the cells on a Leica DMRA fluorescent microscope using a Chroma FITC filter set no. 41001. Images are acquired using SPOT RT software (Diagnostic Instruments, Sterling Heights, MI) and further processed using Adobe Photoshop 5.5.

3.4. Fluorescence Activated Cell Sorter

1. Wash stable cell lines in the T-25 tissue culture flask with 4 mL of PBS.
2. Add 0.5 mL trypsin-EDTA and incubate at 37°C with 5% CO_2 until the cell monolayer is resuspended.
3. Once cells have detached, add 4.5 mL DMEM 10% FBS to inactivate trypsin.
4. Count cells with a hemocytometer and dilute to a density of 5×10^5 cells in 500 μL DMEM 10% FBS.
5. Add 500 μL of cells to a 5-mL polystyrene round-bottom tube from Falcon for FACS analysis.
6. FACS analysis was performed at the Comprehensive Cancer Center Flow Cytometry shared resource at the Duke University Medical Center.
7. FACS analysis is performed on a Beckon Dickinson FACS Caliber machine with a 488-nm laser.
8. Mean EGFP is the average emission in relative fluorescence units (RFU) of approx 10,000 cells that reside within a gated value set at a threshold above background fluorescence.

3.5. Measuring Exon IIIb Inclusion Using pGIIIb Plasmids

The plasmid pEGFPN1 (Clontech, Palo Alto, CA), which can drive EGFP expression in both DT3 and AT3 cells, was used to construct the pG family of alternative splicing reporter vectors. Cells transfected with either pEGFP-N1 or pGInt expressed high levels of EGFP as detected by fluorescence microscopy and FACS (**Fig. 4A** and data not shown). The high expression of EGFP corresponded to efficient splicing of the intron in pGInt transcripts, leading to the conclusion that pGInt could report on the constitutive removal of the pI-12 intron.

The authors sought to construct plasmids that could report on regulated splicing events. To this end, the intron in pGInt was interrupted with the rat FGF-R2 exon IIIb and its flanking intronic splicing silencers, the upstream intronic splicing silencer (UISS) and the downstream intronic control element (ICE) (***11***; *see* **Materials** and **Figs. 2** and **5A**). If UISS and ICE function properly, exon IIIb will be silenced (skipped) and the predominant mature transcript will encode EGFP. If, on the other hand, UISS and/or ICE are compromised, exon IIIb will be included, the EGFP ORF will be disrupted, and EGFP expression will be diminished. When pGIIIb was stably transfected into DT3 cells, high levels of fluorescence were observed (**Fig. 5B**; *see* **Note 15**). Deletion of either UISS or ICE, which has been shown to dramatically increase the amount of exon IIIb inclusion ***(11,16)***, led to decreased EGFP expression (**Fig. 5B**). To confirm the results of fluorescence microscopy, the levels of EGFP fluorescence were quantified by FACS (**Fig. 5C**). The authors directly examined the alternative splicing of the pGIIIb reporters using RT-PCR analysis and demonstrated an excellent correlation between the alternative splicing pattern and the levels of EGFP fluorescence (**Fig. 6**; *see* **Note 16**). These data demonstrate that the pGIIIb vectors can accurately report on the silencing of exon IIIb.

3.5.1. Advantages of Frt/flp Vectors

Although the pGIIIb vectors were a good first step, they suffered from several limitations. First, the level of EGFP expression varied significantly from cell to cell in the selected population. Indeed, a considerable pool of cells expressed very low levels of EGFP. Second, the expression of EGFP from pGInt (or pEGFPN1) was much higher for DT3 cells than for AT3 cells (data not shown). In order to correct these problems, the authors decided to transport reporters into the pcDNA5/FRT/TO plasmid system (*see* **Subheading 2.2.2.**). The site-specific integration of the splicing reporters into the FRT site in Flp-In 293 T-Rex cells (**Subheading 2.2.2.**) leads to high levels of EGFP expression in the presence of tetracycline, this expression was more homogenous than that obtained with conventional transfection methods (**Fig. 7**). Analysis of cells harboring pcDNA5/FRT/TO-GIIIb vectors revealed a 20-fold decrease in

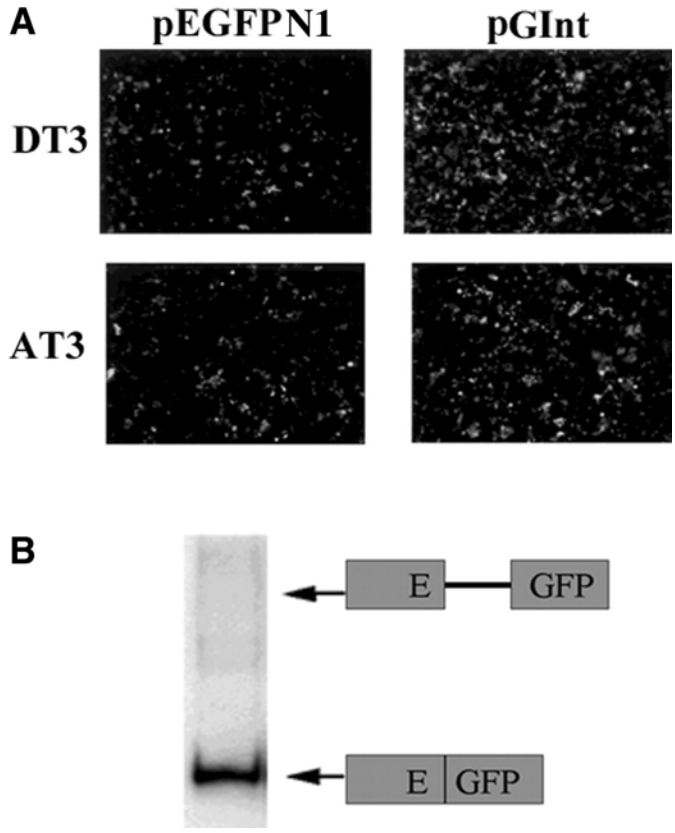
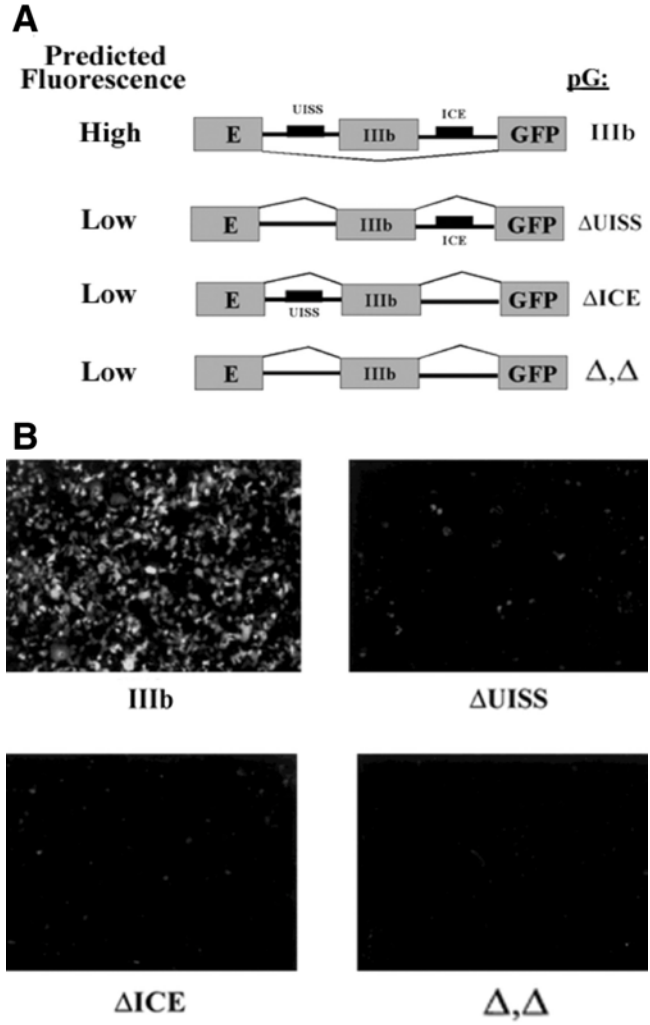


Fig. 4. The placement of an intron in EGFP results in complete splicing in vivo and high level of GFP fluorescence. (**A**), DT3 and AT3 cells were transfected with both EGFP vector as well as the newly created pGInt vector and subject to stable selection using geneticin. High levels of fluorescence were seen in both cell lines using either vector. The exposure time for the images presented here has been normalized to the pEGFP fluorescence in DT3 cells and thus may be compared. The increase in GFP fluorescence observed from the intron containing EGFP vector was apparent in this experiment and repeated in subsequent experiments. (**B**), The results of RT-PCR analysis using exonic primers that flank the intron insertion of pGInt. Total splicing of the pI-12 intron was observed in DT3 cells, as well as AT3 cells (not shown).

Fig. 5. (*see opposite page*) The pGIIIb reporter plasmids successfully report the splicing regulation of the UISS and the ICE intronic splicing silencers. (**A**), Four splicing reporters were constructed containing various deletions of the intronic splicing silencers located in the UISS or ICE. (**B**), These four reporters were transfected into DT3 cells and then were subject to stable selection. The cells were then analyzed for GFP fluorescence. Near identical results were seen with the transfection of AT3 cells (not shown). (**C**), The FACS analysis of the fluorescing cells of (**B**) quantified GFP levels.


A
Predicted Fluorescence
pG:
High
E
UISS
IIIb
ICE
GFP
IIIb
Low
ΔUISS
Low
ΔICE
Low
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IIIb
ΔUISS
ΔICE
Δ,Δ
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1400
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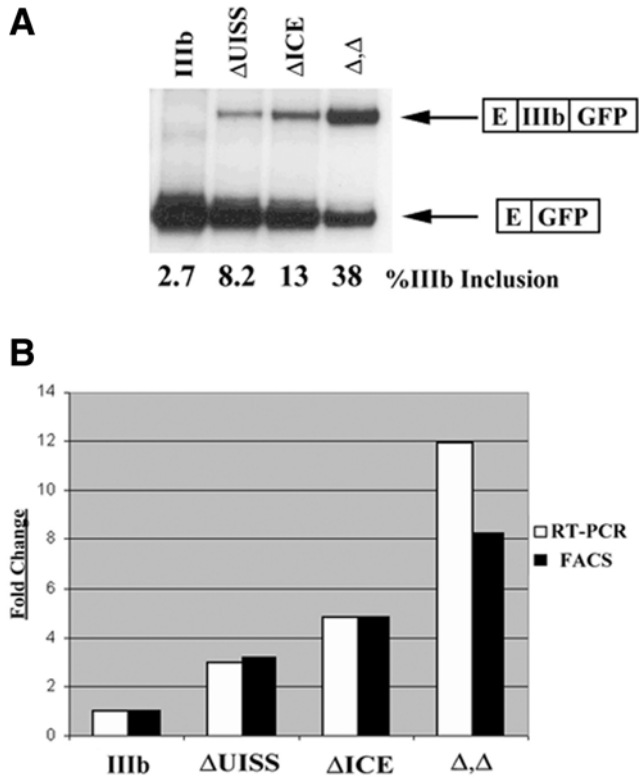

Fig. 6. The levels of fluorescence of DT3 cells stably transfected with pGIIIb reporters correlates with the results of RT-PCR analysis of the in vivo splicing patterns. (**A**), The results of RT-PCR analysis using EGFP specific primers demonstrate increased exon IIIb inclusion as the silencer sequences are deleted. (**B**), The fold change in exon IIIb inclusion compared with the pGIIIb is plotted against the fold change in FACS analysis for the same constructs. The fold changes are consistent with each other with the exception of pG ΔΔ (*see* **Note 16**).

EGFP expression when the UISS and ICE silencer elements were deleted (**Fig. 7**). These and other data (not shown) provide convincing evidence that these FRT vectors are superior splicing reporters in living cells. Presently, the authors are constructing DT3 and AT3 cells bearing single FRT sites to carry out a similar analysis in these cell lines.

4. Notes

1. FGFR2 alternative splicing may represent a unique case, since the most important *cis*-acting elements are localized within the introns. In cases in which exonic

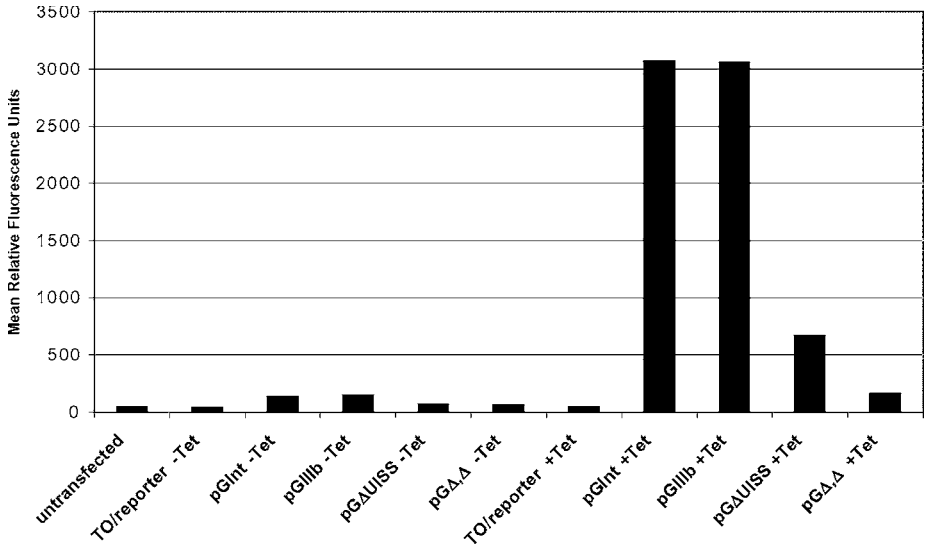

Fig. 7. The pcDNA5/FRT/TO-G plasmids provide and efficient and facile system to study silencing of exon IIIb in 293 T-Rex cells. The mean EGFP fluorescence is plotted vs the HEK293 cells grown in the absence (–) or presence (+) of 12.5 μg/mL tetracycline and harboring the indicated pcDNA5/FRT/TO-G plasmid in the unique genomic FRT site.

splicing enhancers (ESE) or exonic splicing silencers (ESS) are critically important, it will be necessary to further engineer the reporter systems.

2. It is important to note that these reporters do not directly assay the splicing of FGF-R2 transcripts, but rather report on the activity of *cis*-acting elements of alternative splicing.
3. Currently, the authors are constructing a parallel series of reporters based on firefly and renilla luciferase genes.
4. When working with pEGFPN1 there seems to always be a band at 500 nt that copurifies full length plasmid from maxi/minipreps; therefore, the uncut vector must be run next to anything that is to be screened for cloning purposes.
5. The specific location at which to divide the coding region of EGFP was chosen based on a division that would result in consensus splice junction sequences. The pI-12 intron was specifically engineered to contain multiple cloning sites in the center to facilitate the insertion of other exonic and intronic sequences. This intron contains a highly optimized branchpoint and polypyrimidine tract as well as perfect consensus matches at both the 5' and 3' splice sites. Sequences encoding amino acids 1–35 of EGFP were PCR-amplified using pEGFPN1 as a template and primers 5'GFP-F (5'-CCGGCTCGAGGCCACCATGG TGAGCAAGGG-3') and 5'GFP-R (5'-CCGGGAATTCGGATCCATACTCACCT CGCCCTCG-CCGGAC ACGC-3'). These primers were designed with overhangs such that

they generated a product containing an *Xho*I restriction site at the 5' end and *Eco*RI and *Bam*HI sites at the 3' end, as well as a consensus-matching 5' splice site. Sequences encoding amino acids 36–239 of EGFP were PCR-amplified using pEGFPN1 as a template and primers 3'GFP (5'-CCGGGGGCCC TTTCCTTTTT TTTCCTCAGG GCGATGCCAC CTACGGCAA-3') and 3'GFP-R (5'-CCGGGCGGCC GCTTTACTTG TACAGCTCGT CC-3'). These primers included overhangs that generated a product containing an *Apa*I site, a polypyrimidine tract, a branchpoint, and a 3' splice site ahead of the second half of the EGFP coding region. The multicloning site of the intron of pI-12 ***(12)*** was inserted between the *Bam*HI site and the *Apa*I site of the intron, generating an intron with a final length of 131 nt.

6. In the absence of tet repressor, the pcDNA5/FRT/TO will behave as CMVp-driven mammalian expression vector. In addition, there are other pcDNA5/FRT vectors available that have different features than pcDNA5/FRT/TO (*see* http://www.invitrogen.com for additional details).
7. *See* http://www.invitrogen.com for more details on the pcDNA5/FRT/TO backbone and its use.
8. The tet-inducible system used here is extremely sensitive to the presence of low levels of tetracycline. Therefore, if tight control of the CMVp/2X Tet Op promoter is desired, it is important that the FBS used during cell culture lack any tetracycline. The authors have used two sources of tet-free FBS with good success (BD Clontech, cat. no. 8630-1 and Hyclone, Inc., cat. no. SH30070.03T).
9. Falcon six-well plates were used for transfection because non-Falcon plates resulted in detachment of AT3 cells following the transfection protocol.
10. Linearization of plasmids at the ApaL1 site probably led to loss of CMV promoter activity in a significant percentage of transfected cells, which may be because of trimming or recombination of the linearized plasmids after transfection into cells.
11. The authors have found that the degree of confluence of the cells is not critical for these transfections. The transfection efficiency may vary, but hygromycin/blasticidin-resistant colonies will be produced at various cell densities. If optimization of stable cell production is critical, then the transfection reagent used and complete transfection protocol should be optimized. This may involve altering the pOG44:pcDNA5FRT/TO ratio during transfection to increase the number of stable integrants produced.
12. During transfection, all antibiotics, including blasticidin, zeocin, penicillin, and streptomycin, should be left out. The presence of antibiotics during transfection can lead to cell death.
13. When dispersing the transfection mixture, use a back-and-forth motion instead of a rotary motion to avoid piling the FuGENE6/DNA complexes in the middle of the flask.
14. The colonies are dispersed and replated in a smaller flask in order to increase the growth rate of the stably transfected cells.
15. The same results were obtained in AT3 cells. In DT3 cells, exon IIIb is silenced in pGIIIb because the cell-type-specific activators IAS2 and ISAR are not included in the vector.

16. When the fold change in the levels of exon IIIb inclusion for reporters with deletions relative to the pGIIIb is plotted against the fold change in fluorescence, as measured by FACS, of the reporters with deletions relative to pGIIIb, a correlation can be seen. In fact, the fold changes were identical for the pGΔUISS and the pGΔICE. The pGΔ,Δ fold changes were not in complete agreement, although the same trend was observed. The reason for this minor discrepancy is not known, but it may arise from a competitive advantage for the exon IIIb-skipped product in RT-PCR (Wagner et al., manuscript in preparation) or to a lower than expected level of the transcripts that include the IIIb exon, because it is likely subject to nonsense mediated decay (NMD) ***(17)***.

Acknowledgments

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4

Tobramycin Affinity Tag Purification of Spliceosomes

Klaus Hartmuth, Hans-Peter Vornlocher, and Reinhard Lührmann

Summary

The ability to isolate native ribonucleoprotein (RNP) particles is of fundamental importance in the study of processes such as pre-messenger RNA (mRNA) processing and translation. We have developed an RNA affinity tag that allows the large-scale preparation of native spliceosomes in a solid-phase assembly scheme. A tobramycin-binding aptamer cotranscriptionally added to the 3' end of the pre-mRNA is used to bind the pre-mRNA to tobramycin immobilized on a matrix. Incubation of the pre-mRNA thus immobilized allows the assembly of spliceosomes, which can be released from the matrix under native conditions by competition with tobramycin. Further density-gradient centrifugation affords highly purified spliceosomes suitable for the characterization of associated proteins by mass spectrometry as well as for studies using biochemical and biophysical methods. Although the method was developed for the preparation of spliceosomes, it is likewise applicable to the preparation of other RNP particles.

Key Words

RNA affinity tag; tobramycin; splicing; pre-mRNA; RNP; spliceosome; pre-spliceosome; aminoglycoside antibiotic; aptamer; solid-phase spliceosome assembly; glycerol-gradient centrifugation.

1. Introduction

The splicing of pre-messenger RNA (mRNA), which yields mRNA, is catalyzed by the spliceosome, which is composed of U snRNPs (U1, U2, U4/U6.U5) and a large number of proteins (for review, *see* **refs. *1–4***). The spliceosome is a highly dynamic molecular machine, which forms anew on each intron in a process termed the spliceosomal cycle. During this cycle, distinct subcomplexes, namely complexes E, A, B, and C, can be discerned in vitro (for review, *see* **ref. *3***). The A complex—also called the prespliceosome—is composed of pre-mRNA, U1 and U2 snRNPs, and a large number of additional non-snRNP proteins ***(5)***. Integration of the U4/U6.U5 tri-snRNP into the A

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complex leads to formation of the precatalytic B complex. A hallmark feature of this complex is the presence of all U snRNAs. Subsequent dynamic RNA–RNA rearrangements ***(2)*** result in the formation of the C complex, which lacks U1 and U4 snRNA. The first step of pre-mRNA splicing occurs concomitantly with formation of the C complex, and the C complex itself then catalyzes the second step. The spliceosome finally disintegrates, releasing the mRNA and intron-lariat products as RNP particles.The isolation of defined functional stages of the spliceosomal cycle is of fundamental importance for understanding the molecular biology and biochemistry of pre-mRNA splicing. Recently, a number of affinity-based selection procedures have been developed to this end. One such procedure uses a pre-mRNA into which MS2 coat protein binding sites have been inserted. An MS2:maltose binding fusion protein is then used as an affinity tag to isolate spliceosomal complexes from a solution-phase splicing reaction through reversible interaction with an amylose resin ***(6–8)***. Another approach is the immunoaffinity selection of spliceosomes with a peptide antibody directed against a protein that is known to associate with the spliceosome at a defined assembly stage. Spliceosomes can then be released form the antibody by competition with the peptide. This approach was recently shown to be a powerful tool for the isolation of functional activated mammalian spliceosomes ***(9)***. We have developed a method for affinity purification of spliceosomes by using an RNA affinity tag ***(10)***. The tag is a tobramycin-binding aptamer that is added cotranscriptionally to the pre-mRNA. The tagged pre-mRNA binds to a tobramycin matrix with high specificity and can be released by competition with tobramycin. In the method outlined below, this pre-mRNA is used in a solid-phase assembly procedure for the preparation of spliceosomes. Major advantages of the method are the fact that it uses readily available, inexpensive reagents, and the ease with which the procedure is conducted. Furthermore, the procedure can be scaled up to any desired level.

1.1. The Tobramycin RNA Affinity Tag

Generally, an RNA affinity tag for use in the isolation of native RNP particles should conform to the following criteria: (1) the sequence must be short; (2) it must have a high affinity for a given ligand immobilized on a solid support; (3) the binding of the RNA affinity tag to the immobilized ligand must be stable under the conditions of the reaction studied; and (4) the interaction of the tagged RNA must be reversible under conditions that preserve the integrity of the RNP under study. Numerous short RNA aptamers are known that bind aminoglycosidic antibiotics reversibly, with moderate to high affinities ***(10–15)***. For our studies, we focused on the 40-nt-long tobramycin-binding J6f1 RNA aptamer ***(10)*** because of its high affinity for tobramycin (K_D approx 5 n*M*) at 145 m*M* KCl and 1.5 m*M* Mg^{2+}.

The J6f1 aptamer was attached to the 3' end of the pre-mRNA by cloning it into the transcription template (**Fig. 1A** and **B**; *see* **Note 1**). The pre-mRNA thus modified was bound to a tobramycin matrix, with high specificity, through the attached tobramycin aptamer (**Fig. 1C**). Furthermore, it was efficiently processed in a standard in vitro splicing reaction (**Fig. 1D**). However, we were unable to select the pre-mRNA from the solution-phase splicing reaction by passing it over a tobramycin matrix (*see* **Note 2**). We therefore devised a solid-phase spliceosome assembly procedure (**Fig. 2A**). Here, the pre-mRNA is first immobilized on the matrix, and then spliceosome assembly is initiated. After completion of the reaction, nonspecific components are washed from the matrix and spliceosomes are released by competition with tobramycin. **Figure 2B** shows the kinetics of a typical splicing reaction taking place on the matrix. A dramatic reduction of the reaction velocity is observed in comparison with the splicing reaction in solution (**Fig. 1D**). This is inferred from the appearance of intermediates (e.g., the "exon 2-intron" lariat) and products (mRNA and intron lariat). By choosing the appropriate reaction time, large amounts of a specific spliceosomal complex can thus be prepared by scaling up the solid-phase splicing reaction. Because we were interested in the spliceosomal A complex, we chose to terminate the reaction after 45 min, well before the first appearance of intermediates at around 90 min (**Fig. 2B, lane 2**).

1.2. Fractionation of Spliceosomes by Glycerol Gradient Centrifugation

The crude eluate from the tobramycin matrix consists of a mixture of spliceosomal complexes with the A complex as the predominant species. In addition, minor nonspliceosomal contaminants are present. A further fractionation is therefore required if pure spliceosomal A and/or B complexes are to be prepared. For this purpose, the eluate from the tobramycin matrix is fractionated by ultracentrifugation on a glycerol gradient (*see* **Note 3**). This affords a clean separation of the spliceosomal A and B complexes and the lesser contaminating proteins. The assignment of the spliceosomal A and B complexes to particular fractions is done by first inspecting the RNA pattern of the individual fractions (**Fig. 5B**). The position of the spliceosomal A complex can be narrowed down further by performing psoralen crosslinking ***(16)*** on the gradient fractions (**ref. *5***; *see* **Note 4**).

2. Materials

2.1. Materials and Basic Buffers

1. Oligonucleotide primers.
2. pGEM-3Zf(+) vector (Promega).
3. pMINX clone with the MINX pre-mRNA ***(17)***.

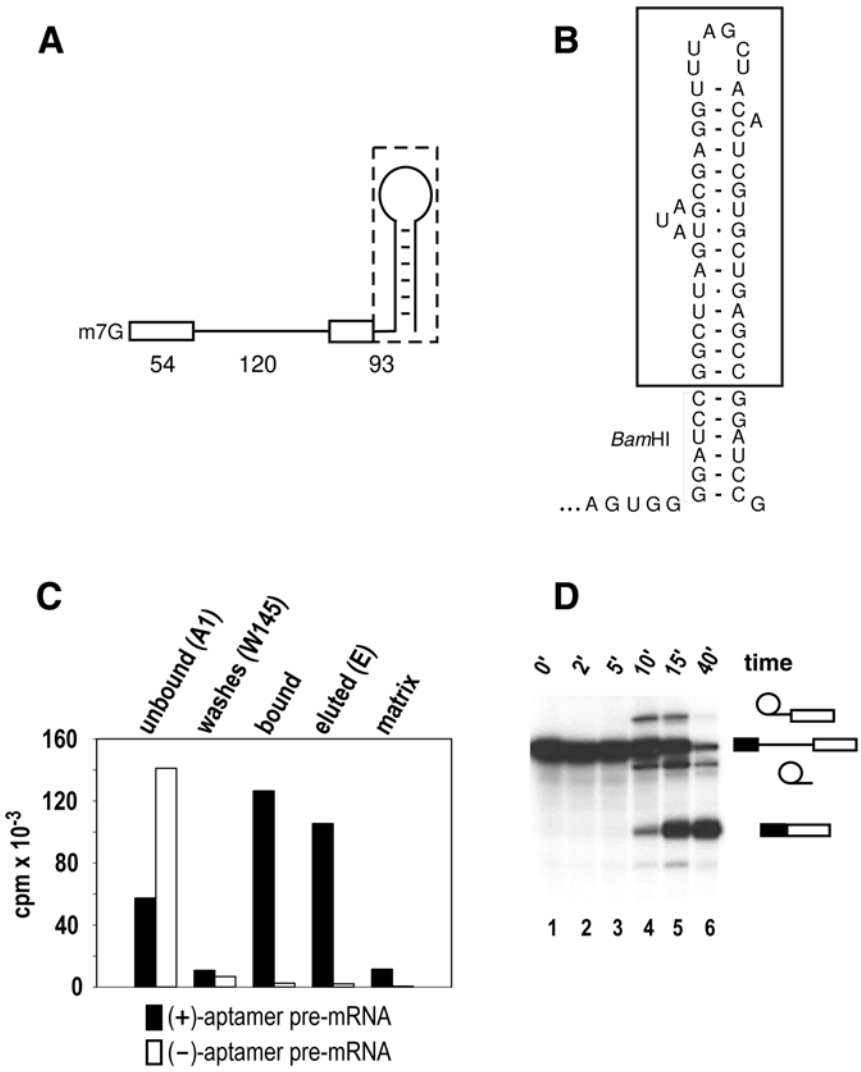

Fig. 1. **(A)**, Diagrammatic representation of the pre-mRNA. The lengths of exons (boxes) and the intron (line) are indicated. The dashed box highlights schematically the J6f1 tobramycin-binding aptamer attached to the 3' end. **(B)**, Secondary structure of the 3' end of the pre-mRNA. The tobramycin aptamer attached to the 3' end of the pre-mRNA is enclosed in the box and flanking sequences are shown. The *Bam*HI site, used to cleave the J6f1 aptamer from the transcription template (*see* **Subheading 3.1.2.**) is indicated. **(C)**, Specificity of the interaction of the tobramycin-aptamer-tagged pre-mRNA with the tobramycin matrix. The radioactivity of the fractions described in the text (*see* **Subheading 3.3.2.**) is shown. Fractions referred to as

4. Restriction enzymes, *Taq* polymerase, T7 RNA polymerase, RNase-free DNase I.
5. Agarose gel and DNA-sequencing equipment.
6. m^7GpppG (Kedar sc, Warsaw, Poland).
7. S-300 HR spin columns (Amersham Biosciences).
8. Coupling buffer: 0.2 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3 (NaOH).
9. Tobramycin (Fluka); it is dissolved in coupling buffer (buffer 8) at a final concentration of 100 m*M*. The solution is stable at –20°C.
10. NHS-activated Sepharose, fast flow (Amersham Biosciences).
11. 10% NaN_3.
12. 10 mg/mL tRNA (*Escherichia coli*; Roche).
13. 20 mg/mL Bovine serum albumin (BSA) (Roche).
14. Gradient mixer (Bio Comp).
15. HeLa cell nuclear extract, prepared according to Dignam ***(18)*** in Dignam's buffer D (20 m*M* HEPES-KOH, pH 7.9; 100 m*M* KCl; 1.5 m*M* $MgCl_2$; 0.2 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0; 0.5 m*M* dithiothreitol (DTT); 0.5 m*M* PMSF; 10% glycerol); *see* **Note 5** for a standard activity assay.
16. 0.1 *M* ATP.
17. 0.85 *M* Creatine phosphate.
18. Hybridization oven.
19. Rotating device for slow head-over-tail inversion of tubes.
20. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
21. Denaturing PAGE equipment.
22. CE buffer: 10 m*M* cacodylic acid-KOH, pH 7.0; 0.2 m*M* EDTA, pH 8.0.
23. 1 *M* Tris-HCl, pH 8.1, at 21.5°C (add 22.5 mL of 37% HCl for each 0.5 L of 1 *M* Tris base).
24. 1 m*M* HCl.
25. 1 *M* $CaCl_2$.
26. 1 *M* $MgCl_2$.
27. 0.5 *M* DTT.
28. Phosphate-buffered saline (PBS): 130 m*M* NaCl, 20 m*M* potassium phosphate, pH 8.0.
29. PBS containing 0.02% NaN_3.
30. 1% NP-40 (Nonidet P-40).
31. 10X G75: 200 m*M* HEPES-KOH, pH 7.9; 750 m*M* KCl, 15 m*M* $MgCl_2$.
32. 10% Glycerol (v/v) in 1X G75.
33. 30% Glycerol (v/v) in 1X G75.

Fig. 1. (*continued*) "bound" and "matrix" correspond to the amounts of the different RNAs that remained bound to matrix after washing and elution, respectively. **(D)**, Kinetics of the solution-phase splicing reaction of the pre-mRNA (*see* **Note 5**). The RNA was analyzed on a 10% polyacrylamide/8.3 *M* urea gel.

2.2. Buffers That Must Be Freshly Prepared Before Use

1. Blocking buffer A: 0.2 *M* $NaHCO_3$, 0.1 *M* NaCl, 1 *M* ethanolamine, pH 8.0 (HCl).
2. 4X Binding buffer (4X BP): 80 m*M* Tris-HCl (pH 8.1 at 21.5°C), 4 m*M* $CaCl_2$, 4 m*M* $MgCl_2$, 0.8 m*M* DTT (*see* **Note 6**).
3. Blocking buffer B: 1X BP, 300 m*M* KCl, 0.1 mg/mL tRNA, 0.5 mg/mL BSA, 0.01% NP-40.
4. Binding buffer: 1X BP, 145 m*M* KCl, 0.1 mg/mL tRNA.
5. 145 m*M* KCl washing buffer (W145): 1X BP, 145 m*M* KCl, 0.1% NP-40.
6. Elution buffer (E145T): 1X BP, 5 m*M* tobramycin, 145 m*M* KCl, 2 m*M* $MgCl_2$.
7. 75 m*M* KCl washing buffer (W75): 1X BP, 75 m*M* KCl, 0.1% NP-40.

3. Methods

3.1. Template Construction

The generation of the transcription template for the synthesis of the tobramycin aptamer-tagged pre-mRNA is outlined in **Subheadings 3.1.1.** and **3.1.2.** This comprises cloning of the J6f1 tobramycin aptamer into the 3' end of the pre-mRNA template and large-scale polymerase chain reaction (PCR) amplification of the transcription template suitable for synthesis of the tagged pre-mRNA (**Fig. 2**). Unless otherwise stated, all molecular–biological procedures should be performed according to **ref. *19*** or, when a commercial kit is used, according to manufacturers' instructions.

3.1.1. Cloning Procedures

The J6f1 aptamer ***(10)*** was attached to the 3' end of the pre-mRNA transcription template (*see* **Note 7**) in two steps. A pre-mRNA coding sequence was brought under the control of the T7 RNA polymerase promoter by first excising the *Eco*RI–*Bam*HI fragment from the pMINX plasmid originally described by Zillmann et al. ***(17)***. The fragment was then transferred to the pGEM-3Zf(+) vector, resulting in the pGEM-MINX plasmid. The following oligonucleotides were used to insert the J6f1 aptamer: HPV23 GATCCGGCTT AGTATAGCGAGGTTTAGCTACACTCGTGCTGAGCCGGATCCGCATG and HPV24 CGGATCCGGCTCAGCACGAGTGTAGCTAAACCTCGCTAT ACTAAGCCG containing the aptamer coding and noncoding sequences, respectively. The two oligonucleotides were annealed and cloned through their sticky *Bam*HI/*Sph*I ends into *Bam*HI/*Sph*I-linearized pGEM-MINX, yielding pGEM-MINX-T5. The correctness of the complete construct was verified by DNA sequencing.

Fig. 2. *(see opposite page)* **(A),** Outline of spliceosome assembly on the matrix. T, tobramycin. **(B)**, Kinetics of the solid-phase spliceosome assembly. The reaction was performed for the times indicated under the conditions outlined in **Subheading 3.4.** except that the reaction was scaled down by a factor of two. RNA was extracted

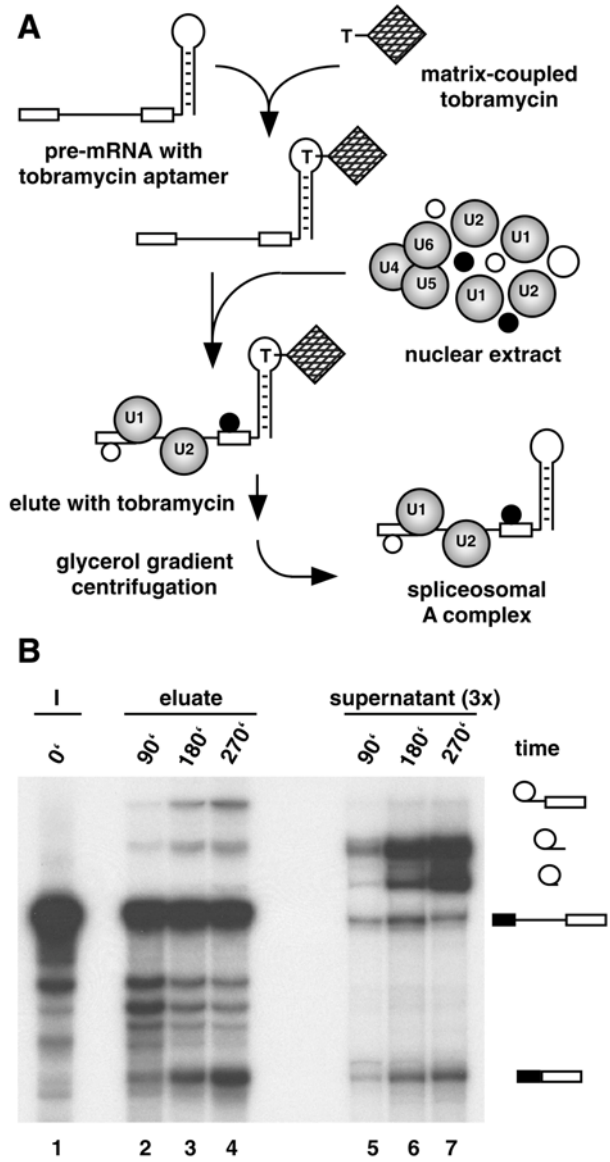

Fig. 2. *(continued)* (*see* **Subheading 3.5.**) and analyzed on a denaturing 15% polyacrylamide/8.3 *M* urea gel. To visualize the minor RNA species present in the supernatant, three times more supernatant was analyzed than eluate. The position of the pre-mRNA and splicing intermediates/products are indicated on the right. I, input pre-mRNA. (Reprinted with permission from **ref. *5***, copyright 2002, National Academy of Sciences.)

3.1.2. PCR Amplification of the Transcription Template

The transcription template for the (+)-aptamer pre-mRNA is generated in a PCR by using the pGEM-MINX-T5 template and the oligonucleotides HPV24 (*see* **Subheading 3.1.1.**) and M13f (GTAAAACGACGGCCAGT). The latter oligonucleotide primes upstream of the T7 promoter sequence. Ten 100-µL PCRs are performed (conditions: 94°C, 30 s; 30 cycles of 94°C, 30 s, 62°C, 30 s, 68°C, 30 s; final 68°C, 2 min), using 2 µg/mL template and 1 µ*M* of each oligonucleotide. The PCR product is fractionated by 1% agarose gel electrophoresis and further purified (QIAquick Gel extraction kit, Qiagen). To generate the template for the (–)-aptamer, pre-mRNA control used in **Subheading 3.3.2.**, the (+)-aptamer pre-mRNA template is cleaved with *Bam*HI (**Fig. 2A**).

3.2. RNA Synthesis

The (+)-aptamer and (–)-aptamer pre-mRNAs are synthesized with T7 RNA polymerase (MegaScript, Ambion) from the templates prepared in **Subheading 3.1.2.** Capping of the transcripts is achieved by inclusion of 5 m*M* m^7GpppG and lowering the GTP concentration to 1.5 m*M*. ATP, CTP, and UTP are each added, to a concentration of 7.5 m*M* of each, and the RNA was tracer-labeled by adding [α-^{32}P] (3000 Ci/mmol) to a concentration of 0.23 µ*M*. After transcription (approx 6 h) and DNase I treatment, transcripts are purified by overnight precipitation at –20°C in the presence of 2.5 *M* LiCl and subsequent spin-column chromatography (S-300 HR column, Pharmacia). The RNA is dissolved at approx 10 pmol/µL in CE buffer and stored at –20°C.

3.3. Preparation and Testing of the Tobramycin Matrix

Subheading 3.3.1. details the preparation of the tobramycin matrix in an adaptation of the protocol by Wang and Rando ***(11)***, and **Subheading 3.3.2.** outlines the procedure that is used to test the binding activity of the matrix with the two RNAs prepared according to **Subheading 3.2.**

3.3.1. Preparation of the Tobramycin Matrix

Freshly prepare blocking buffer A (buffer 1, **Subheading 2.2.**). The procedure is conveniently performed in a 12-mL capped tube, and centrifugation is performed in a minifuge. All manipulations are performed at 4°C unless otherwise stated.

1. Wash 2 mL (packed volume) NHS-activated Sepharose (fast flow) four times with 9 mL of 1 m*M* HCl by gentle resuspension, brief centrifugation at 250*g* (1 min), and by carefully decanting the supernatant.
2. Prepare 1 mL of a 5-m*M* tobramycin solution by mixing 50 µL of tobramycin in coupling buffer with 950 µL of coupling buffer. Then add this mixture to the matrix (*see* **Note 8**). Incubate by head-over-tail rotation overnight.

3. Centrifuge at 250*g* (5 min), remove the supernatant, and add 8 mL of blocking buffer A. Incubate by head-over-tail rotation for 2 h at room temperature.
4. Centrifuge at 250*g* (5 min), decant the supernatant, and wash three times with PBS and twice with PBS containing NaN_3. Store the tobramycin matrix in 2 mL of PBS containing NaN_3 at 4°C. The tobramycin matrix is stable for approx 3 mo.

3.3.2. Testing of the Tobramycin Matrix

This section describes the procedure for testing the tobramycin matrix. Furthermore, the specificity of the interaction between the tobramycin aptamer on the RNA and the matrix-bound tobramycin is assayed. It is crucial for the solid-phase splicing procedure that the RNA only binds through the tobramycin aptamer to the tobramycin on the matrix, and that levels of non-specific interactions are below background (*see* **Note 6** concerning the importance of pH in the binding). To test this, the (+)- and (–)-aptamer substrates prepared in **Subheading 3.2.** are required. Specificity is only given when the (+)-aptamer RNA binds and the (–)-aptamer RNA fails to bind.

Buffers 2–6 (*see* **Subheading 2.2.**) should be freshly prepared. The procedure comprises the following: preblocking the tobramycin matrix (hereafter referred to as matrix) with tRNA and BSA (**steps 1** and **2**); binding of the RNA and subsequent washing of the matrix to remove nonspecifically bound RNA (**step 3**); and finally elution of the bound RNA (**step 4**). RNA is traced by counting the radioactivity in the various fractions and the experiment is performed in duplicate for each of the two RNAs. All steps are performed at 4°C.

1. Take an aliquot of the matrix corresponding to 15 µL of packed volume of beads for each assay and collect the beads by centrifugation.
2. Block the matrix by resuspension in 250 µL of blocking buffer B with head-over-tail rotation overnight.
3. For one matrix aliquot, prepare 400 µL of binding buffer containing 60–80 pmol of RNA (*see* **Note 9**). Save 5 µL (sample A0) and then add the mixture to the collected matrix. Incubate by head-over-tail rotation for 1–1.5 h, collect the matrix, save 5 µL of the supernatant (sample A1), and then wash the matrix three times with 1 mL of the W145 buffer. Save the supernatants of the washes in one tube (sample W145). Determine the amount of RNA bound to the matrix.
4. To elute the bound RNA, add 50 µL of E145T elution buffer to each matrix aliquot and incubate by head-over-tail rotation for 10 min, then collect the matrix by centrifugation. The supernatant (sample E) contains the eluted RNA. Determine the amount of RNA left on the matrix.

Count all fractions saved (samples A0, A1, W145, and E) and determine the proportion of RNA in each. Specificity with the above RNAs (i.e., (+)-aptamer vs (-)-aptamer pre-mRNAs) has been achieved when 60–70% of the tagged RNA, but less than 2% of the untagged RNA, is bound to the matrix (**Fig. 1C**). Furthermore, more than 80% of the immobilized RNA must be released upon elution in order for the solid-phase assembly scheme to work.

3.4. Solid-Phase Splicing

Buffers 2–7 (*see* **Subheading 2.2.**) must be freshly prepared. The procedure for isolation of spliceosomes by splicing on immobilized pre-mRNA is subdivided into three steps: production of the matrix-bound pre-mRNA (**step 1**); initiating and performing the splicing reaction (**step 2**); and washing the matrix and elution of the matrix assembled spliceosomes (**step 3**). During the whole procedure, small aliquots are saved to monitor the RNA content of each fraction.

Because the (–)-aptamer pre-mRNA does not bind to the matrix under the conditions outlined in **Subheading 3.3.2.**, a mock incubation with this pre-mRNA has no value. However, to control for nonspecific binding of nuclear extract components to the matrix during the procedure, it is essential to perform a mock assembly by omitting the pre-mRNA in **step 1** (**Fig. 3**). The steps of the procedure are as follows:

1. Prepare the matrix-bound pre-mRNA exactly as detailed in **Subheading 3.3.2., steps 1–3**. For a standard experiment, 4X 15 µL matrix-bound pre-mRNA is prepared (*see* **Note 9**). The same amount of matrix material is required for a mock assembly. Each aliquot is sufficient for one 1.5-mL splicing reaction (*see* **Note 10**).
2. On ice, prepare a 6-mL splicing mix, consisting of 35% HeLa cell nuclear extract (*see* **Note 9**) supplemented with 25 m*M* KCl, 3 m*M* $MgCl_2$, 2 m*M* ATP, and 20 m*M* creatine phosphate. Gently mix and distribute 1.5 mL onto each matrix aliquot. Fix the tubes securely to the rotating wheel of a hybridization oven set at 30°C. Incubate for the required time with rotation (approx 150 rotations/min); a 45-min incubation is sufficient to allow the assembly of large amounts of spliceosomal A complex (*see* **Note 11**).
3. At the end of the incubation the reaction mixtures are placed on ice. All further operations are performed at 4°C. The matrix is collected and the reaction supernatant is saved (fraction SN). The matrix is now washed three times with 750–800 µL aliquots of W75 buffer. For ease of elution the matrix aliquots are combined in pairs during the first and second washes, by adding the W75 buffer to only one tube and transferring the matrix material together with the W75 buffer to its twin tube. The washes are saved separately (W75, fractions 1–3). Assembled spliceosomes are released by adding 250 µL of E145T elution buffer to the single matrix aliquot and further incubation with head-over-tail rotation for 10 min at 4°C. The matrix is collected and the supernatant containing the assembled spliceosomes (fraction E) is used for further analysis (*see* **Subheading 3.5.**) and fractionation (*see* **Subheading 3.6.**).

Aliquots are withdrawn from all fractions and their radioactivity is measured. The amount of RNA in each fraction is determined. It is convenient to prepare a balance sheet listing the volumes next to amounts of RNA in each fraction.

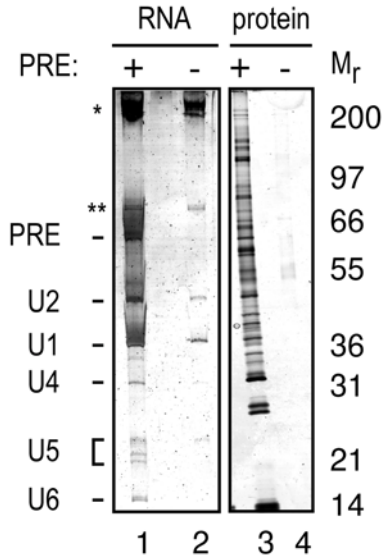

Fig. 3. RNA and protein content of splicing complexes eluted with tobramycin. Solid-phase splicing was performed for 45 min at 30°C in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of pre-mRNA, and the complexes were subsequently eluted with tobramycin. RNA and protein were recovered, analyzed by PAGE, and visualized by silver staining. (Reprinted with permission from **ref. *5***, copyright 2002, National Academy of Sciences.)

A comparison of the eluates from two parallel assembly reactions, in the presence of the same matrix but with and without pre-mRNA, is shown in **Fig. 3**. This demonstrates clearly the high level of purity obtained in the eluted spliceosomes. **Figure 4** shows the RNA (**A** and **B**) and protein (**C**) analysis of the fractions collected during the actual purification procedure: the reaction supernatant (SN), the 75-m*M* KCl washes (W75), and the eluate (E).

3.5. Protein and RNA Analysis

1. Aliquots of equal volume are taken from the reaction supernatant (SN), the three washes (W75 1–3), and the eluate (E). The volume to be taken is chosen in such a way that approx 1 pmol RNA is withdrawn from the eluate. After one phenol/chloroform extraction, the RNA is precipitated from the aqueous phase by adding ethanol, and the protein from the organic phase by adding acetone.
2. The RNA is analyzed by denaturing PAGE on a 15% polyacrylamide/8.3 *M* urea gel and the protein by SDS-PAGE on a 10%/13% step gel. Both RNA and protein gels are stained with silver ***(20)*** and dried. The RNA gel is also autoradiographed.

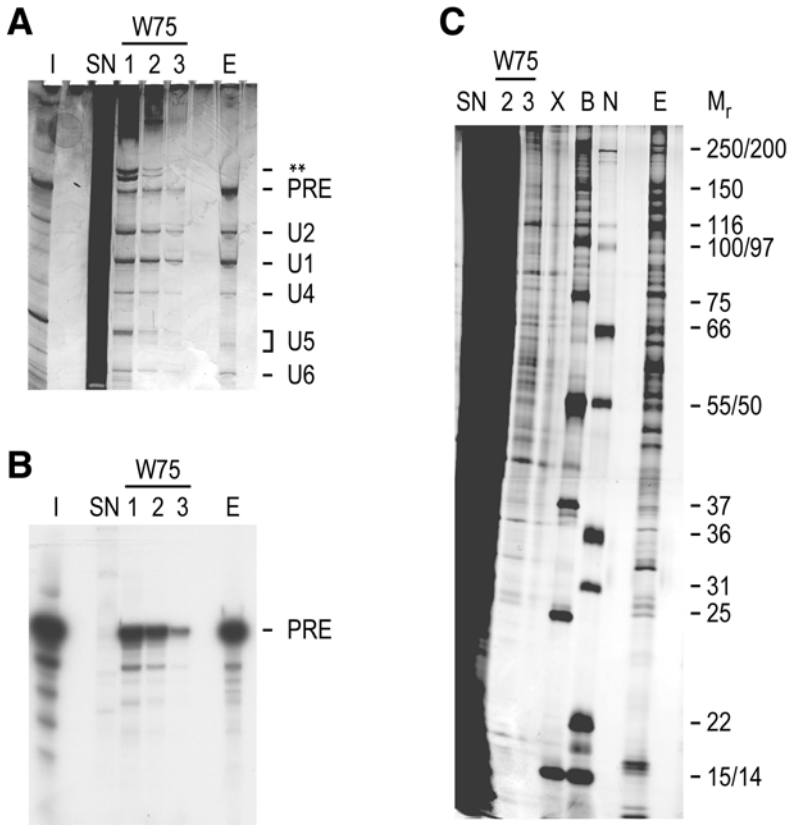

Fig. 4. Purification of spliceosomes assembled on the matrix. The gels show the RNA (**A,** silver stain), pre-mRNA (**B,** autoradiography of **A**), and protein (**C**) analysis of the different fractions in the course of purification of spliceosomes. I, input pre-mRNA (A0; *see* **Subheading 3.3.2.**). SN, reaction supernatant; W75, 75 m*M* washes 1, 2, and 3; E, eluate. In **(C)**, the W75 first wash was not analyzed, and the lane marked X is spillover from W75 fraction 3. B, Bio-Rad broad-range molecular-weight marker (250, 150, 100, 75, 50, 37, 25, and 15 kDa); N, Novex Mark12 wide-range protein standard (200, 116, 97, 66, 55, 36, 31, 22, and 14 kDa).

3.6. Glycerol-Gradient Fractionation of Assembled Spliceosomes

The assembled spliceosomes present in the eluate are further fractionated by glycerol-gradient centrifugation to separate the different spliceosomal complexes (*see* **Note 3**). One gradient is used to analyze the eluate from one assembly reaction (6 mL). Eluate (220 μL) is loaded onto each gradient, and the remainder is used for protein/RNA analysis (*see* **Subheading 3.5.**). We use the

Sorvall TH660 rotor (equivalent to the Beckman SW60 rotor), with a nominal capacity of 4.2 mL per tube. All operations are performed at 4°C.

1. First add DTT, to a final concentration of 0.5 m*M*, to aliquots of the 10% and 30% glycerol solutions, and then use these to prepare a gradient in a BioComp gradient mixer (SW60, 10–30% v/v, glycerol program). Let the gradient equilibrate at 4°C for at least 1 h.
2. Remove the rubber cap from the gradient tube, remove 220 μL from the top of the gradient and then carefully layer 220 μL of the eluate on top of the gradient. Centrifuge for 1 h 45 min at 448,579*g*.
3. Manually fractionate the gradient from top to bottom in 175-μL fractions.
4. Use 100 μL of each fraction for first determining the RNA content by measuring the cpm and then, with the same aliquot, for the protein/RNA analysis described in **Subheading 3.5.** The remaining 75 μL of each fraction can be used for psoralen crosslinking analysis ***(5)***.
5. For the gradient profile, plot the quantity of RNA against the fraction number.

A typical gradient profile is show in **Fig. 5A**, and the RNA distribution across the gradient is shown in **Fig. 5B**. Fractions 9–13 contain exclusively pre-mRNA, U1, and U2 snRNA. Through our previous psoralen crosslinking analysis, fractions 11–13 could be assigned unequivocally to the A complex ***(5)***. These fractions contain only pre-mRNA, U1, and U2 snRNA in approximately equal stoichiometric amounts. The spliceosomal B complex migrates in fractions 16–18. It is readily apparent from **Fig. 5B** that these fractions contain equal stoichiometric amounts of pre-mRNA, U1, U2, U4, U5, and U6 snRNAs, a hallmark of the intact spliceosomal B complex.

4. Notes

1. Depending on the experimental context, the aptamer may have to be inserted at different positions in the RNA under study. Although we had variable success when it was inserted into positions within the intron of the pre-mRNA (H.-P. Vornlocher, unpublished), a 5' position was found to be optimal for the isolation of the mRNP from a glycerol-gradient-fractionated solution-splicing reaction similar to the one shown in **Fig. 1D** (C. Merz, personal communication).
2. The inability to affinity-select spliceosomes from a solution reaction may be owing to masking of the aptamer tag and/or the tobramycin matrix by factors present in the nuclear extract. We observed that the affinity selection is somewhat improved (15% recovery) after fractionation of the splicing reaction mixture either by glycerol-gradient centrifugation or by Superdex 200 (Amersham Biosciences) column chromatography (C. Merz, personal communication).
3. In principle, any procedure based on separation by size could be used. However, gel filtration on Sephacryl S500 ***(21)*** was impracticable owing to the dilution effect of the sample (not shown) and because the A, B, and C complexes migrate as a single peak. Furthermore, the spliceosomal A complex was found to be unstable when subjected to size-exclusion chromatography on Sephacryl S400 ***(7)***.

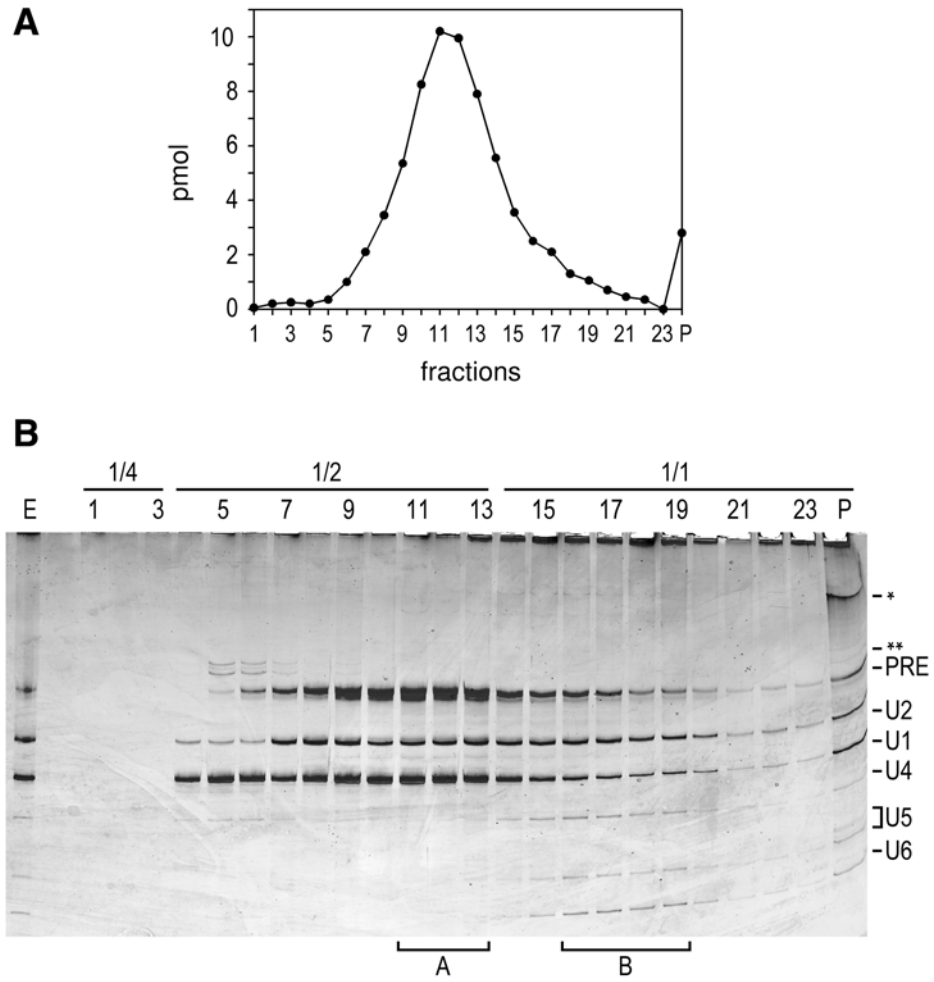

Fig. 5. Gradient fractionation of spliceosomes assembled on the matrix. **(A)**, Distribution of the pre-mRNA, expressed as pmol, across the gradient. The fraction numbers are indicated above the lanes; P, pellet fraction. **(B)**, RNA composition of the gradient fractions. Above the lanes, the amount of RNA analyzed from the total RNA recovered from a 100-μL gradient fraction is expressed as a the fraction of the total. Fractions corresponding to the spliceosomal A and B complex are indicated at the bottom. The RNA species are labeled on the right. Contaminating RNAs are indicated with asterisks.

4. Psoralen crosslinking is used to detect RNA–RNA interactions diagnostic for a particular spliceosomal complex. A description of the method is outside the scope of the present chapter on the isolation of spliceosomes, and the reader is referred to **ref. *5*** for details.
5. For a standard splicing reaction in vitro, we use protocol 7 of **ref. *22***. As this protocol is exhaustive, it is not reproduced here in detail. Briefly, pre-mRNA synthesis is essentially as described in **Subheading 3.2.**, except that (1) the concentration of cold UTP is lowered to 100 μ*M*; (2) [α-^{32}P]UTP is added to 0.825 μ*M* in a 20-μL transcription reaction, and (3) the transcript is gel-purified on a 9.6% polyacrylamide/8.3 *M* urea gel. A 100-μL in vitro splicing reaction is set up essentially as described in **Subheading 3.4.**, **step 2**, except that pre-mRNA is added to a concentration of 50–60 fM and incubation takes place at 30°C without agitation. For each time point in **Fig. 1D** a 15-μL aliquot is withdrawn after the specified time and placed on ice. After treatment with proteinase K ***(19)***, RNA is recovered by phenol/chloroform extraction and analyzed as in **Subheading 3.5., step 2**.
6. The most critical parameter for obtaining specificity in binding is the correct pH. The aminoglycosidic antibiotic tobramycin itself is positively charged at neutral pH ***(23–26)***. The pK_a values of the primary amino groups are (positions in brackets; refs. ***23*** and ***24***): 7.6 (6'), 8.6 (2'), 6.2 (3), 7.4 (1), 7.4 (3"). The molecule is only close to neutrality at a pH of around 9. Hence a basic pH is required to suppress non-specific binding of the RNA to matrix. The Tris buffer used (pH 8.1 at 21.5°C) will attain the correct pH upon equilibration at 4°C in the blocking, binding, washing and elution buffers (buffers 2–7, **Subheading 2.2.**). To facilitate handling, a 4X BP without DTT may be prepared and kept at 4°C. DTT (2 m*M*) is then added just before use.
7. In the context of the RNA under study, it is essential that the tobramycin aptamer folds correctly. Partial complementarities with other regions of the RNA are best excluded by using the RNA folding program "mfold" (at http://www.bioinfo.rpi.edu; **refs. *27*** and ***28***). All calculated substructures should have the aptamer correctly folded.
8. A number of different tobramycin concentrations, ranging from 0.05 to 10 m*M*, were tested in the matrix coupling reaction. Concentrations below 5 m*M* dramatically reduced the binding capacity of the aptamer-RNA to the matrix, probably because of a reduced coupling efficiency of tobramycin to the matrix. Although higher concentrations somewhat improved the binding efficiency of the aptamer-RNA, they were not used, because we reasoned that too many binding sites on the matrix could lead to a molecular crowding effect and thus compromise the assembly of macromolecular structures such as spliceosomes.
9. The three critical parameters for the solid-phase assembly of spliceosomes are (1) the RNA concentration on the matrix, (2) the HeLa cell nuclear extract concentration, and (3) the time of incubation at 30°C (*see* **Note 11**). The values given for each parameter are derived mainly from optimization experiments. In the solid-phase assembly procedure, a HeLa cell nuclear extract should be used that

processes at least 80% of the pre-RNA when assayed in a standard solution splicing reaction (*see* **Fig. 1D** and **Note 5**) at an optimized concentration. Using this condition (35% nuclear extract in most cases), it was found that the optimal pre-mRNA concentration is 22–25 n*M*, that is, 33–38 pmol of pre-mRNA bound to a 15-µL matrix aliquot. Not all pre-mRNA added is eventually bound to the matrix and that minor variations in binding efficiency is observed in different tobramycin matrix preparation. Hence, the amount of pre-mRNA to use in the initial binding (60–80 pmol) has to be calibrated experimentally to the desired value of 22–25 n*M* in the final reaction. A lower amount of RNA (10 or 20 pmol/15-µL bead aliquot) affects the overall yield of spliceosomes, whereas a higher amount (50–75 pmol/15-µL bead aliquot) affects the yield of spliceosomal B complex.

10. The amount of splicing mix added to one 15-µL bead aliquot can be decreased. However, when using less than 1 mL of splicing mix/15-µL bead aliquot the overall yield of spliceosomes was found to be reduced.
11. It is essential that the splicing reaction *per se* has not yet started when the aim is the preparation of the spliceosomal A and/or B complexes. In the glycerol gradient the C complex would migrate close to the B complex and the mRNP close to the A complex (K. Hartmuth, unpublished data).

Acknowledgments

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5

Using Single-Strand Conformational Polymorphism Gel Electrophoresis to Analyze Mutually Exclusive Alternative Splicing

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Summary

Single-strand conformational polymorphism analysis has been used successfully to identify single nucleotide changes within sequences based on the fact that multidetection enhancement gels will separate molecules based on their conformation rather than their size. We have expanded the utility of this technique to analyze easily the alternative splicing of pre-mRNAs containing multiple mutually exclusive exons of the same size. We have used this technique to study the *Caenorhabditis elegans let-2* gene containing two alternative exons and the *Drosophilia melanogaster Dscam* gene, which contains 12 mutually exclusive exons. The ease and the quantitative nature of this technique should be very useful.

Key Words

Alternative splicing; single-strand conformational polymorphism (SSCP); exons.

1. Introduction

Many genes contain mutually exclusive, alternatively spliced exons. The vast majority of such genes have only two alternative exons. Some genes, however, can contain considerably more. For instance, the *Drosophila Dscam* gene contains a cluster of 48 mutually exclusive exons ***(1)***. In most cases, adjacent mutually exclusive exons are nearly identical in size and most likely arose by tandem exon duplication ***(2)***. Alternative splicing is commonly examined by performing reverse-transcription polymerase chain reaction (RT-PCR) with primers complementary to the flanking constitutive exons and resolving the products on either an agarose gel, or a denaturing polyacrylamide gel. How-

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ever, these approaches are usually not adequate to analyze the alternative splicing of messenger RNAs (mRNAs) derived from genes containing mutually exclusive exons because the alternative exons are similar or identical in size.To analyze the alternative splicing of pre-mRNAs encoded by this class of genes, we have developed a method that uses single-strand conformational polymorphism (SSCP) gel electrophoresis. SSCP gel electrophoresis separates DNA molecules based on their conformation rather than their size ***(3)***. This technique frequently is used to detect single nucleotide polymorphisms that cause human diseases. Here, we describe the use of this method to separate RT-PCR products derived from mRNA isoforms encoded by genes containing mutually exclusive alternatively spliced genes. Although this technique can be used for any gene, results obtained for the *Drosophila Dscam* gene and the *Caenorhabditis elegans let-2* gene will be described as examples.

2. Materials

2.1. Equipment

The equipment required for these experiments is typically available in most labs that routinely perform molecular biology experiments.

1. Temperature-controlled power supply. The migration of DNA fragments is exquisitely sensitive to temperature. It is therefore strongly advised that a power supply unit with a temperature control feature be used. We have had much success using the Bio-Rad Power Pac 3000.
2. Electrophoresis unit. The SSCP gels are run as vertical gels. We typically use sets of 3/16"-thick glass plates that are 20 × 45 cm although for some applications 20- × 20-cm glass plates are suitable. One plate in each set should be notched. All gels are assembled using 0.4-mm spacers and sharkstooth combs. These gels can be run in any of a number of electrophoresis units, such as those available from Owl Scientific or Harvard Biological Laboratories Machine Shop. In addition, the gels should be run with a 1/8"-thick aluminum plate clamped to the front of the gel to facilitate cooling.
3. Thermal cycler. This is required for PCR but can also be used for other reaction incubations if needed.
4. Gel drying equipment. The SSCP gels must be dried before exposure to film or phosphorimagery. Therefore, a suitable gel dryer and vacuum pump will be needed. In addition, Whatman 3MM paper backing and plastic wrap will be needed.
5. Phosphorimager. This is necessary if you wish to quantitate the results.

2.2. Supplies

1. Electrophoresis chemicals. Mutation Detection Enhancement Gel Solution 2X concentration (Cambrex, cat. no. 50620), *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED), APS and TBE.

2. Basic molecular biology reagents. Thermostable DNA polymerase (i.e., *Taq*), reverse transcriptase (i.e., Superscript II; Invitrogen), dNTPs, T4 polynucleotide kinase, γ-[^{32}P]ATP, and manufacturer-supplied reaction buffers.
3. DNA. Oligonucleotides used for the RT-PCR reactions must be synthesized. We have had much success using oligos that have been desalted but not HPLC purified or gel purified.
4. Cloning vector. To easily clone the RT-PCR products, we use the pCRII-TOPO-TA vector from Invitrogen.

2.3. Solutions

1. TBE: For 20 L of a 5X stock, use 605.7 g Tris, 292.5 g boric acid, and 37.2 g ethylenediamine tetraacetic acid (EDTA).
2. Stop solution: 95% formamide, 10 m*M* NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol.
3. Elution buffer: 300 m*M* sodium acetate, pH 6.1, 0.2% sodium dodecyl sulfate, 1 m*M* EDTA.

3. Methods

The methods described below discuss (1) the isolation of total RNA, (2) performing reverse transcription reactions, (3) 5' end labeling of an oligonucleotide primer, (4) using PCR to generate products labeled with [^{32}P] on one strand only, (5) separating these PCR products on SSCP gels, and (6) determining the identities of each band on the gel.

3.1. RNA Collection

Total RNA can be collected from your desired source (whole organisms, dissected tissue, cultured cells, etc.) by using any procedure that yields intact RNA. We typically use a LiCl/Urea method for isolating RNA from *Drosophila* ***(4)***, and Trizol™ reagent (Invitrogen) for isolating RNA from cultured cells.

3.2. Reverse Transcription

Once the RNA has been isolated, it must be converted into cDNA before amplification by PCR. Although this could be performed by using either an oligo-dT primer or a gene-specific primer, we have had the most consistent success using random hexamer primers, as indicated below:

1. Set up a 20-µL reaction containing 1 µL of RNase inhibitor (Invitrogen), 1 µL of random hexamers (265 ng/µL stock), 10 µL RNA (5 µg total RNA and H_2O to volume), 4 µL First Strand Buffer (provided with Superscript II), 2 µL dithiothreitol (0.1 *M*), 1 µL dNTPs (10 m*M*), and 1 µL Superscript II (Invitrogen).
2. Incubate the reaction at 42°C for 1 h.
3. The reaction can then be immediately used as a template for PCR (*see* **Subheading 3.4.**) or stored at –20°C indefinitely.

3.3. End-Labeling Primer

To visualize the PCR products on the SSCP gel, the DNA must be radiolabeled. More specifically, only one strand of the DNA should be labeled as the top and bottom strands will have different conformations and therefore will migrate differently in the SSCP gel (*see* **Note 1**).

1. Assemble a 20-µL reaction containing 1 µL 10 µ*M* oligonucleotide, 4 µL 5X forward reaction buffer, 2 µL γ–[^{32}P]ATP (3000 Ci/mmol; 10 mCi/mL), 12µL H_2O, and 1 µL T4 polynucleotide kinase.
2. Incubate the reaction at 37°C for 30 min.
3. While the kinase reaction is incubating, prepare a gel-filtration column that will be used to remove the unincorporated nucleotides. Add approx 1.5 mL of a 50% slurry of Sephadex G-25 (Sigma) that has been swollen in TNE (10 m*M* Tris-HCl, pH 8.0; 50 m*M* NaCl; 0.5 m*M* EDTA) into a disposable chromatography column (Bio-Rad, cat. no. 732-6008).
4. Snap off the bottom stopper from the column, place the column in a 1.5-mL Microfuge tube and allow the excess buffer to flow out of the column by gravity by lightly packing the column.
5. Once the buffer has stopped coming out, place a 1.5-mL microcentrifuge tube on the bottom of the column and place the column and microcentrifuge tube into a 50-mL conical tube and spin at 50*g* for 2 min in a Sorvall ST-H750 rotor to pack the column.
6. Remove the buffer that eluted from the column and centrifuge the column at 675*g* for 2 min to remove any residual buffer.
7. Once the kinase reaction is complete, add 30 µL of TNE to the reaction and carefully place the entire volume of the kinase reaction on the bed of the column.
8. Centrifuge the column at 675*g* for 4 min. The eluate contains the [^{32}P]-labeled primer that will be used directly in **Subheading 3.4.** below.

3.4. PCR

Once the reverse transcription reaction (*see* **Subheading 3.2.**) is complete and the primer is end-labeled (*see* **Subheading 3.3.**), PCR is performed to generate products specifically labeled on one strand. Our PCR reactions were typically assembled in 0.2- or 0.5-mL tubes as described below. However, reaction conditions should be optimized on a case-by-case basis.

1. Assemble a 50-µL reaction containing 3 µL cDNA from **Subheading 3.2.** or a 25 ng/µL solution of a cDNA clone from **Subheading 3.6.**, 1 µL dNTPs (10 m*M*), 1 µL of 5'-end-labeled primer from **Subheading 3.3.**, 4 µL 5' primer (2 µ*M*), 4 µL 3' primer (2 µ*M*), 5 µL 10X PCR buffer without $MgCl_2$, 1.5 µL $MgCl_2$ (50 m*M*), 0.25 µL *Taq* DNA polymerase (1 U/µL), and 30.25 µL H_2O.
2. The reactions are then cycled 35 times at 94°C for 45 s, 55°C for 45 s, 72°C for 1 min (extension time is determined as 1 min/kb), followed by a 3-min incubation at 72°C.

3.5. SSCP Gel

The next step is to run the SSCP gel. This involves the following three steps: (1) pouring the SSCP gel, (2) preparing the samples for electrophoresis, and (3) running the gel.

3.5.1. Pouring the SSCP Gel

1. As with all acrylamide gels, it is important to use very clean glass plates. After thoroughly cleaning the glass plates, treat the notched plate with GelSlick (Cambrex, cat. no. 50640), followed by a further washing with ethanol.
2. Assemble the plates with 0.4-mm spacers and clamp together.
3. For a 20-cm by 45-cm gel, prepare 50 mL of gel solution containing 12.5 mL of MDE solution, 6 mL of 5X TBE, 31.5 mL of H_2O, 200 μL of 10% ammonium persulfate, and 20 μL of TEMED.
4. Pour the gel solution into the gel plates with care being taken to avoid trapping bubbles inside.
5. Once the glass plates are filled with the gel solution, insert a sharkstooth comb upside down to form a flat surface that will be used to generate the wells.
6. Let the gel polymerize for 1 h.
7. Remove the clamps, bottom spacer, comb, and rinse the well with 0.6X TBE to remove any gel fragments or unpolymerized acrylamide.
8. Wash off the comb and insert into the gel with the teeth facing the gel surface. Insert the comb approx 1 mm into the gel.
9. Clamp the gel to the electrophoresis assembly and fill the top and bottom buffer tanks with 0.6X TBE. Be certain to remove any air bubbles from underneath the gel.

3.5.2. Preparing the Samples

1. Combine 10 μL of SSCP stop solution with 2 μL of the PCR from **Subheading 3.3.**
2. Heat the reactions at 100°C for 2 min to denature the DNA.
3. Place the reactions immediately on ice until ready to load gel.
4. Carefully load 1.5 μL of each sample into the wells created by the sharkstooth comb.

3.5.3. Running the SSCP Gel

One of the most important variables influencing how the PCR products will migrate in the gels is temperature (*see* **Note 2**). Therefore, it is important to use a power supply that monitors the gel temperature and allows for gels to be run at a constant temperature. Although the optimal temperature must empirically be determined on a case-by-case basis, we have found that 25°C and a maximum power of 8 W works well for many applications. In addition, the length of the electrophoresis run will depend on both the size of the DNA fragments being analyzed and their degree of separation. To analyze *Dscam* alternative splicing, where there are 12 different PCR products that are approx 500 bp, the

gels are run for 24 h (**Fig. 1**). To analyze *let-2* alternative splicing, where there are only two PCR products that are approx 150 bp, the gels are run for 6 h (**Fig. 2**).

3.6. Determining the Identity of Bands

An important step when using this SSCP technique is determining where each product will run within the gel under the chosen conditions (*see* **Note 3**). Unfortunately, there is no *a priori* way of predicting the mobilities of different PCR products and this must therefore be performed by generating a set of standards or markers. This can be accomplished by two different methods. First, PCR products obtained using the primers used in **Subheading 3.4.** can be cloned in bulk and individual clones sequenced until one of each variant is obtained. Alternatively, after resolving the RT-PCR products on an SSCP gel, each band can be excised from the gel, amplified by PCR, cloned, and sequenced. Once cDNA clones representing each variant are obtained, they can be used as PCR templates and the resulting products will serve as migration markers on the SSCP gels. Below is a protocol for cloning the DNA bands from the gel.

1. Align a film to the dried gel to determine the location of each band. Mark the band with a pen.
2. With a scalpel blade, excise the gel fragments along with the 3MM Whatman paper backing.
3. Soak the gel slice in 500 µL of elution buffer overnight at room temperature.
4. Briefly centrifuge the tubes to collect the excised Whatman paper and gel in the bottom of the tube. Remove the elution buffer to a separate tube.
5. Precipitate the DNA with 2.5 vol of ethanol and 1 µL of 1 mg/mL glycogen as a carrier. Wash the pellet with 75% ethanol and resuspend the pellet in 10 µL of H_2O.
6. Amplify the DNA fragments isolated from the gel by PCR using 1 µL of the resuspended DNA as a template in reactions identical to those described in **Subheading 3.3.**
7. Clone the PCR products into the pCRII-TOPO-TA vector from Invitrogen as described by the manufacturer.
8. Sequence individual clones to determine their corresponding spliced isoform.
9. Use the cloned cDNAs as templates in PCR (**Subheading 3.3.**) to generate mobility standards for the SSCP gels.

4. Notes

1. For reasons unknown to us, we have always found that PCR products in which the 5' primer is labeled produce much sharper bands than products in which the 3' primer is labeled. However, we suggest that separate reactions should initially be performed in which either the 5' or 3' primers are labeled to determine which one produces a more satisfying pattern on the gel.
2. If the temperature of the room you are running the gel in is close to the temperature limit you set the power supply to, it is possible that once the gel reaches the

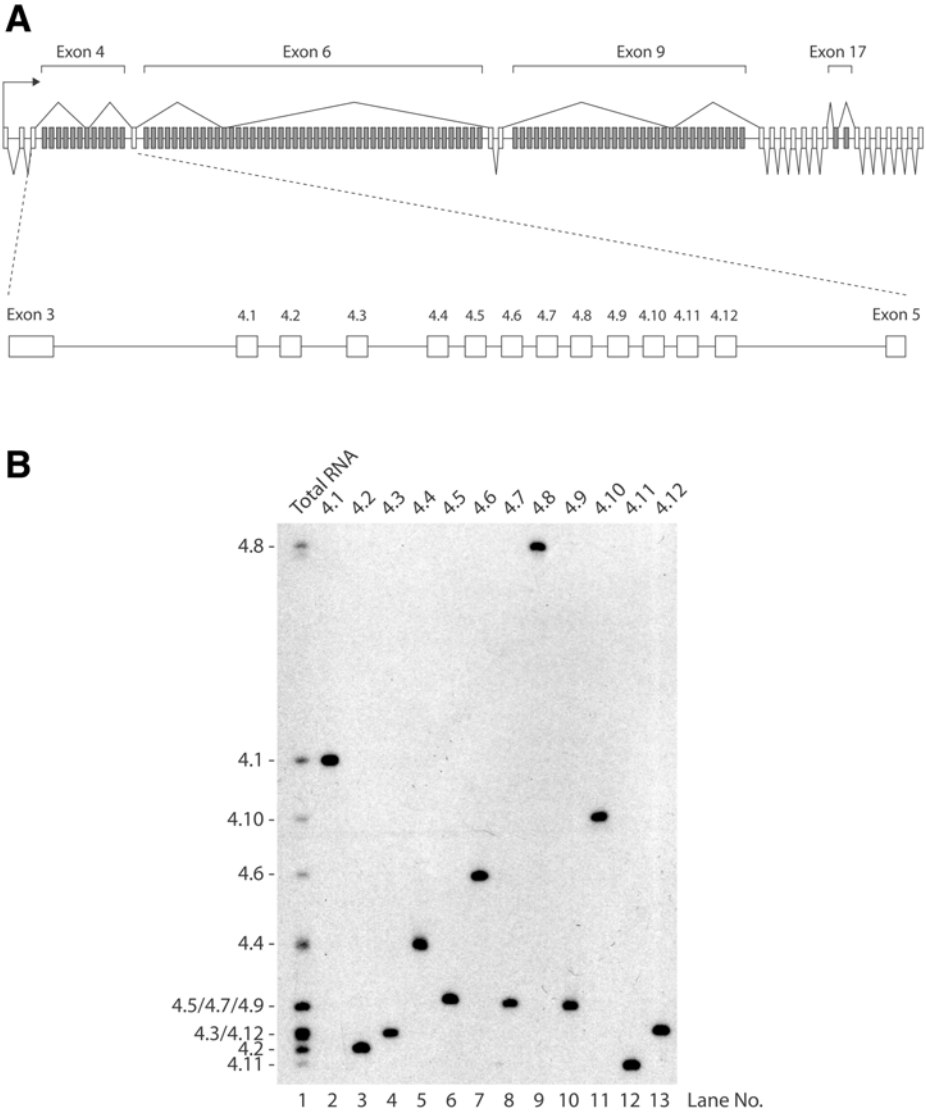

Fig. 1. Example of an SSCP gel used to analyze alternative splicing of the *Drosophila Dscam* pre-mRNA. **(A)**, The organization of the *Dscam* gene and a larger view of the exon 4 cluster. **(B)**, Lane 1, RT-PCR reaction using total RNA from an adult fly as a template. Lanes 2–13, mobility standards generated by PCR reactions using *Dscam* cDNA clones containing the indicated exon 4 variant as templates. Reprinted from **ref. *4*** with permission from the Genetics Society of America.

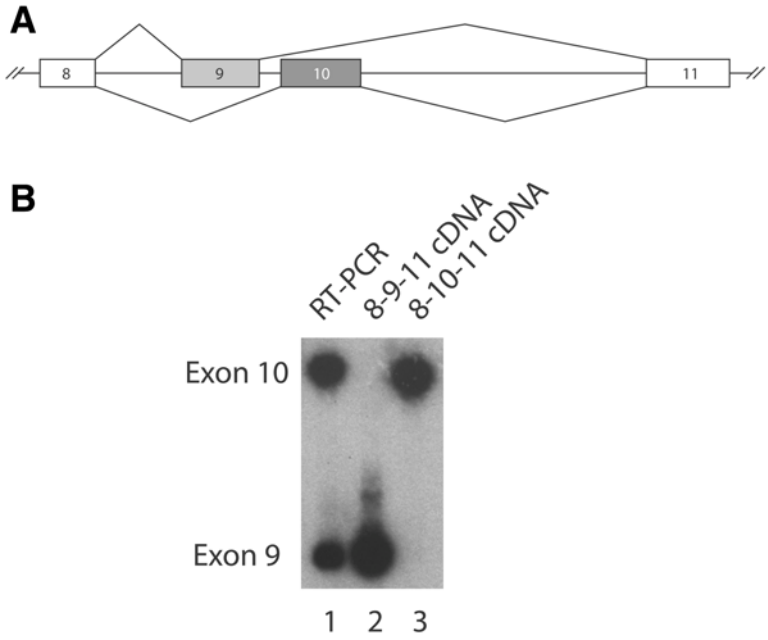

Fig. 2. Example of an SSCP gel used to analyze alternative splicing of the *C. elegans let-2* pre-mRNA. **(A)**, The organization of the alternatively spliced region of the *let-2* gene. (**B**), Lane 1, RT-PCR reaction using total RNA from a collection of adult hermaphrodite worms. Lanes 2 and 3, mobility standards generated by PCR reactions using let-2 cDNA clones containing the indicated alternative exon.

set temperature, the power will be reduced to such an extent that the gel will run extraordinarily slow. To circumvent this problem, we occasionally set a bucket filled with dry ice in front of the electrophoresis apparatus and use a fan to blow air across the dry ice and onto the front of the gel. This technique usually allows the power to return to normal while maintaining the desired gel temperature. We have also tried running the gels in a cold room but find that this tends to result in smeared bands.

3. It is important to run mobility standards after obtaining clones. You cannot rely on sequence information from excised bands alone, as we find that there is frequently cross-contamination from band to band.

Acknowledgments

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6

Methods for the Analysis of Adenosine-to-Inosine Editing in RNA

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Summary

In this work we describe methods for the analysis of RNAs that have been edited by the double-strand RNA-specific adenosine deaminase, ADAR. These RNAs contain inosine residues that can be detected and quantified by a variety of approaches, including base hydrolysis and thin-layer chromatography, reverse transcription polymerase chain reaction, primer extension, and inosine-specific base cleavage. The most common method for the analysis of editing will be described here. This method involves complete hydrolysis of edited RNAs to nucleoside monophosphates, followed by separation of the products using thin-layer chromatography.

Key Words

RNA editing; editing; inosine; adenosine deaminase; deaminase; dsRNA; thin-layer chromatography.

1. Introduction

Within cells of higher eukaryotes, long double-strand RNA (dsRNA) molecules in the nucleus are extensively edited by adenosine deaminases that act on RNA (ADARs). This editing results in the hydrolytic deamination of adenosine residues to inosine residues, and up to 50% of the A residues on each duplex RNA strand can be edited. In contrast to their action on long dsRNA molecules, ADARs can edit very specific sites on substrates that form short duplexes or that have optimized primary and secondary structures for such recognition. Inosines base-pair with cytosines instead of uridines and are recognized by the translation machinery as if they were guanosines.

Numerous methods have been developed to study adenosine to inosine (A-to-I) editing events in messenger RNAs (mRNAs). In this chapter, we

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describe in some detail the most common of these methods (complete hydrolysis of RNA followed by thin-layer chromatography [TLC]) but also describe in outline form the basic strategies behind a number of other important methods to detect and characterize editing.

2. Materials

2.1. Preparation of dsRNA

1. α-[^{32}P]ATP, 10 mCi/mL, 800 Ci/mmol.
2. Phage T3, T7, or SP6 RNA polymerases used for making dsRNA substrates in vitro (Promega). 5X transcription buffer supplied by the manufacturer.
3. Rnasin™ 40 U/μL (Promega).
4. 100 m*M* Dithiothreitol.
5. 10X NTP mix: 5 m*M* GTP, 5 m*M* CTP, 5 m*M* UTP, 0.5 m*M* ATP, in 0.01 *M* Tris-HCl, pH 8.0.
6. Phenol:chloroform:isoamyl alcohol (25:24:1).
7. 0.3 *M* Sodium acetate, pH 5.0.
8. RNase-free DNase (RQ1 DNase, Promega).
9. RNA dye mixture: 90% formamide, 50 m*M* Tris-HCl, pH7.5, 1 m*M* ethylenediamine tetraacetic acid (EDTA), 0.1% bromophenol blue, 0.1% xylene cyanol FF.
10. RNA elution buffer: 0.1% sodium dodecyl sulfate, 0.3 *M* sodium acetate, pH 5.5.
11. 20X SSC: 3 *M* NaCl, 0.3 *M* sodium citrate, prepared in sterile DEPC-treated water.
12. 5% denaturing polyacrylamide gel (acrylamide: bis, 19:1, 7 *M* urea).
13. 10 μg/μL Glycogen (Roche).

2.2. In Vitro Editing by ADAR

1. ADAR enzyme is either the recombinant protein, or is provided in cellular extracts prepared by the method of Dignam ***(1)***.
2. 5X ADAR assay buffer: 200 m*M* Tris-HCl, pH 7.9; 25 m*M* EDTA; 125 m*M* KCl; 50 m*M* NaCl; 5.5 m*M* $MgCl_2$, 25% (v/v) glycerol.
3. 1 mg/mL Acetylated bovine serum albumin (BSA) (Gibco-BRL).

2.3. Hydrolysis and TLC

1. Nuclease P1 powder (Pharmacia).
2. 10 mg/mL Proteinase K in water (Sigma).
3. Proteinase K buffer: 0.2 μg/μL proteinase K, 50 m*M* Tris-HCl, pH 7.5; 50 m*M* NaCl; 5 m*M* EDTA; 0.5% SDS.
4. Nuclease P1 buffer: 30 m*M* potassium acetate, pH 5.3; 10 m*M* $ZnSO_4$.
5. Cellulose polyester TLC plates (Sigma-Aldrich).
6. Saturated $(NH_4)_2SO_4$.
7. 10 m*M* Sodium acetate (pH 6.0).
8. Isopropanol.
9. TLC solvent: saturated $(NH_4)_2SO_4$, 10 m*M* sodium acetate, pH 6.0; and isopropanol, 79:19:2.

3. Methods

3.1. Preparation of dsRNA Substrate

1. pBlueScript plasmid (5 μg) containing any cloned insert of 100–800 bp is digested with restriction endonucleases in 50 μL of digestion buffer in two separate reactions, such that the plasmid is linearized at either end of the insert (*see* **Note 1**).
2. Each digestion product is extracted by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vortexing for 30 s.
3. The aqueous phase is recovered by centrifugation for 1 min at 12,000*g* and is diluted to 300 μL with 0.3 *M* sodium acetate, pH 5.0, followed by precipitation with 2 vol of ethanol.
4. DNA pellets are dissolved in 50 μL of DEPC-treated water to a final concentration of 0.1 mg/mL.
5. In vitro transcription reactions are prepared from each template such that either phage T3 or phage T7 RNA polymerase copies the entire plasmid insert sequence. DNA (2 μL) is added to reaction mixes of final volume of 20 μL containing 4 μL of 5X transcription buffer, 2 μL of 100 m*M* dithiothreitol, 2 μL 10X NTP mix, 20 μCi α-[^{32}P]ATP, and 1 μL (20 U) T3 or T7 RNA polymerase (*see* **Note 2**).
6. The reactions are terminated after 1 h at 37°C by addition of 1 μL RNAse-free DNase (RQ1 DNase from Promega), followed by further incubation for 15 min at 37°C.
7. RNA dye mixture (5 μL) is added to the reaction and incubated at 90°C for 3 min. The entire reaction mixture is then loaded onto a 5% denaturing polyacrylamide gel.
8. Electrophoresis is performed for 1 h at 600 V. The radioactive band is identified by exposing the wet gel (wrapped in plastic wrap) to X-ray film for 1 min. The gel piece containing the RNA band is excised using a new razor blade, chopped into small pieces, and placed into RNA elution buffer overnight with gentle shaking at room temperature.
9. The eluted material is extracted once with phenol:chloroform, and 5 μg of glycogen is added to the aqueous phase before the RNA is precipitated by addition of 2.5 vol of ethanol. The RNA is recovered by centrifugation at 12,000*g* for 15 min at 4°C.
10. The RNA pellet is dissolved in 20 μL of RNAse-free water, and 1 μL is removed for liquid scintillation counting (*see* **Note 3**).

3.2. Hydrolysis and TLC

The most common way to assay the promiscuous editing of long dsRNA duplexes is complete hydrolysis of the substrate, followed by TLC to resolve IMP from AMP. In this method, dsRNA substrates are prepared such that internal A residues are radiolabeled. After editing reactions, the RNAs are digested to mononucleotides using ribonuclease P1 and products resolved by TLC, separating [^{32}P]AMP residues from [^{32}P]IMP residues, which have been created by deamination.

3.2.1. In Vitro Editing of dsRNA by ADAR

1. An equal molar amount of gel purified [^{32}P]ATP radiolabeled sense and antisense RNAs (approx 50 fmol of each) are annealed in 20–30 μL of 2X SSC buffer by heating at 95°C for 2 min then cooling slowly to room temperature for at least 1 h. This material is then used in editing reactions with either cell extracts or purified ADAR enzyme.
2. [^{32}P]ATP-labeled dsRNA (10 fmol or 1×10^5 cpm) is added to a 50-μL reaction containing 1X assay buffer, 150 μg/mL acetylated BSA, 1 m*M* dithiothreitol, 4 U of Rnasin, and 30 ng of recombinant ADAR1 or ADAR2 and incubated at 30°C for 2 h.
3. 10 μL of 1X proteinase K buffer is then added to the reaction and incubation allowed to proceed for an additional 30 min at 37°C.
4. The reaction mixture is extracted with phenol/chloroform and the RNA is precipitated by ethanol and analyzed by TLC as described below.

3.2.2. TLC Analysis

The method for RNA digestion and TLC analysis is essentially as described by Wagner et al. ***(2)***.

1. In vitro ADAR-edited RNA as above, is resuspended in 10 μL of Nuclease P1 buffer and digested with 1.5 μg of nuclease P1 for 1.5 h at 45°C.
2. The samples are then loaded on a cellulose polyester TLC plate (Sigma) and resolved in TLC solvent. The sample volume for each reaction is approx 10 μL; however, this cannot all be spotted on the TLC plate at once. The sample should be spotted approx 1.5–2.0 cm from the bottom of the plate. It is preferable to spot the sample 2–3 μL at a time, allowing it to air-dry (or using a blow dryer) between applications. This results in smaller, less diffuse chromatography spots.
3. The plates are then placed in an air-tight vessel containing approx 0.5-cm deep TLC solvent at the bottom. Nonradioactive 5'-IMP and 5'-AMP are loaded as markers and can be visualized under 260 nm wavelength UV light.
4. Chromatography is allowed to proceed until the solvent has risen approx 75% of the way to the top of the plate (2–3 h).
5. Autoradiography is performed at –80°C using an intensifying screen, and quantitation of each spot can be determined by PhosphorImager analysis or by scraping spots identified by autoradiography into liquid scintillation vials for direct determination of radioactivity (*see* **Note 4**).

3.3. Other Methods for Analyzing Edited RNA

3.3.1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A second approach involves the use of RT-PCR to amplify edited sequences. This method differs from the first method previously described in that it can be easily applied both to promiscuously edited substrates as well as to substrates that are edited at only one or a few precise sites. RNA to be analyzed is subjected to RT-PCR to convert sequences into dsDNA. After the RT-PCR step,

there are numerous additional steps of analysis that can be carried out. In one approach, one can examine the DNA products for the appearance or disappearance of a restriction enzyme cleavage site (e.g., *see* **ref. *3***). In another approach, one can directly sequence the products to look at the frequency of A-to-I conversion at particular sites. This method can not only detect evidence of editing, appearing as mixed A and G peaks but also can provide evidence of the fraction of RNAs in the original population that are edited at a particular site (e.g., *see* **ref. *4***). Additionally, one can clone and sequence pools of PCR products to look at the general pattern of editing events observed (e.g., *see* **ref. *5***). An example of this sort is outlined in **Fig. 1**. Here, RT-PCR primers are chosen so as to amplify all RNAs of interest in the population, whether or not editing has occurred. The RT primer is chosen to bind to a region of about 15 nt that contains no A residues on the template strand. Thus, this primer lacks T residues. Priming using this oligonucleotide should be insensitive to the presence or absence of editing in substrate RNA molecules. Similarly, the second primer, used for the PCR reaction, lacks A residues. RT-PCR will lead to a mixed population of products, some of which are not edited and others that are. If these products are to be analyzed by cloning and sequencing ***(5)***, one must appreciate the fact that, although a single band may appear by gel electrophoresis, the product is not homogeneous; rather, it is often a very complex mix of many thousands of differently edited sequences. If the PCR is conducted past the logarithmic amplification stage, then these products may be difficult to clone because they may consist primarily of mismatched heteroduplexes instead of perfect duplexes.

3.3.2. Primer Extension Approaches

A third general way to examine editing is to use a modification of the primer extension technique to reveal specific editing sites. One approach of this type is outlined in **Fig. 2**, and specific conditions for this method can be found in numerous publications (e.g., *see* **refs. *6*** and ***7***). The basic strategy here is to anneal a short 5'-labeled DNA oligonucleotide primer to target RNA molecules just downstream of where a suspected editing site occurs. The oligonucleotide is used as a primer for reverse transcriptase, and cDNA sequences are prepared and analyzed by PAGE. The important difference between the specific approach here and other primer extension methods is the inclusion of one specific dideoxynucleoside triphosphate, for example, ddCTP in place of dCTP (or the omission of dCTP completely if the primer binding site is only a few nucleotides downstream of the putative editing site). In this way, cDNA synthesis stops at precisely the positions corresponding to Gs or Is on the template strand. Editing substrates will yield a different pattern, with a new and smaller band corresponding to the position of the altered base.

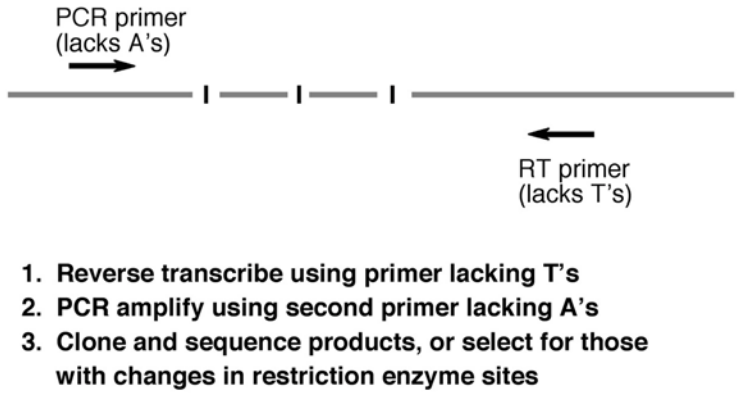

Fig. 1. An RT-PCR assay for editing (adapted from **ref. *6***). RT-PCR primers are chosen so as to amplify all RNAs of interest in the population, whether or not editing has occurred. The RT primer is 15–18 nt in length and contains no T residues. Priming using this oligonucleotide should be insensitive to the presence or absence of editing in substrate RNA molecules. The second primer, used for the PCR reaction, lacks A residues. RT-PCR will lead to a mixed population of products, some of which are not edited and others which are. Editing can be monitored by examination of the RT-PCR product for the gain or loss of a diagnostic restriction enzyme cleavage site or by cloning and sequencing products of the reaction.

3.3.3. Inosine-Specific Cleavage

The fourth, and relatively new, approach involves inosine-specific cleavage of edited RNAs ***(8)***. Inosine-specific cleavage is performed by reacting RNA with glyoxal, stabilizing the glyoxal adducts with borate, and then treating the RNA with ribonuclease T1. In this method, glyoxal treatment is used to form stable adducts with guanosines but not with inosines. Glyoxal-modified guanosines are resistant to RNase T1 cleavage, whereas unmodified guanosines are efficiently cleaved. Thus, glyoxalated RNA is primarily cut only after inosine residues by T1 nuclease. A detailed description of this method can be found in Morse and Bass ***(8)***, and the overall strategy is outlined in **Fig. 3**. After T1 cleavage, PAGE bands that appear uniquely in RNAs that have been glyoxal-treated can be excised from the gels and characterized further. A recent example of the successful application of this technique is the identification of numerous new editing sites in the 3'-untranslated regions of human brain mRNAs ***(9)***.

3.3.4. Protein–RNA Interactions

Finally, the possibility exists that inosine-containing RNAs might be detected, isolated and analyzed by virtue of their ability to interact with inosine-binding

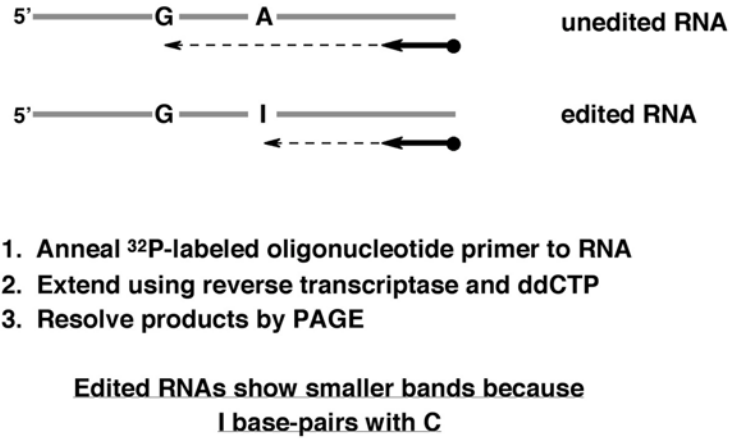

Fig. 2. A primer extension assay for editing. The basis of the method is that edited RNAs have a slightly different sequence and will therefore show unique bands in the presence of ddCTP. In this approach, a short 5'-labeled DNA oligonucleotide primer is annealed to target RNA molecules just downstream of where a suspected editing site occurs. The oligonucleotide is used as a primer for reverse transcriptase. Transcription is performed in the presence of a chain-terminating nucleoside triphosphate, ddCTP. cDNA sequences are prepared and analyzed by PAGE. In this way cDNA synthesis stops at precisely the positions corresponding to Gs or Is on the template strand. Edited substrates will yield a different pattern, with a new and smaller band corresponding to the position of the altered base.

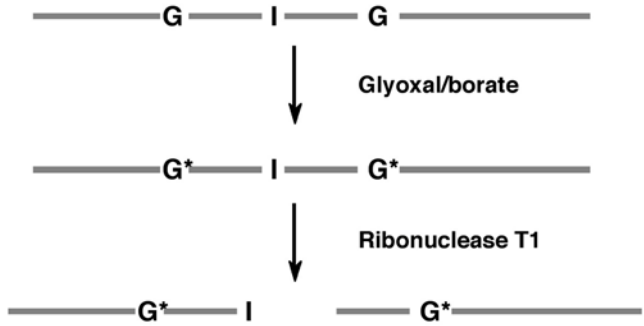

Fig. 3. Inosine-specific cleavage of RNA. In this method, RNA is treated with glyoxal/borate, which modifies guanosines so that they are resistant to ribonuclease treatment. Glyoxal forms stable adducts with guanosines but not with inosines. These glyoxalated guanosines cannot be cleaved by ribonuclease T1, whereas unmodified guanosines can. After ribonuclease T1 cleavage, PAGE bands that appear uniquely in RNAs that have been glyoxal-treated can be excised from the gels and characterized further.

protein(s). It was recently reported that promiscuously edited RNAs bind tightly and specifically to the nuclear protein $p54^{nrb}$ ***(10)***. This strong association might be exploited via affinity methods to select from complex RNA mixtures those molecules that contain numerous inosine residues. This approach is in principle more comprehensive than those previously described, and could help in the discovery of new in vivo editing targets, especially those that derive from sense–antisense RNA interactions in the nucleus.

4. Notes

1. All the reagents and buffers are made with care to eliminate general RNAse contamination. When practical, buffers should be autoclaved or treated with DEPC.
2. All of the RNA substrates used for editing assay should be gel-purified after in vitro transcription. When annealing the sense–antisense strands, it is important to keep the two strands in high concentration to get higher percentage yield of dsRNA. Sometimes it is necessary to perform annealing for longer time or purify dsRNA by native gel electrophoresis.
3. It is important to point out that when performing ADAR in vitro assays with dsRNA, care must be taken to ensure that results fall within the linear range of the in vitro activity of the ADAR enzymes. Thus, different enzyme concentrations should be used. It has been reported that the assay is quite sensitive to the ratio of enzyme to substrate, and ADAR is sensitive to substrate inhibition ***(11)***.
4. Essentially, the TLC assay can also be used to detect site-specific editing. In this case, the IMP spot will have much lower intensity than the AMP spot even the editing is very efficient, since only one of the multiple adenosines can be edited in the substrate. Longer exposure times may be required to detect the signal. A site-specific labeling strategy has been developed to increase the sensitivity of this method ***(12)***.

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7

Assaying Nuclear Messenger RNA Export in Human Cells

Bryan R. Cullen

Summary

This chapter describes a simple method for the analysis of nuclear messenger RNA (mRNA) export in human cells in culture. The assay described relies on the observation that mRNA molecules containing an intron are generally retained in the nucleus until splicing is completed. Upon sequestration of the *cat* indicator gene in a single intron located 5' to an mRNA cap site, CAT protein expression becomes dependent on the specific recruitment of a nuclear RNA export factor to the unspliced *cat* RNA via an inserted RNA binding site. This site can be a natural, high affinity RNA target for the nuclear export factor or alternately the export factor can be tethered to the unspliced *cat* mRNA by fusion to a heterologous RNA binding domain.

Key Words

HIV-1; mRNA; nuclear export; Rev; RNA binding; Tap.

1. Introduction

Xenopus oocyte microinjection provides a powerful technique to identify the factors affecting nuclear RNA export efficiency and has been invaluable in defining several RNA export pathways. However, recent evidence clearly demonstrates that export factors can be recruited to messenger RNA (mRNA) during both transcription and 3'-end processing ***(1,2)***, and therefore it is probable that the nuclear export of microinjected mRNA molecules that have been synthesized and labeled in vitro does not fully recapitulate the processes that regulate the export of endogenously transcribed mRNAs. To address this concern, it is important to develop assays that can detect the nuclear export of specific mRNAs in human cells.

The initial impetus for efforts to develop assays that measure mRNA export efficiency in human cells arose from research into the nuclear mRNA export

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factors produced by several complex retroviruses, including the Rev protein encoded by Human Immunodeficiency Virus Type 1 (HIV-1; reviewed in **ref. *3***). HIV-1 proviral transcription gives rise to only a single initial mRNA species, and the nine gene products encoded by HIV-1 are then translated from fully spliced and partially spliced derivatives of this genome-length transcript, which is itself also expressed in the cytoplasm. However, cells express proofreading factors that inhibit the nuclear export of intron containing mRNAs, that is, generally pre-mRNAs. To overcome this problem, HIV-1 produces a nuclear RNA export factor termed Rev that binds to a *cis*-acting RNA target site, the Rev response element (RRE), present in all incompletely spliced HIV-1 mRNAs, thus mediating their nuclear export and cytoplasmic expression ***(3)***. Rev itself is encoded by a fully spliced HIV-1 mRNA.

To measure Rev function, Malim et al. ***(4)*** developed an HIV-1-based indicator construct, termed pgTat, that contains only two of the many HIV-1 splice sites, that is, the splice sites that flank the two coding exons of the HIV-1 Tat protein. The single intron present in pgTat, derived from the HIV-1 *env* gene, naturally contains the RRE. In the absence of Rev, only full-length Tat, encoded by a fully spliced mRNA, is detected in pgTat-transfected cells. However, Rev coexpression induces the nuclear export of the unspliced mRNA encoded by pgTat, which encodes a truncated form of Tat. Rev function can therefore be readily detected by quantitation of the relative level of expression of full-length vs truncated Tat in pgTat-transfected cells. Although the pgTat assay has proven very useful for functional analysis of not only the Rev/RRE axis but also of other retroviral nuclear export factors, it nevertheless requires Western analysis using an antiserum to Tat that is not invariably available.

To address this concern, Hope et al. ***(5)*** developed an indicator plasmid termed pDM128 that, although in principle quite similar to pgTat, is nevertheless even easier to assay. To develop pDM128, Hope et al. ***(5)*** retained the two HIV-1 splice sites used in pgTat. However, they deleted most of the Tat coding exon located 5' to the 5' splice site and replaced the 5' end of the *env* open reading frame, located inside the intron and proximal to the 5' splice site, with the bacterial chloramphenicol acetyl transferase (*cat*) gene (**Fig. 1**). In my laboratory, pDM128 was subsequently modified by insertion of the SV40 origin of replication and the cytomegalovirus immediate early (CMV-IE) promoter by insertion of a polylinker within the intron 3' to the *cat* gene, and finally by insertion of the genomic rat preproinsulin II poly(A) addition site ***(6)***. The resultant indicator plasmid is termed pDM128/PL (**Fig. 1**).

In both the original pDM128 plasmid and in the pDM128/PL derivative, the *cat* gene is sequestered within an HIV-1-derived intron. Because intact introns, as noted above, inhibit nuclear egress via the cellular mRNA nuclear export pathway, the unspliced mRNA encoded by pDM128/PL is not normally able to reach the cytoplasm, and CAT protein expression is, therefore, minimal. How-

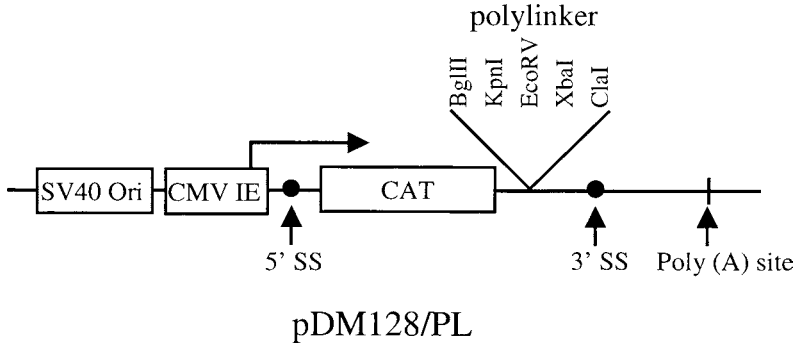

Fig. 1. Schematic of the pDM128/PL indicator plasmid (*see* text for detailed description). The polylinker provides unique sites for insertion of candidate RNA export elements. The backbone of the plasmid is derived from pBR322 and encodes ampicillin resistance in bacteria. 5'SS, 5' splice site; 3'SS, 3' splice site; SV40 Ori, SV40-derived origin of DNA replication; CMV IE, cytomegalovirus immediate early promoter; CAT, chloramphenicol acetyl transferase gene; Poly(A) site, poly(A) addition site derived from the genomic rat preproinsulin II gene.

ever, if a *cis*-acting RNA sequence that can recruit a nuclear RNA export factor is inserted into the polylinker present in pDM128/PL, then the unspliced mRNA is exported, resulting in high levels of CAT expression. For example, insertion of the HIV-1 RRE will generate high levels of CAT activity, but only in cells expressing the HIV-1 Rev protein (**Fig. 2**).

2. Materials

1. The pDM128/PL indicator plasmid and appropriate derivatives thereof.
2. Human 293T cells (*see* **Note 1**).
3. Tissue culture medium: Dulbecco's modified eagle medium, containing 5% fetal calf serum, supplemented with the antibiotics gentamycin and fungizone.
4. 2.5 *M* $CaCl_2$: 183.7 g of calcium chloride dihydride adjusted to 500 mL with distilled water. Filter-sterilize and store aliquots at –20°C.
5. 2X HeBS: 16.4 g NaCl, 11.9 g HEPES acid, 0.21 g Na_2HP0_4 adjusted to 1 L with distilled H_2O. Adjust pH to precisely 7.05 with NaOH. Filter-sterilize and store aliquots at –20°C.
6. TEN buffer: 0.1 *M* NaCl, 1 m*M* ethylenediamine tetraacetic acid, 10 m*M* Tris-HCl, pH 7.5.
7. Platform mixer.
8. Temperature-adjustable waterbath.
9. CAT buffer: 2 mg/mL chloramphenicol in 100 m*M* Tris-HCl, pH 7.5 containing 1.5 μL/mL of [^{3}H]acetyl coenzyme A (Perkin-Elmer, cat. no. NET290L).
10. Econofluor (Packard).
11. Scintillation counter.

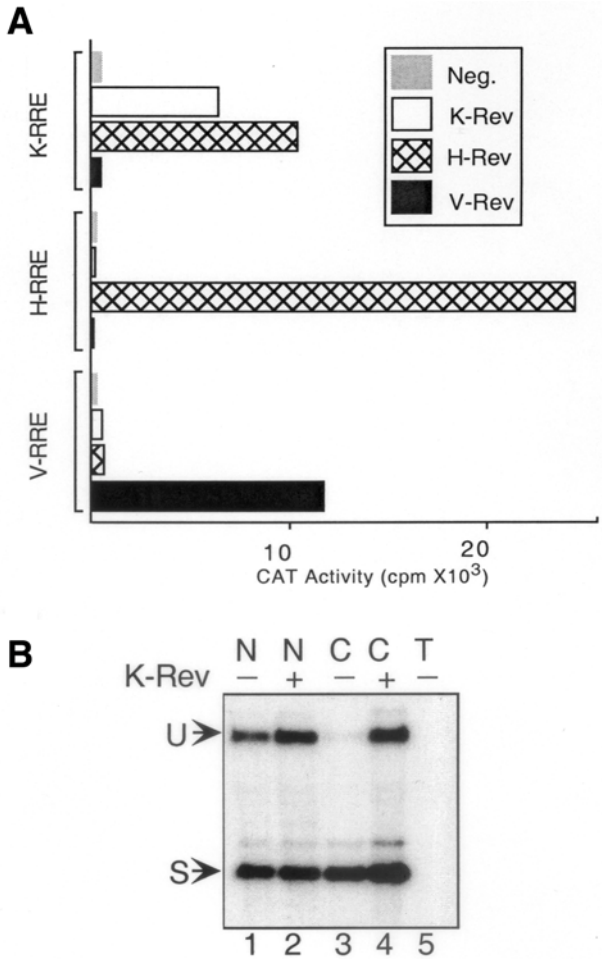

Fig. 2. Visualization of nuclear mRNA export using the pDM128/PL indicator plasmid. **(A)**, 293T cells were transfected with pDM128/PL derivatives containing the indicated viral RREs together with pBC12/CMV derivatives encoding the indicated Rev proteins. The parental pBC12/CMV plasmid served as the negative control. CAT activities were measured at approx 48 h after transfection. **(B)**, Cytoplasmic (C) and nuclear (N) RNA was isolated from 293T cells transfected with pDM128/K-RRE in the presence (+) or absence (–) of a K-Rev expression plasmid. The RNA protection assay probe used detects both the unspliced (U) and spliced (S) RNA encoded by pDM128/K-RRE. T, total RNA from mock transfected 293T cells (reproduced from Yang et al. [1999] *Proc. Natl. Acad. Sci. USA* **96,** 13,404–13,408, by permission of the publisher).

3. Methods

The methods described below outline (1) the derivation of appropriate indicator and effector plasmids, (2) transfection of 293T cells and, (3) lysis of the transfected cells and measurement of induced CAT enzyme levels.

3.1. Indicator and Effector Plasmids

The pDM128/PL plasmid does not contain any *cis*-acting target sequence for a nuclear mRNA export factor and can therefore be used as a negative control. The polylinker present in pDM128/PL contains five unique restriction enzyme sites that lend themselves to the insertion of an RNA export element (**Fig. 1**). RNA elements that have been inserted into pDM128/PL in this laboratory include the RREs encoded by HIV-1 (here termed the H-RRE) by the related sheep lentivirus visna maedi virus (V-RRE) and by the unrelated human endogenous retrovirus K (K-RRE; **ref. 7**). These three RNA elements all interact with their cognate viral nuclear export factor (here termed H-Rev, V-Rev, and K-Rev, respectively, *see* **Fig. 2**). Alternatively, retroviral RNA export whose function does not depend on any viral protein can be inserted into pDM128/PL, such as the constitutive transport element found in Mason Pfizer Monkey Virus, which directly interacts with the cellular Tap nuclear RNA export factor ***(8)***. Finally, if an RNA target for a candidate nuclear RNA export factor is not known, then the factor can be expressed fused to a heterologous RNA binding domain and the matching RNA target inserted into pDM128/PL. For example, a pDM128/PL derivative containing copies of the phage MS2 operator RNA sequence gives rise to readily detectable levels of unspliced *cat* mRNA nuclear export and expression when coexpressed with fusion proteins consisting of either HIV-1 Rev or human Tap linked to the phage MS2 coat protein RNA binding domain ***(9,10)***.

With the exception of RNA elements, such as the constitutive transport element, that respond to endogenously expressed human proteins, most pDM128/PL derivatives are only active when coexpressed with a particular effector protein, such as HIV-1 Rev. These effector proteins can be expressed using essentially any commercially available expression plasmid. The parental expression plasmid favored in this laboratory is pBC12/CMV, which is available on request, as are its relevant derivatives.

3.2. Transfection of 293T Cells

1. One day before transfection, plate 3×10^5 293T cells into each well of a tissue culture plate bearing six 35-mm wells. Add 2 mL of tissue culture medium at 37°C.
2. Add 25 ng of the pDM128/PL plasmid, or a derivative, together with 500 ng of an effector plasmid (or an appropriate negative control plasmid) to a microcentrifuge tube (*see* **Notes 2** and **3**). Increase volume to 90 µL by the addition of sterile

distilled H_2O. Add 10 μL of 2.5 *M* Ca_2Cl and pipet up and down to mix. Add 100 μL of 2X HeBS and vortex immediately. Incubate for 30 min at room temperature. Add mixture drop-wise to a tissue culture well, covering the entire surface area. Precipitate should be visible in 1 h or less (*see* **Note 4**).

3. Incubate for 24 h at 37°C, then aspirate medium from each dish and replace with 2 mL of fresh tissue culture medium.
4. Incubate for a further 20 to 24 h at 37°C and then aspirate the medium from each dish and add 1 mL of TEN buffer (*see* **Note 5**). Swirl plate gently on a platform mixer for up to 5 min to loosen all cells from dish. Transfer each sample to a microcentrifuge tube, centrifuge briefly to pellet cells, aspirate TEN buffer and resuspend cells in 200 μL of 100 m*M* Tris-HCl, pH 7.5. Freeze-thaw samples three times using a dry ice/ethanol bath and a warm water bath. Centrifuge for 5 min in a microcentrifuge and transfer supernatant media to a fresh tube. Heat at 65°C for 10 min in an adjustable water bath (*see* **Note 6**).
5. Perform two-phase liquid CAT assay as described by Neuman et al. ***(11)***. Place 50 μL of each sample in a scintillation vial. Prepare CAT buffer fresh and add 200 μL to each sample in scintillation vial. Mix gently. Add 2 mL of Econofluor scintillation cocktail and determine [^{3}H] counts in a scintillation counter. Incubate at room temperature, counting at regular intervals, until the most positive sample is between 25,000 and 50,000 counts. The level of [^{3}H] counts detected accurately measures the level of CAT enzyme activity in the sample, which in turn reflects the efficiency of nuclear export of the unspliced CAT mRNA encoded by the particular pDM128/PL derivative (*see* **Notes 7** and **8**).

3.3. Representative Data

Figure 2A shows representative data obtained using the above protocol ***(7)***. In this instance, the three pDM128/PL-derived indicator constructs used contain either the K-RRE, the H-RRE, or the V-RRE inserted in a sense orientation into the polylinker. These indicator constructs were transfected into 293T cells together with effector constructs, derived from pBC12/CMV that express K-Rev, H-Rev, or V-Rev. The parental pBC12/CMV expression plasmid served as a negative control. As may be readily observed, the pDM128/H-RRE plasmid only gave rise to CAT activity when cotransfected with an H-Rev expression plasmid, whereas pDM128/V-RRE only responded to V-Rev. Conversely, the pDM128/K-RRE indicator plasmid was activated not only be the cognate K-Rev protein but also by the heterologous H-Rev protein. Therefore, this assay clearly demonstrates the sequence specific nature of the interaction of these viral mRNA export factors with their RNA response elements (**Fig. 2A**).

To confirm that this assay was in fact measuring nuclear mRNA export, we also performed an RNAase protection assay using nuclear and cytoplasmic RNA fractions derived from additional 293T cells transfected in parallel (**Fig. 2B**). Only the unspliced (U) mRNA encoded by pDM128/K-RRE is predicted to encode CAT and this mRNA is only expected to reach the cytoplasm when

K-Rev is coexpressed, as is indeed observed (compare lanes 3 and 4, **Fig. 2B**). However, the unspliced mRNA encoded by pDM128/K-RRE is readily detectable in the nucleus regardless of K-Rev expression (lanes 1 and 2, **Fig. 2B**). Finally, the spliced (S) mRNA encoded by pDM128/K-RRE, which does not encode CAT, is able to reach the cytoplasm independently of K-Rev expression (**Fig. 2B**). Therefore, the RNA analysis shown in **Fig. 2B** confirms that the indicator gene assay presented in **Fig. 2A** is indeed detecting the nuclear export of the otherwise nuclearly sequestered unspliced CAT mRNA encoded by pDM128/K-RRE. Similar RNA analyses confirming the general validity of the pDM128 assay as a measure of mRNA export efficiency have been presented in several other manuscripts ***(5**,**8**,**9**,**12)***.

4. Notes

1. Other cell types, including nonhuman cells, can be used successfully in place of 293T cells ***(6**,**10)***. However, human 293T cells are easy to transfect and give a very low basal activity upon transfection with the parental pDM128/PL plasmid.
2. We use a low level (25 ng) of the pDM128/PL-derived indicator plasmid in each transfection. In our hands, using a greater amount decreases the signal to background ratio.
3. Although not mentioned in this basic protocol, we generally add 25 ng of an internal control plasmid encoding β-galactosidase (pBC12/CMV/(β-gal) to each transfection ***(6)***. Because β-galactosidase is heat labile, activity must be measured using an aliquot of the 293T cell lysate that has not been subjected to incubation at 65°C. Heating at 65°C reduces the background activity observed in this CAT assay.
4. We describe a standard calcium phosphate transfection procedure for 293T cells as this works reliably and is very inexpensive. However, commercially available transfection reagents, such as lipofectamine and fugene, also work perfectly well.
5. CAT activity is first detected at approx 20 h after transfection and continues to increase until approx 72 h after transfection. Later or earlier harvest is therefore possible.
6. Heating the cell lysate can occasionally cause turbidity. This is not a problem, but the samples can be clarified by centrifugation if desired.
7. The two-phase liquid CAT assay ***(11)*** is favored in this laboratory because it is reliable, simple and inexpensive. However, other assays for CAT activity, including nonradioactive assays, can be used if desired.
8. The two-phase liquid CAT assay becomes nonlinear at levels higher than 50,000 cpm, and counts above this level should therefore be avoided.

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8

RNA Unwinding Assay for DExD/H-Box RNA Helicases

Stephanie S. Tseng-Rogenski and Tien-Hsien Chang

Summary

The evolutionarily conserved DExD/H-box proteins are essential for all RNA-related biological processes. They are thought to modulate the structure and function of specific RNAs and/or ribonucleoprotein particles by using their intrinsic RNA-dependent ATPase activities to achieve the desired conformational changes. A number of DExD/H-box proteins have been shown to unwind short RNA duplexes in vitro, a hallmark of the so-called RNA helicases or unwindases. However, some are unable to do so, perhaps because of requirements for cofactors. Here, we present a "solid-state" method that may allow investigators to overcome such problems.

Key Words

DExD/H-box protein; RNA helicase; RNA unwinding; mRNA splicing; RNA-dependent ATPase.

1. Introduction

The ubiquitous DExD/H-box proteins are enzymes that participate in essentially all RNA-related biological processes, such as nuclear pre-messenger RNA (mRNA) splicing, ribosomal biogenesis, mRNA export, translation, and RNA turnover ***(1**,**2)***. They are often referred to as RNA helicases or RNA unwindases because a number of them can unwind short RNA duplexes in vitro ***(3–11)***. At least one of them, NPH-II, has been shown to unwind RNA duplexes in a processive and directional manner ***(12)***. Thus, the prevailing hypothesis is that DExD/H-box proteins directly bind to and unwind specific RNA duplexes by harnessing energy from ATP hydrolysis in a manner similar to that of the better studied DNA helicases ***(3**,**12**,**13)***. However, given that RNA duplexes in vivo are rarely longer than approx 10 contiguous base pairs and that they are often stabilized by RNA-binding proteins ***(14**,**15)***, RNA rearrange-

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ments mediated by DExD/H-box proteins may not simply involve duplex unwinding. In fact, studies on translation initiation factor eIF4A have raised the possibility that the DExD/H-box proteins may perform functions distinct from RNA unwinding, which include mediating large scale RNA structural rearrangements, disrupting protein–RNA or protein–protein interactions, and functioning as fidelity sensors in RNA–RNA interactions and rearrangements ***(16,17)***. Indeed, recent works from several laboratories appear to confirm that the functions of the DExD/H-box proteins are diverse ***(18–21)***.

Therefore, it is important for investigators to bear in mind that not all DExD/H-box proteins are capable of unwinding RNA duplexes in vitro. There are at least two possible explanations for this observation. First, some DExD/H-box proteins are predicted to work in a highly specific environment and to act on a particular RNA substrate. For example, the yeast splicing factor Prp28p has failed to unwind RNA duplexes in vitro thus far ***(22)***, perhaps because Prp28p must work in the spliceosome and act on an RNA duplex formed between the 5' end of U1 snRNA and pre-mRNA 5' splice site ***(15,21)***. Second, some DExD/H-box proteins are known or thought to work in concert with cofactors. For example, the RNA unwinding activity of eIF4A is greatly stimulated by eIF4B ***(6,23)*** or by its presence in the eIF4F complex ***(6,24,25)***. Such requirements for cofactors have also been suggested for the yeast and human Dbp5p because the purified recombinant Dbp5p could not unwind RNA duplexes, but the immunoprecipitated Dbp5p could do so ***(5,26)***.

Here, we describe an RNA unwinding assay developed for examining the RNA unwinding activity of Dbp5p , aiming to overcome its potential requirement for cofactors. This "solid-state" method involves first immobilizing the cytosolic Dbp5p on a solid support and then assaying for its RNA unwinding activity. We believe that this method can be easily adapted for studying other DExD/H-box proteins ***(26)***.

2. Materials

2.1. Supplies

1. Plasmid pSP65H.
2. *Bam*HI restriction enzyme (New England Biolab) and SP6 RNA polymerase (GibcoBRL).
3. Acrylamide, *bis*-acrylamide, and urea (all from Roche Biochemicals); gel electrophoresis equipment.
4. IgG-Sepharose (Pharmacia).
5. 100 m*M* nucleoside triphosphates (guanosine triphosphate [GTP], adenosine triphosphate [ATP], UTP, and CTP; Pharmacia).
6. $\alpha[^{32}P]$-UTP (3000 Ci/mmol, DuPont-NEN).
7. Phenol–chloroform mix, pH 4.7 (Fisher).
8. Sorbitol (Sigma).

9. Zymolyase (100T; Seikagaku America).
10. 500 m*M* acetic acid, pH 3.4.
11. Rnasin™ (Promega).
12. Leupeptin (Roche Biochemicals).
13. Aprotinin (Roche Biochemicals).
14. Pepstatin (Roche Biochemicals).
15. Glass dounce homogenizer (Kontes).
16. All other chemicals were obtained from Sigma unless stated otherwise.

2.2. Buffers

1. SB buffer: 1.2 *M* sorbitol, 10 m*M* potassium acetate, pH 7.5.
2. 10X SP6 buffer: 400 m*M* Tris-HCl, pH 7.5; 60 m*M* $MgCl_2$; 40 m*M* spermidine.
3. Elution buffer: 500 m*M* sodium acetate, pH 5.2; 1 m*M* ethylenediamine tetraacetic acid (EDTA); 2.5% phenol-chloroform, pH 4.7.
4. YPD media: 1% yeast extract, 2% peptone, 2% dextrose (Becton Dickinson).
5. HMS buffer: 25 m*M* HEPES-KOH, pH 7.5, 250 m*M* sucrose, 5 m*M* EDTA, 1 m*M* DTT, 150 m*M* KCl, 1 m*M* PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 m*M* pepstatin. Protease inhibitors were from Roche Biochemicals.
6. HMC sucrose: 25 m*M* HEPES-KOH, pH 7.5; 5 m*M* EDTA, 0.5 *M* sucrose.
7. 10X Unwinding buffer: 170 m*M* HEPES-KOH, pH 7.5; 10 m*M* $MgCl_2$; 20 m*M* dithiothreitol (DTT); 10 m*M* spermidine; 3% PEG8000.
8. 1X Unwinding buffer (17 m*M* HEPES-KOH, pH 7.5; 1 m*M* $MgCl_2$; 2 m*M* DTT; 1 m*M* spermidine; 0.3% PEG8000).
9. Gel loading solution: 95% formamide (Fisher), 10 m*M* EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue.
10. Stop buffer: 10 mg/mL proteinase K, 10% sodium dodecyl sulfate.

3. Methods

3.1. Preparing [^{32}P]-Labeled RNA Duplexes

To test RNA unwinding activity of Dbp5p, the plasmid pSP65H (kindly provided by Dr. N. Sonenberg; **Fig. 1A**) containing an RNA substrate insert at the *Sma*I site is used to synthesize RNA substrates. Transcription of the *Bam*HI-linearized pSP65H using SP6 RNA polymerase yields an RNA transcript that can self anneal into a stable RNA duplex containing ten alternate G/C base pairs flanked by single-stranded regions of 53 nt at the 5' end and 21 nucleotides at the 3' end (**Fig. 1B**). We used this standard RNA substrate for routine RNA unwinding assays. RNA duplexes with only 3' overhangs can also be generated by transcribing the *Eco*RI- or *Sac*I-linearized pSP65H using T7 RNA polymerase (**Fig. 1A**).

3.1.1. In Vitro Transcription of pSP65H

1. A 40-μL reaction containing 2 μL of 0.5 *M* DTT, 4 μL of 10X NTP mix (5 m*M* GTP, 5 m*M* ATP, 5 m*M* CTP, 1 m*M* UTP), 4 μL of 10X SP6 buffer, 4 μL of *Bam*HI-

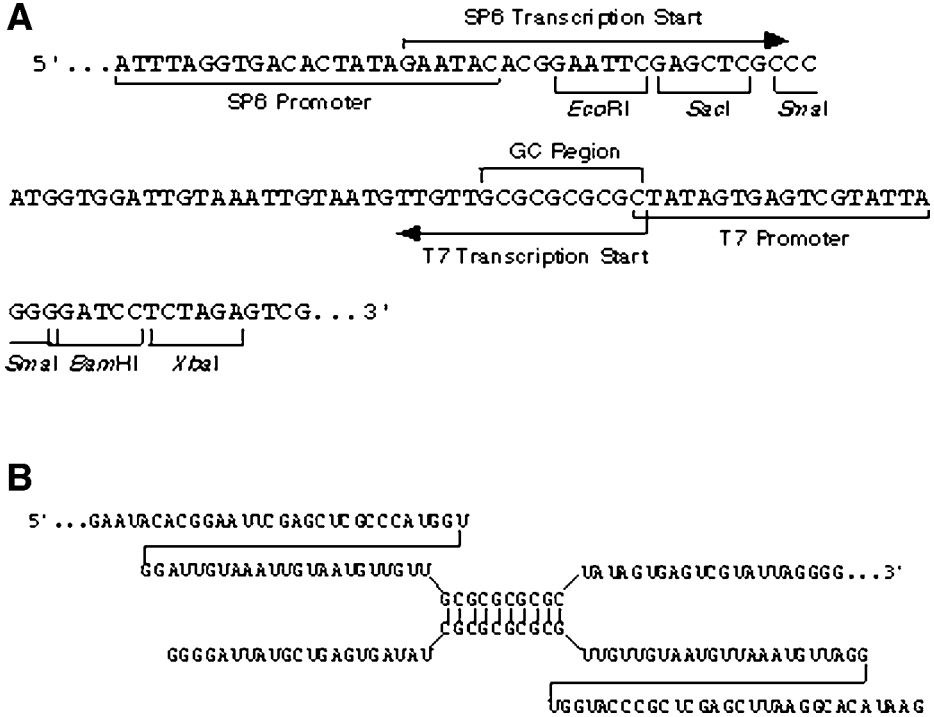

Fig. 1. DNA template and RNA duplex for RNA unwinding assay. **(A)**, DNA template used in SP6 in vitro transcription. SP6 and T7 promoters, their respective transcriptional start sites, relevant restriction sites, and the central GC sequence for forming a stable RNA duplex are indicated. **(B)**, Sequence and the predicted structure of the standard RNA duplex used for RNA unwinding assay. Note that the central 10-bp GC duplex is flanked by single-stranded 5' and 3' overhangs.

linearized pSP65H plasmid (0.5 mg/mL), 2 μL of RNasin (40 U/μL), 3.2 μL of SP6 polymerase (15 U/μL), 8 μL of α-[^{32}P]-UTP (3000 Ci/mmol), and 12.8 μL of diethyl-pyrocarbonate (DEPC)-treated H_2O is prepared. The reaction is incubated at 37°C for 2 h and then terminated by adding 14 μL of gel loading solution.

2. The sample is boiled for 3 min, chilled on ice, loaded onto an 8% polyacrylamide (19:1 crosslinking)-8 *M* urea gel (0.5-mm thick) and electrophoresed until the bromophenol blue dye migrated to two thirds of the gel distance.
3. The gel apparatus is disassembled and the gel was left on one glass plate. The gel on the glass plate is wrapped with plastic wrap and then exposed to an X-ray film for approx 1 min (or until the signal corresponding to the transcription product is seen clearly).
4. The position of the transcription product (which migrates very close to xylene cyanol) on the gel is determined by superimposing the gel and the film, the gel slice is then excised and placed into a microcentrifuge tube.

5. To recover the transcripts, the gel slice is minced using a flame-sealed yellow tip and incubated with 0.5 mL of elution buffer at 4°C with rocking on a nutator overnight.
6. The next day, the gel suspension is filtered through a disposable spin column (Poly-Prep chromatography columns; Bio-Rad). The elution solution containing the radiolabeled transcript is collected and extracted with 0.5 mL of phenol-chloroform, pH 4.7. After centrifugation, 0.4 mL of top fraction was transferred to a well-siliconized microcentrifuge tube and radiolabeled transcripts are recovered by ethanol precipitation.
7. The RNA pellet is rinsed with 70% ethanol, dried and dissolved completely in 20 μL of 100 m*M* KCl. Special care should be taken at this step to assess the transcript recovery by monitoring the remaining radioactivity in the empty tube using a Geiger counter (Model 3; Ludlum).

3.1.2. Purification of RNA Duplex

1. To promote the formation of the RNA duplex, the RNA sample is placed into 1 L of boiling water for 3 min. The flame is then extinguished and the water containing the RNA sample is allowed to cool for the next 1.5 h to promote annealing.
2. The annealed RNA sample is mixed with 5 μL of 10X unwinding buffer, loaded onto a 10% native gel (19:1), and electrophoresed at 200 V in the cold room until the bromophenol blue dye reaches the bottom of the gel (approx 2.5 h).
3. The dsRNA is recovered as described in **steps 3–7** of **Subheading 3.1.1.** and dissolved in 20 μL of DEPC-treated H_2O from which 1 μL is used for determine recovery of radiolabeled RNA by scintillation counting. The duplex RNA is adjusted to 50 pmol/μL and stored in aliquots at –20°C.

3.2. Preparing Dbp5p-Enriched Cytosolic Fraction

Yeast Dbp5p was previously localized to the cytoplasmic side of the nuclear pore complex and also to cytoplasm in general ***(5,27)***. To assay for Dbp5p's RNA unwinding activity, the Dbp5p-enriched cytosolic fraction is prepared from a diploid strain expressing a functional recombinant Dbp5p-Protein A fusion ***(5)***.

1. The diploid strain YTC41 is grown in 250 mL of YPD medium at 30°C to 0.7 OD_{600} unit.
2. Cells are collected by centrifugation (3300*g*; Sorvall GSA rotor) for 5 min, washed once with 10 mL of 0.5% β-mercaptoethanol, harvested, and resuspended in 10 mL of SB buffer. Ten milligrams of zymolyase is then added to the cell suspension and the mixture is incubated on a nutator at 37°C for 30 min.
3. The resulting spheroplasts are harvested by low-speed centrifugation (2400*g*; Sorvall SS34 rotor) for 1 min at room temperature, gently washed with 10 mL of YPD/1.2 M sorbitol, and regenerated in 250 mL of YPD/1.2 M sorbitol at 30°C for 1 h with slow shaking (120 rpm). The purpose of this step is to allow spheroplasts to recover from zymolyase-induced stress, which may result in mislocalization of proteins of interest.

4. All steps hereafter are performed at 4°C with prechilled buffer, pipets, pipet tips, and centrifuge rotors.
5. The regenerated spheroplasts are harvested by low-speed centrifugation (2400*g*; Sorvall SS34 rotor) for 1 min, gently washed once with 10 mL of HMS buffer, collected again by centrifugation, and resuspended in 2 mL of HMS buffer containing 0.1% NP-40.
6. The spheroplasts are then transferred into a prechilled glass dounce homogenizer on ice and broken using 12 up-and-down strokes with the glass pestle.
7. The resulting crude lysate is quickly loaded onto two pre-chilled 5-mL HMC sucrose cushions and centrifuged at 7670*g* (Sorvall SS34 rotor) for 5 min at 4°C.
8. The cytosolic fraction was then recovered from the top layer and its protein concentration measured.

3.3. RNA Unwinding Assay

3.3.1. Preparation of Dbp5p-Protein A-Bound IgG-Sepharose

1. To prepare the matrix for immobilizing Dbp5p-protein A, 200 μL of IgG-Sepharose is first rinsed with large amount of DEPC-treated H_2O to remove ethanol and then washed sequentially with 0.5 mL of 500 m*M* acetic acid, pH 3.4, 1 mL of HMS buffer, 0.5 mL of 500 m*M* acetic acid (pH 3.4), and then three times of 1 mL of HMS buffer. Each wash consisted of 5 min rocking on a nutator at 4°C.
2. The washed IgG-Sepharose is resuspended in an equal bed volume of HMS Buffer and divided into 40-μL aliquots.
3. 5–20 μL of cytosolic fraction (protein concentration = 3 mg/mL) is incubated with 20 μL (bed volume) of IgG-Sepharose at 4°C for 3 h.
4. The mixture is extensively washed eight times with 1 mL of HMS buffer containing 0.05% NP-40, followed by three additional washes with 1X unwinding buffer.

3.3.2. RNA Unwinding Reaction

1. A typical RNA unwinding reaction contains the following ingredients: 1X unwinding buffer, 1 m*M* ATP, 0.1 m*M* GTP, 5% glycerol, 150 m*M* KCl, 40 U RNasin, 10 μg tRNA and 50 fmol [^{32}P]labeled dsRNA substrates (*see* **Note 3**). Typically, a 20-μL unwinding reaction is assembled, added to the washed beads, and incubated at 37°C for 20 min.
2. The reaction tube is then centrifuged in a microcentrifuge for 1 min at top speed and 10 μL of supernatant is removed and transferred to a new tube containing 1 μL of stop buffer. The tube is then incubated at 37°C for 20 min to terminate the reaction.
3. Reactions are analyzed by loading the recovered sample onto a 10% native polyacrylamide (19:1) gel and electrophoresed at 200 V in cold room until the bromophenol blue dye reaches the bottom of the gel (about 2.5 h).
4. After electrophoresis, the gel is exposed to an X-Ray film overnight at –80°C with an intensify screen. A typical result is shown in **Fig. 2**.

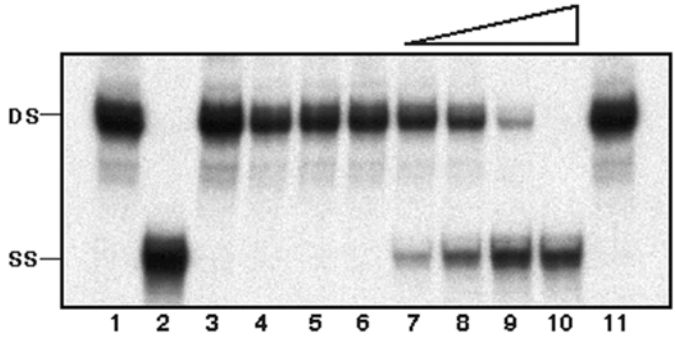

Fig. 2. RNA unwinding activity of immunoprecipitated Dbp5p-Protein A. Increasing amounts of the cytosolic fraction isolated from a DBP5-Protein A diploid strain were incubated with IgG-Sepharose (lanes 7–10) and the corresponding immunoprecipitates were assayed for RNA unwinding activity at 37°C using the standard RNA substrate. Cytosolic fractions from an isogenic wild-type strain (lane 4) or from the DBP5-Protein A strain but omitting ATP (lane 5) or Mg^{2+} (lane 6) in the unwinding assay were used as controls. Neither IgG-Sepharose (lane 3) nor protein A-Sepharose (lane 11) alone can unwind the RNA duplex. Double-stranded RNA substrate (DS; lane 1) can be denatured into the single-stranded form (SS) by boiling (lane 2).

4. Notes

1. To strengthen the argument that the observed RNA unwinding activity is derived from Dbp5p *per se*, instead of from some unknown coprecipitated proteins, we carried out the RNA unwinding assay using cytosolic fractions prepared from a dbp5-2-Protein A temperature-sensitive strain. We showed that, in this case, the IgG-Sepharose-associated RNA unwinding activity was active at 30°C but was completely inactivated by preincubating the reaction at 37°C for 15 min ***(5)***. Thus, the observed RNA unwinding activity is unlikely to have resulted from other factors coprecipitated with Dbp5p.
2. The *Escherichia coli* overproduced human Dbp5p (hDbp5p), like its yeast counterpart, cannot unwind RNA duplexes by itself ***(5,26)***. A variation of the described protocol has been successfully applied to demonstrate the RNA unwinding activity of hDbp5p ***(26)***. In this case, hDbp5p was immunoprecipitated from HeLa cell S-100 extracts using anti-hDbp5p antibodies coupled to protein A-Sepharose. The observed RNA-unwinding activity was not detected when preimmune serum was used in the control experiments.
3. The amount of tRNA used in the unwinding reaction may need to be optimized. We found a positive correlation between quantitative recovery of the input RNA duplex substrate and the increasing amount of tRNA (2.5–10 μg), which presumably served to block nonspecific RNase activity in the cytosolic fraction. How-

ever, less unwinding activity was observed when 50 μg of tRNA was used, presumably reflecting nonspecific binding of tRNA to Dbp5p.

4. Freshly prepared cytosolic fraction is always preferred. RNA unwinding activity of Dbp5p appeared to drop precipitously upon freezing the cytosolic fraction.

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9

Approaches for Monitoring Nuclear Translation

Francisco J. Iborra, Dean A. Jackson, and Peter R. Cook

Summary

The nuclear membrane is the defining feature of eukaryotes. It divides the cell into two functionally specialized compartments, and it is widely assumed that translation is restricted to only one: the cytoplasm. However, recent results suggest that some translation takes place in nuclei closely coupled to transcription. Various labeling techniques are described that enable nascent peptides to be labeled and then localized wherever they might be in the cell.

Key Words

Autoradiography; biotin; biotin-lys-tRNA; BODIPY-lys-tRNA; immunofluorescence; immunogold labeling; saponin; transcription; translation.

1. Introduction

The nuclear membrane is the defining feature of eukaryotes. It divides the cell into two functionally specialized compartments, and it has been widely assumed that translation is restricted to only one: the cytoplasm. However, recent results suggest that some translation takes place in nuclei ***(1,2)***. The evidence is of various types. First, the components required for translation can be found in nuclei. Second, although most nascent peptides are found in the cytoplasm, some can be found in nuclei. Different approaches were used to label and then localize the nascent peptides including incubating living cells with radiolabeled amino acids and then autoradiography, or incubating permeabilized cells (or nuclei) with biotin-lysine-tRNA (or BODIPY-lysine-tRNA) before immunolocalization using fluors and light microscopy (or gold particles and electron microscopy). Third, the production of the nascent peptides in the nucleus is closely coupled to transcription because inhibiting the nuclear transcription immediately inhibits the production of the nascent nuclear peptides. Fourth, the nascent nuclear peptides (and various components of the

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translation machinery) are found within a few nanometers of nascent nuclear RNA. Here, we describe various labeling techniques that enable nascent peptides to be labeled and then localized, whether they are in the nucleus or cytoplasm.

2. Materials

2.1. Growth Media

1. Dulbecco's modified Eagle medium (DMEM; Invitrogen, cat. no. 41966-029).
2. DMEM without leucine and MEM without leucine (these are no longer available; alternatives can be made by adding the appropriate supplements to DME/F12HAM without L-leucine, L-lysine, L-methionine, calcium chloride, magnesium sulfate, magnesium chloride from Sigma, cat. no. D9785).

2.2. Detergents Used for Permeabilization

1. Digitonin (Sigma, cat. no. D 1407).
2. Lysolecithin (Sigma, cat. no. L 4129).
3. Saponin (Sigma, cat. no. S-7900 or S-4521).
4. Triton X-100 (Pierce, cat. no. 28314).

2.3. Specialized Chemicals

1. Adenosine triphosphate (ATP), CTP, guanosine triphosphate (GTP), UTP (from Ultrapure NTP set Amersham International, cat. no. 27-2025-01) used for transcription reactions.
2. Biotin-lysine-tRNA from brewer's yeast (Roche, cat. no. 1 559 478).
3. BODIPY-lysine-tRNA (FluoroTect™ $Green_{Lys}$; Promega, cat. no. L5001).
4. Dextrans (40, 70, 500 kDa) conjugated with FITC (Sigma, cat. no. FD-40S, FD-70S, FD-500S).
5. Diethylpyrocarbonate (Sigma, cat. no. D5758).
6. Fluorescein-12-ATP (New England Nuclear, cat. no. NEL439).
7. Gelatin (from cold water fish skin; Sigma, cat. no. G-7765).
8. Human placental ribonuclease inhibitor (Amersham International, cat. no. 799 025).
9. LR White (Agar Scientific, cat. no. 14380).
10. Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710-S).
11. Phenylmethanesulphonyl fluoride (Sigma, cat. no. P7626).
12. Phosphocreatine disodium salt (Sigma, cat. no. P7936).
13. Poly-L-lysine (Sigma, cat. no. P4707).
14. SYTO-16 (Molecular Probes, cat. no. S-7578).
15. TOTO-3 (Molecular Probes, cat. no. T-3604).
16. tRNA (Sigma; Type XI: from bovine liver, cat. no. R-4752).
17. Uranyl acetate (Agar Scientific, cat. no. R1260A).

2.4. Radiolabels and Amino Acid Mixtures

1. L-[4,5-^{3}H]lysine (86 Ci/mmol; Amersham International, cat. no. TRK520).
2. Amino acids minus lysine (Roche, cat. no. 1 559 478).
3. L-[4,5-^{3}H]leucine (147 Ci/mmol; Amersham International, cat. no. TRK510).

4. Amino acids minus leucine (available in the rabbit reticulocyte lysate system from Amersham International, cat. no. RPW3150).
5. L-[^{35}S]methionine (0.5 Ci/mmol; Amersham International, cat. no. SJ123).
6. Amino acids minus methionine (available in the rabbit reticulocyte lysate system from Amersham International, cat. no. RPW3150).

2.5. Enzymes, Proteins, and Inhibitors

1. Aminoacyl-tRNA synthetase (Sigma; from bakers yeast, cat. no. A-6302).
2. Bovine serum albumin (BSA), essentially free of fatty acids and γ-globulin (Sigma, cat. no. A-7030).
3. Creatine phosphokinase (Sigma, cat. no. C-3755).
4. DNase, RNase-free (Boehringer, cat no. 1119915).
5. Human placental ribonuclease inhibitor (10 U/mL; Amersham International, cat. no. 27-0815-01).
6. Protease inhibitors cocktail for mammalian cells (Sigma, cat. no. P-8340).
7. Puromycin (Sigma, cat. no. P-7255).

2.6. Antibodies

1. Donkey anti-mouse IgG conjugated with Cy3 (Affinipure grade, Jackson ImmunoResearch, cat. no. 115-165-071).
2. Goat anti-mouse IgG conjugated with 5- or 10-nm gold-particles (British BioCell International, cat. nos. EM.GAM5 and EM.GAM10).
3. Mouse anti-biotin (Jackson ImmunoResearch, cat. no. 200-002-096).

2.7. Sundries

1. Coverslips, no. 1.5, glass, 16-mm diameter (Agar Scientific, cat. no. L4098-2).
2. Emulsion (dipping) film (Ilford K.5 supplied by Agar Scientific, cat. no. P9281).
3. Filter paper, Whatman no. 1 (Whatman International Ltd, cat. no. 1001 090).
4. Glass fiber discs (GF/C; Whatman International Ltd, cat. no. 1822 025).

2.8. Buffers

All buffers used up to fixation were ice cold unless stated otherwise. Where analysis of RNA is critical, RNAse-free distilled H_2O should be used. This can be prepared by treated with diethylpyrocarbonate; add 0.5 mL of diethylpyrocarbonate to 500 mL of distilled H_2O, mix, stand at 37°C overnight, and autoclave.

1. Physiological buffer (PB): PB is 100 m*M* potassium acetate, 30 m*M* KCl, 10 m*M* Na_2HPO_4, 1 m*M* $MgCl_2$, 1 m*M* Na_2ATP (Sigma Grade I), 1 m*M* dithiothreitol, and 0.2 m*M* phenylmethylsulphonylfluoride (pH 7.4). As the acidity of ATP batches varies, 100 m*M* KH_2PO_4 (usually 1/100 vol) can be added to adjust the pH. When triphosphates were added to PB, extra $MgCl_2$ was added in an equimolar amount (*see* **Note 1**).
2. PB*: PB* is PB plus human placental ribonuclease inhibitor (10 U/mL).
3. PB-BSA and PB*-BSA: These contain 100 mg/mL BSA.

4. PBS+: This is PBS plus 1% BSA and 0.2% gelatin; where indicated the pH was adjusted to 8.0.
5. PB-diluted: This is 1 vol of PB mixed with 2 vol of distilled H_2O.

2.9. Translation Mixtures

Different mixtures are used, depending on whether intact or permeabilized cells are to be labeled, which tag is incorporated into the nascent peptides, and on which localization approach is used (as discussed in **Subheading 3.**).

2.9.1. Labeling Intact Cells

Cells are grown in media containing the appropriate radiolabeled amino acid, plus the other unlabeled amino acids (*see* **Subheading 2.4.**).

2.9.2. Labeling Isolated Nuclei or Permeabilized Cells

The translation mixture should contain the appropriate tagged precursor, the other amino acids, ATP, GTP, and an energy-regenerating system. Our basic translation mixture contains PB*-BSA (which contains ATP; *see* **steps 1** and **3** in **Subheading 2.8.**), creatine phosphokinase (20 U/mL; *see* **Subheading 2.5.**), 2.5 m*M* phosphocreatine (*see* **Subheading 2.3.**), 0.25 m*M* GTP (*see* **Subheading 2.3.**), 0.5 mg/mL tRNA (*see* **Subheading 2.3.**), 200 U/mL aminoacyl-tRNA synthetase (*see* **Subheading 2.5.**), protease inhibitors for mammalian cells (*see* **Subheading 2.5.**), and the various supplements indicated. In addition, $MgCl_2$ is always added to ensure that it is in equimolar amounts to any nucleotide triphosphates present in the reaction. As nuclear translation is closely coupled to transcription, translation is improved by adding the 0.25 m*M* CTP and UTP required for transcription to this mixture. When synthetases were omitted the biotin incorporation falls by only 22%.

3. Methods

Various related strategies are available for localizing nascent peptides. All rely on tagging nascent peptides with a label, and then measuring the relative concentrations of tagged peptides in the nucleus and cytoplasm. The strategies differ in the type of tag (e.g., radiolabel, biotin, fluor) and localization approach (e.g., biochemical fractionation, autoradiography, immunolabeling coupled with light microscopy, immunogold labeling and electron microscopy). This gives a wide range of possible combinations and therefore only selected examples are given here.

3.1. Growth in a Radiolabeled Amino Acid, Cell Fractionation, and Scintillation Counting

Here, cells are grown in a radiolabeled amino acid and nuclei isolated; then, the amount of radiolabel in the whole cells and the isolated nuclei are compared.

3.1.1. Cell Growth

10^7 HeLa cells were grown in suspension in 50 mL of MEM without leucine for (5 min), pelleted, and regrown in 0.5 mL of the same medium with [^{3}H]leucine (147 Ci/mmol; 1 mCi/mL); after 10 s, 50 mL of ice-cold PBS was added and the cells pelleted. Essentially, the same procedure is used for attached cells growing in the appropriate medium, but centrifugation is not required.

3.1.2. Isolation of Nuclei

Many approaches are available for isolating nuclei from mammalian cells; almost all use hypotonic (i.e., unphysiological) buffers and must be adapted to the particular cell used. In our study ***(1)***, we isolated nuclei from HeLa cells as follows:

1. 4–20 × 10^7 cells are washed in PBS, resuspended in 10 mL PB*-diluted, and incubated (2 min; 37°C) to swell them.
2. 40 mL of PB*-diluted is added, and swollen cells are re-incubated (15 min) at 4°C.
3. The cells are broken using 10–15 strokes with a Dounce homogenization to release 95–99% nuclei (assayed by phase-contrast microscopy).
4. Triton X-100 is added to 0.25% (v/v).
5. After 5 min, spin nuclei (250 g; 5 min) through PB* with 10% glycerol, and resuspend pellet gently in PB*-BSA. The resulting nuclei can then be checked for ribosomal contamination (*see* **Note 2**) and for the integrity of the nuclear membrane (*see* **Note 3**).

3.2. Isolated Nuclei or Permeabilized Cells, "Run-On" Translation in Radiolabeled Precursors, and Scintillation Counting or Gel Electrophoresis

Here, permeabilized cells or nuclei isolated as in **Subheading 3.1.2.** are incubated in a "translation mixture" containing all the components required for translation (including a radiolabeled precursor), and the amount of radiolabel incorporated into peptides is measured by scintillation counting or autoradiography after gel electrophoresis.

3.2.1. Cell Permeabilization

Once nuclei have been isolated as in **Subheading 3.1.2.**—or cells permeabilized as described next—endogenous precursor pools are depleted by washing, and exogenous precursors must be added back to a concentration that gives the required rate of elongation. In the absence of a natural precursor, a modified precursor that is recognized as a substrate by the endogenous enzymes is then incorporated relatively efficiently. Cells can be permeabilized in many different ways, for example, using proteins (streptolysin O, α-toxin) or detergents ***(3)***, which include (rough concentrations indicated): Triton X-100 (0.02–0.05%),

digitonin (0.01–0.02%), lysolecithin (0.02–0.05%), and saponin (0.01–0.02%). We currently use saponin because it is sufficiently gentle that lysis is easily controlled; moreover, it preserves nuclear structure well ***(4)***. We also use this reagent in the presence of BSA to improve the structure further, but then higher concentrations of saponin must be used. Both saponin and BSA are mixtures of biomolecules that vary from batch to batch. Therefore, we routinely titrate saponin levels and choose those that give 95% lysis (assessed as below) with each batch of BSA.

1. Prepare a twofold dilution series of detergent in PB*-BSA.
2. Assess the level of permeabilization using trypan blue exclusion. Add 50:l 1% trypan blue in PB*-BSA to a coverslip; after 2 min, inspect by light microscopy; score the percentage of permeabilized, dark blue cells.
3. Choose the detergent concentration that permeabilizes >95% cells. If cells detach from coverslips during washing, use a lower concentration of detergent. Excess saponin also reduces translational activity as progressively more ribosomes are lost from the cytoplasm. It can also disrupt the integrity of the nuclear envelope (*see* **Note 3**).

3.2.2. A Basic "Translation Mixture" for "Run-On" Translation

The translation reaction involves incubating nuclei or permeabilized cells with the appropriate labeled precursor, the other amino acids, ATP, GTP, and an energy-regenerating system. Our basic translation mixture contains PB*-BSA (which contains ATP), creatine phosphokinase (20 U/mL), 2.5 m*M* phosphocreatine, 0.25 m*M* GTP, 0.5 mg/mL tRNA, 200 U/mL aminoacyl-tRNA synthetase, protease inhibitors for mammalian cells, and the various supplements indicated. In addition, $MgCl_2$ is always added to ensure that it is in equimolar amounts to any nucleotide triphosphates present in the reaction. Because nuclear translation is closely coupled with transcription, translation is improved by adding the 0.25 m*M* CTP and UTP required for transcription to this mixture. When synthetases were omitted the biotin incorporation falls by only 22%.

3.2.3. Translation Reaction Using Isolated Nuclei or Permeabilized Cells

Isolated nuclei or permeabilized cells are incubated in the basic translation mixture (**Subheading 3.2.2.**) supplemented with the appropriate labels as required; at the end of the reaction, samples are denatured, nascent peptides precipitated on to filter discs, washed thoroughly to remove unincorporated label, and the amount of radiolabel measured by scintillation counting ***(5)*** or by autoradiography after resolving nascent peptides by gel electrophoresis ***(1)***.

1. 2X Concentrates of permeabilized cells (8–50 × 10^6/mL) or nuclei (4–20 × 10^7/mL) in suspension and the basic translation "mixture" (plus any supplements and

inhibitors as required) are preincubated separately (3 min, 27.5°C). In general, the supplements include one radiolabeled amino acid, plus all the other unlabeled amino acids. Examples of such supplements include the following: (1) 5 μ*M* L-[4,5-^{3}H]lysine (86 Ci/mmol) + 50 μ*M* amino acids minus lysine, (2) 5 μ*M* L-[4,5-^{3}H]leucine (147 Ci/mmol) + 50 μ*M* amino acids minus leucine, (3) 1 μ*M* biotin-lysine-tRNA from brewer's yeast + 5 μ*M* L-[4,5-3H]leucine (147 Ci/mmol) + 50 μ*M* amino acids minus lysine, and (iv) 200 μCi/mL L-[^{35}S]methionine (0.5 Ci/mmol) + 50 μ*M* amino acids minus methionine.

2. The two are mixed, and incubated together (27.5°C) for the appropriate time.
3. For scintillation counting, reactions are stopped by transferring 100-μL samples to 350 μL 2% sodium dodecyl sulfate plus 50 μL 5 *M* NaOH. After incubation at 37°C for at least 30 min, 100 μL of this mixture is spotted on to glass fiber discs, the discs washed successively in 5% trichloroacetic acid (10 changes), ethanol (2 changes), and ether dried and their radioactivity estimated by scintillation counting.
4. For gel electrophoresis and autoradiography, the transcription reaction can contain [^{35}S]methionine. Then, nascent [^{35}S]peptides in 250-μL reaction mixture are removed and added to 10 mL of PB* to stop the reaction. After pelleting, cells or nuclei are rewashed in 10 mL of PB* to remove most unincorporated label. Now, DNA in the pellet of cells or nuclei is removed. Cells or nuclei are resuspended in 150 μL of PB*-diluted plus 1 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 25 U/mL human placental ribonuclease inhibitor, 100 U/mL RNase-free DNase, and protease inhibitors; after incubation (37°C, 10 min) the digestion is stopped by adding 100-μL sample buffer used for electrophoresis. [^{35}S]proteins from 10^5 cells or 3×10^5 nuclei are run on a 10–20% gradient polyacrylamide gel and an autoradiograph of the gel prepared ***(4)***.

3.3. Incorporation of Radiolabels Assessed by Autoradiography

Autoradiography can be used to detect sites of translation after incubating cells with radiolabeled amino acids (e.g., [^{3}H]leucine). This approach has some disadvantages:

1. It is rather specialized and technically demanding.
2. Detection of translation sites, rather than distant sites where the translation products might accumulate subsequently, requires very short pulses as the translation rate is so rapid (peptides are extended by approx 5 residues/s in vivo). Because the cellular pools of unlabeled amino acids are relatively high, little radiolabel is incorporated during short pulses, necessitating lengthy autoradiographic exposures. This problem can be mitigated by the use of isolated nuclei or permeabilized cells, where cellular pools are washed away (**Subheadings 3.1.2.** and **3.2.1.**).
3. The path-length of the β-particles emitted by ^{3}H is so long that autoradiographic grains often lie hundreds of nanometers away from the incorporation site.
4. Labeling other antigens is technically difficult.

3.3.1. Whole Cells

Procedures for labeling translation sites require various manipulations and are applied with difficulty to cells free in suspension; therefore, an approach involving cells attached to coverslips is described.

1. Wash glass coverslips in 70% ethanol for 15 min, rinse twice with distilled H_2O, drain, blot dry with Whatman no. 1 filter paper and heat sterilize (180°C dry heat for 12 h). If the cells tend to detach from the coverslip during subsequent manipulation, coverslips can be coated with gelatin or poly-L-lysine before plating to improve cell attachment.
2. Place dry coverslips in tissue-culture dishes.
3. Seed cells in the dish and allow to adhere. Best results are obtained with well-spread cells, covering 30–50% of the coverslip during labeling.
4. Grow cells in DMEM overnight.
5. Substitute the media for DMEM without leucine, incubate for at least 1 h and then add 50 μ*M* L-[4,5-^{3}H]leucine (147 Ci/mmol) and incubate for 2 min or longer.
6. Fix in 4% paraformaldehyde in 250 m*M* HEPES pH 7.4, 20 min at 4°C.
7. Wash in 5% TCA, rewash in H_2O, and dry.
8. Cover with emulsion (dipping) film, expose for 3 d, and develop.
9. Count grain numbers over nucleus and cytoplasm.

3.3.2. Permeabilized Cells

1. Incubate permeabilized cells (prepared as described in **Subheading 3.2.1.**) with translation mixture supplemented with [^{3}H]lysine (100 μCi/mL; 86 Ci/mmol) + 50 μ*M* amino acids minus lysine.
2. Fix cells in 4% paraformaldehyde in 250 m*M* HEPES, pH 7.4; 20 min at 4°C.
3. Wash in 5% TCA, rewash in water, and dry.
4. Prepare autoradiographs as in **Subheading 3.3.1.**

3.4. Incorporation of Biotin-Lysine-tRNA Assessed by Immunofluorescence

The immediate precursor—biotin-lysine-tRNA^{Lys}—is accepted by the translation machinery and incorporated into the growing peptide chain ***(6)***. Therefore, translation sites can be localized with high resolution by allowing permeabilized cells to elongate nascent peptides by only a few residues in the presence of biotin-lysine-tRNA^{Lys} and then immunolabeling the resulting biotin peptides with fluors ***(1)***.

1. Cells on coverslips are permeabilized as in **Subheading 3.2.1.**
2. Permeabilized cells are incubated in the basic translation mixture supplemented with 1 μ*M* biotin-lysine-tRNA from brewer's yeast + 50 μ*M* amino acids minus lysine.
3. Samples are fixed (20 min, 4°C) in 4% paraformaldehyde in 250 m*M* HEPES, pH 7.4.

4. Biotin-peptides are now indirectly immunolabeled with a fluor (Cy3 is chosen as an example). Coverslips are incubated (120 min; 20°C) with anti-biotin (5 μg/mL diluted in PBS+), washed in PBS, incubated with donkey anti-mouse IgG conjugated with Cy3 (0.5 μg/mL), and rewashed.
5. Nucleic acids are counterstained with 20 μ*M* TOTO-3, and the sample is mounted in Vectashield.
6. Images are collected using a confocal microscope ***(4)***.
7. The extent of nuclear translation can be estimated by quantitative analysis of the resulting images. Intensities are measured (e.g., using EasiVision or Metamorph software) over the cytoplasm, nucleoplasm, and a cell-free area of the slide (for measurement of background), and data exported to Excel (Microsoft) for background subtraction and analysis. Average intensities (confocal sections) of nucleoplasm and cytoplasm abutting the nucleus are determined, and multiplied by the volume fraction of the two compartments (i.e., 400 and 673 μm^3 in the case described in **ref. *7***) to obtain relative contents.

3.5. Using Permeabilized Cells and BODIPY-Lysine-tRNA

BODIPY-lysine-$tRNA^{Lys}$ also is accepted by the translation machinery and incorporated into the growing peptide chain ***(8)***. Therefore, translation sites can be localized more directly by allowing permeabilized cells to elongate nascent peptides by only a few residues in the presence of this immediate precursor and then viewing the resulting BODIPY-peptides (**Fig. 1**; **ref. *1***).

1. Cells on coverslips are permeabilized as in **Subheading 3.2.1.**
2. Permeabilized cells are incubated in translation mixture supplemented with a 1/10 dilution of BODIPY-lysine-tRNA (FluoroTect™ $Green_{Lys}$) + 50 μ*M* amino acids minus lysine.
3. Samples can now be viewed directly, or fixed (20 min; 4°C) in 4% paraformaldehyde in 250 m*M* HEPES, pH 7.4, and analyzed as in **Subheading 3.4.**

3.6. Incorporation of Biotin-Lysine-tRNA Assessed by Electron Microscopy

Biotin (**Subheading 3.4.**) can also be localized after immunogold labeling using the electron microscope ***(1)***.

1. Permeabilized cells are allowed to extend nascent peptides in biotin-lysine-tRNA and fixed, as in **Subheading 3.2.1.**
2. Samples are embedded in LR White and ultrathin sections cut.
3. Sections are incubated with PBS+ for 30 min.
4. Biotin-peptides are indirectly immunolabeled with mouse anti-biotin (10 μg/mL) in PBS+ for 2 h at 20°C.
5. Wash in PBS and immunolabel with antibodies conjugated with gold-particles (e.g., goat anti-mouse IgG conjugated with 5- or 10-nm particles; 1:25 dilution).
6. After contrasting with uranyl acetate, digital images are collected on an electron microscope ***(7)***.

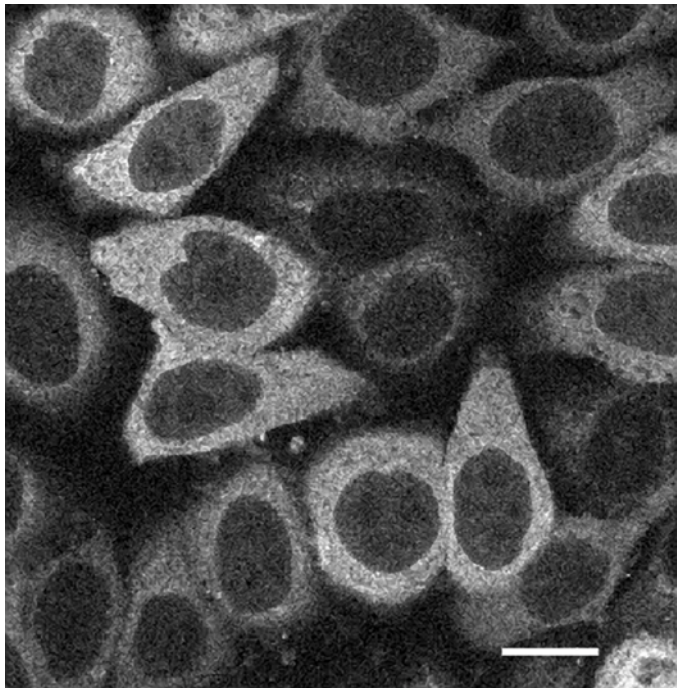

Fig. 1. Translation sites labeled with BODIPY. HeLa cells were permeabilized and allowed to extend nascent polypeptides by approx 20 residues in the presence of BODIPY-lysine-tRNA; after fixation in paraformaldehyde, cells were imaged using a confocal microscope ***(1)***. Nascent BODIPY-peptides are found mainly in the cytoplasm, but some are also found in nuclei. Bar: 10 μm.

4. Notes

1. The concentration of divalent cation must be carefully controlled. As little as 0.5 m*M* free Mg^{2+} causes the visible (by EM) collapse or aggregation of chromatin. Therefore, we use an equimolar Mg/ATP combination so that there is very little free Mg^{2+}; this preserves chromatin structure while supporting the action of Mg-dependent enzymes.
2. The extent of extranuclear ribosomal contamination can be determined (after fixation and staining with osmium) by electron microscopy of Epon sections and the application of standard stereological procedures ***(7)***. For example, nuclei isolated by the procedure described in **Subheading 3.1.2.** are associated with fewer than 5% extranuclear ribosomes seen in whole cells.
3. The integrity of the nuclear membrane in isolated nuclei can be monitored by incubation with a dextran conjugated with fluorescein that is too large to pass through the nuclear pore. For example, 96% nuclei isolated by the procedure described in **Subheading 3.1.2.** excluded a 500-kDa dextran conjugated with

FITC; this makes it unlikely that the nuclear envelope is sufficiently disrupted to allow larger cytoplasmic ribosomes to enter. In addition, 94% nuclei prepared similarly but that were not washed with Triton also excluded a 70-kDa dextran conjugated with FITC—but not fluorescein-12-ATP, which was used as a positive control.

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10

Immunopurification and Analysis of Protein and RNA Components of mRNP in Mammalian Cells

Fabrice Lejeune and Lynne E. Maquat

Summary

We describe a basic, fast, and reliable technique to isolate and characterize ribonucleoprotein (RNP) using antibody to a constituent protein. The antibody serves to immunopurify RNP from total cells or nuclear and cytoplasmic cell fractions under conditions that promote RNP integrity. The presence of other RNP proteins as well as transcripts can then be analyzed by Western blotting and reverse transcription polymerase chain reaction, respectively. RNase treatment before immunopurification can be used to assess the dependence of protein–protein interactions on RNA. We also describe a modification using β-mercaptoethanol that facilitates analyzing proteins that comigrate with antibody light or heavy chains.

Key Words

Immunopurification; mRNP; Western blotting; RT-PCR; RNase treatment; β-mercaptoethanol.

1. Introduction

From pre-mRNA synthesis to mRNA translation, proteins interact with transcripts during and as a consequence of numerous processes. These processes include pre-mRNA capping splicing and 3' end formation and mRNA transport, translation, and decay. A complex of proteins and RNA is called ribonucleoprotein (RNP), and characterizations of the constituent proteins and mRNA of mRNP have rendered great insight into pathways that generate as well as target mRNA (for reviews, *see* **refs. *1*** and ***2***). One of numerous methods to characterize mRNP involves immunopurification (IP) using antibody to a constituent protein. Once immunopurified, mRNP can then be separated into protein and RNA. The proteins can be identified using available antibodies, and the mRNAs can be identified using reverse transcription to synthesize

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cDNA followed by cDNA amplification using mRNA-specific primer pairs and polymerase chain reaction (PCR).

There are five major steps to the IP and analysis of mRNP: (1) cell lysis and extract preparation under conditions that do not dissociate or degrade mRNP, (2) mRNP binding to an antibody that reacts with an mRNP protein followed by capture of the antibody using a matrix (e.g., protein A or G bound to agarose or Sepharose), (3) elution of mRNP (along with antibody) from the matrix, (4) preparation of eluted proteins and RNA for analysis, and (5) protein analysis by Western blotting and RNA analysis by RT-PCR.

We describe here a method that we have used with success ***(3,4)*** to study the multiprotein exon junction complex (EJC) that is deposited 20–24 nucleotides upstream of exon–exon junctions as a consequence of pre-mRNA splicing; the EJC recruits the Upf2 and Upf3/3X, which are required for the mRNA surveillance pathway called nonsense-mediated mRNA decay (for reviews, *see* **refs. *5*** and ***6***; **Fig. 1**).

2. Materials

2.1. Initial Solutions

1. 1 *M* Tris-HCl, pH 7.4, store at 4°C.
2. 5 *M* NaCl, store at 4°C.
3. 20% Nonidet P-40 or Igepal CA 630 (Sigma), store at 4°C.
4. 10% Sodium dodecyl sulfate (SDS), store at room temperature.
5. 0.1 *M* Phenylmethyl sulfonyl fluoride (Sigma), store at 4°C.
6. 0.2 *M* Benzamidine (Sigma), store at 4°C.
7. Glycerol 99% (Sigma), store at room temperature.
8. 0.5 *M* Tris-HCl, pH 6.8, store at 4°C.
9. 1.5 *M* Tris-HCl, pH 8.8, store at 4°C.

2.2. Cell Extract Preparation

1. Phosphate-buffered saline (PBS): 81 m*M* sodium phosphate dibasic anhydrous (Sigma), 15 m*M* potassium phosphate monobasic anhydrous (Sigma), 27 m*M* potassium chloride (Sigma), and 1.4 *M* sodium chloride (Sigma), store at 4°C.
2. NET-2 buffer: 50 m*M* Tris-HCl, pH 7.4; 300 m*M* NaCl; 0.05% NP-40; 1 m*M* phenylmethyl sulfonyl fluoride; 2 m*M* benzamidine. Make fresh and keep on ice.
3. RNasin-RNase inhibitor (Promega).
4. Branson sonifier (Model 450) or comparable sonicator.

2.3. RNase Treatment

1. Ribonuclease A at 10 mg/mL (Sigma), store at –20°C.
2. Bovine serum albumin at 10 mg/mL (New England Biolabs), store at –20°C.

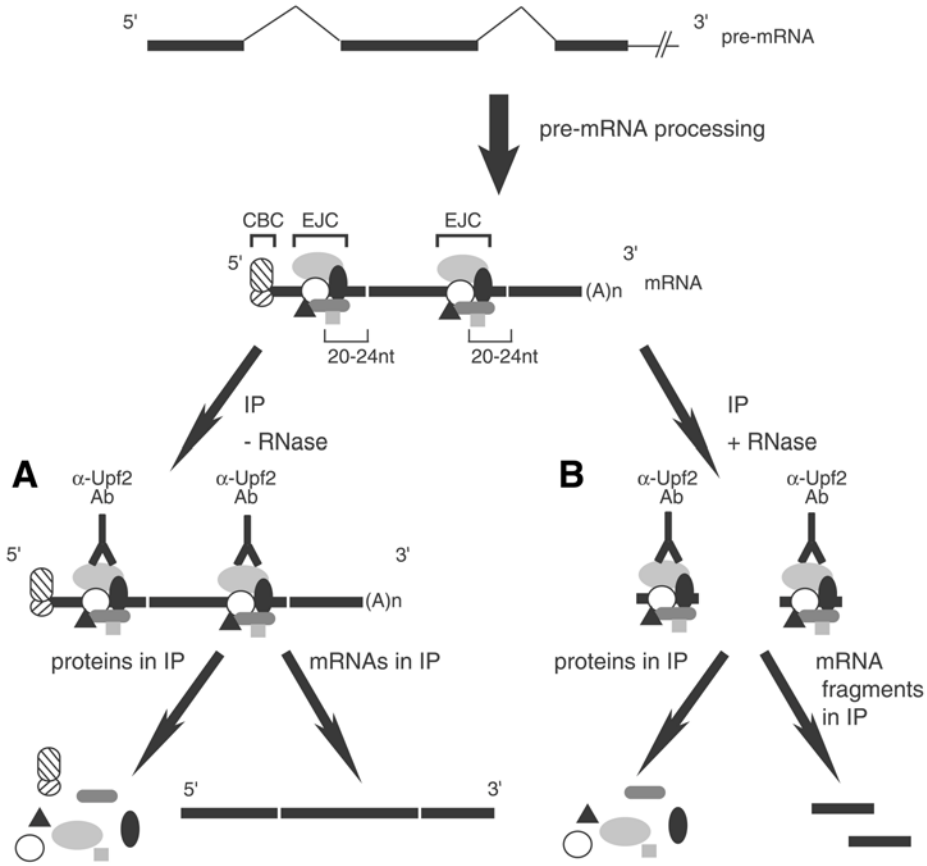

Fig. 1. The principles of mRNP IP. **(A)**, The IP of one component of the EJC, in this case Upf2, leads to the co-IP of all proteins of this complex—even those that do not interact directly with the EJC—provided that mRNP integrity is maintained. For example, mRNP proteins such as the cap binding complex (CBC), which consists of cap binding protein (CBP) 80 and CBP20, will be present in the IP by virtue of their interaction with the same mRNA molecule as the EJC. **(B)**, In contrast, RNase treatment before IP purifies only those proteins that form an interaction with Upf2 that is stable in the absence of mRNA. For example, CBP80 and CBP20 would not be present in the IP.

2.4. Immunopurificaiton (IP)

1. Protein A-agarose (for antibody raised in rabbit; Roche) or protein G-agarose (for antibody raised in mouse; Roche), store at 4°C.
2. Yeast RNA at 100 mg/mL (Sigma), store at –20°C.
3. Sample buffer: 0.1 *M* Tris-HCl, pH 6.8; 4% SDS; 12% β-mercaptoethanol; 20% glycerol, store at room temperature.

2.5. RNA Extraction

1. RQ1 DNase-RNase free (Promega), store at –20°C.
2. Phenol/chloroform (1/1:vol/vol), pH 4.3; protect from light and store at –20°C.
3. Chloroform/isoamyl alcohol (24/1: vol/vol), store at 4°C.
4. Glycogen at 5 μg/μL, store at –20°C.
5. 3 *M* Sodium acetate, pH 5.2, store at 4°C.
6. 100% and 75% ethanol, store at –20°C.

2.6. RT-PCR

1. Superscript reverse transcriptase RNase H^- (Invitrogen), store at –20°C.
2. 5X First Strand Buffer (Invitrogen), store at –20°C.
3. 5 m*M* Deoxynucleotides (Promega), store at –20°C.
4. 100 m*M* Dithiothreitol (Invitrogen), store at –20°C.
5. Random hexamers at 0.5 μg/μL (Promega), store at –20°C.
6. *Taq* polymerase (Promega), store at –20°C.
7. Thermophilic DNA Polymerase 10X buffer (Promega), store at –20°C.
8. 25 m*M* $MgCl_2$ (Promega), store at –20°C.

3. Methods

3.1. Cell Lysis and Extract Preparation

1. For each IP, prepare four 150-mm dishes of adherent mammalian cells (e.g., Cos, Hela, or 293T cells) at 90–100% confluency (*see* **Note 1**). An additional four dishes are generally also prepared as a source of protein and RNA for analysis without IP to assess the IP efficiency.
2. Remove medium, wash cells twice with 5 mL of ice-cold PBS, and transfer cells using a cell lifter to a 50-mL Falcon tube, keeping cells on ice at all times to decrease protein and RNA degradation.
3. Add an additional 1 mL of ice-cold PBS to each dish and pool the residual cells with those removed in **step 2** of **Subheading 3.1.**
4. Pellet cells at 3000*g* for 10 min at 4°C.
5. Carefully remove the supernatant using a Pasteur pipet under vacuum.
6. Resuspend cells in 500 μL of NET-2 buffer (*see* **Notes 2–4**), and transfer to a 1.5-mL microfuge tube.
7. Sonicate cells (40 bursts of 1 s each) to fragment genomic DNA.
8. Pellet cellular debris at 10,000*g* for 10 min at 4°C and transfer the supernatant to a clean 1.5-mL microfuge tube (*see* **Note 5**).
9. At this point, extract from the equivalent of four dishes generally is reserved for the generation and analysis of protein and RNA before IP.

3.2. mRNP Binding to Antibody Followed by Immobilization on Protein A or G Agarose

1. Clear the lysate by rotating end-over-end for 30 min at 4°C with 50 μL of protein A or G agarose to remove nonspecific interactions with protein A or G agarose.

2. Incubate the cleared lysate with antibody for 1 h at 4°C using end-over-end rotation. For each antibody, the amount used should be empirically determined. Also, a comparable amount of a "control" antibody (i.e., one raised in the same organism as was the experimental antibody; *see*, e.g., normal rabbit serum [NRS] in **Figs. 2** and **3**) should be comparably used to control for nonspecific IP.
3. Continue the rotation for an additional 1.5 h at 4°C in the presence of 50 µL of protein A or G agarose that had been preincubated with 2 mg of yeast RNA for 1 h at 4°C. Preincubation, which can be performed in parallel with incubation of the cleared lysate with antibody, serves to preclude nonspecific interactions between protein A or G agarose and RNA in the cleared lysates.
4. Pellet the protein A or G agarose at 3000*g* for 3 min at 4°C.
5. Discard the supernatant and wash the beads with 1 mL of NET-2 buffer (*see* **Note 4**) at least five times, discarding the wash each time.

3.3. mRNP Elution

1. Add 50 µL of sample buffer 2X (*see* **Note 6**) to the protein A or G agarose.
2. Incubate 5 min at room temperature and, subsequently, vortex for 1 min.
3. Pellet the agarose at 10,000*g* for 3 min at 4°C.
4. Collect the supernatant for analysis (*see* below).

Generally, a fraction (1/10) of each IP is sufficient to detect the presence of a cellular protein by Western blotting. The efficiency of IP can be determined by comparing the amount of protein present before IP (**step 9** in **Subheading 3.1.**) relative to the amount of protein present after IP.

3.4. Extraction of RNA From IP

1. Isolate 20 µL of the IP (**step 4** in **Subheading 3.3.**) and add 180 µL of H_2O.
2. Add 100 µL of phenol/chloroform and vortex for 5 min or more.
3. Separate the phases for 5 min at 10,000*g* and 4°C.
4. Collect the upper phase and add 100 µL of chloroform/isoamyl alcohol.
5. Vortex for 5 min or longerand separate the phases for 5 min at 10,000*g* and 4°C.
6. Collect the upper phase and precipitate the RNA in 300 m*M* sodium acetate, pH 5.2, 5–10 µg of glycogen, and 2.5 vol of 100% ethanol for 2 h or longer at –20°C.
7. Pellet the RNA for 30 min at 10,000*g* and 4°C.
8. Wash the pellet with 75% ethanol.
9. Dry the pellet for 3 min under vacuum.
10. Resuspend the pellet in 20.5 µL of H_2O and treat with DNase.

3.5. DNase Treatment

1. Incubate the RNA for 30 min at 37°C in a final volume of 30 µL with 1X RQ1 DNase buffer, 12 m*M* of dithiothreitol, 20 U of RNasin, and 1–2 U of DNase.
2. Add 170 µL of H_2O and extract and precipitate the RNA as described in **steps 2–4** of **Subheading 3.4.**
3. Collect the upper phase and precipitate the RNA in 250 m*M* NaCl and 2.5 vol of 100% ethanol at –20°C for 2 h.

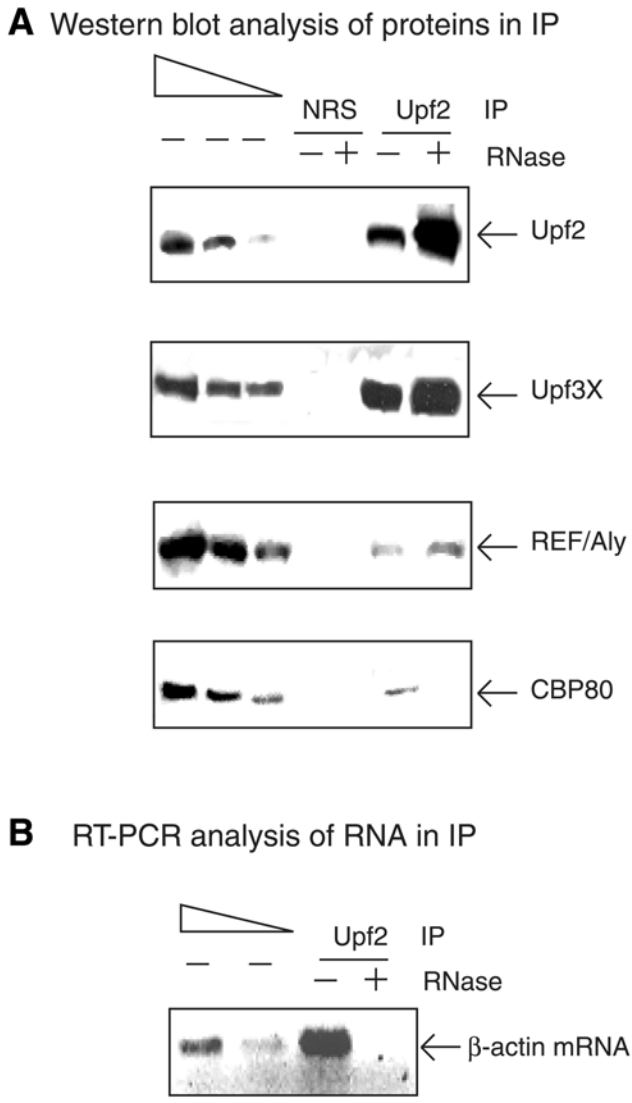

Fig. 2. Analysis of the proteins and mRNA that comprise mRNPs that IP with anti-Upf2 antibody using, respectively, Western blotting and RT-PCR. (**A**), The IP of Upf2 from total Cos-cell extract using anti-Upf2 antibody reveals the presence of Upf2, Upf3X and REF/Aly even in the presence of RNase, indicating that the interaction of Upf2 with Upf3X and REF/Aly is stable in the absence of intact RNA. In contrast, CBP80 is present only in absence of RNase treatment. The absence of Upf2, Upf3X, REF/Aly, and CBP80 in the IP using NRS instead of anti-Upf2 antibody corroborates the specificity of the IP. The three left-most lanes represent twofold dilutions of cellular

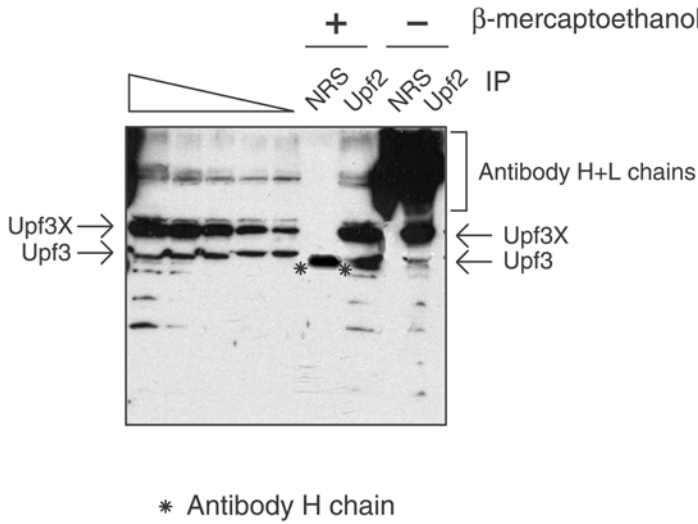

Fig. 3. Analysis of an approx 50-kDa protein after IP. Western blot analysis of Upf3X (which migrates at 68 kDa) and Upf3 (which migrates at 58 kDa) in an IP using anti-Upf2 antibody or, as a control for IP specificity, NRS, where elution from antibody-bound protein A agarose was performed either in the presence (+) or absence (–) of β-mercaptoethanol. In presence of β-mercaptoethanol, the heavy (H) chain of the antibody precludes detection of Upf3. In contrast, in the absence of β-mercaptoethanol, both H and light (L) antibody chains migrate slower than Upf3, allowing for its detection.

4. Pellet the RNA for 30 min at 10,000*g* and 4°C.
5. Wash the pellet with 75% ethanol.
6. Dry the pellet for 3 min under vacuum.
7. Resuspend the pellet in 25 μL of H_2O and calculate the OD at 260 nm.

3.6. Reverse Transcription

1. Denature half of the RNA preparation for 2 min at 95°C and rapidly quench on ice water.
2. Incubate the RNA for 2 h at 37°C with 1X first strand buffer, 10 m*M* of dithiothreitol, 0.5 m*M* of dNTP, 400 ng of random hexamers, 60 U of Superscript reverse transcriptase RNase H^-, and 12 U of RNasin in a final volume of 25 μL.

Fig. 2. (*continued*) protein before IP. These dilutions are used to determine the efficiency of IP, which in this experiment was 10% of cellular Upf2. **(B)**, The efficiency of RNase treatment can be assessed by using RT-PCR to analyze for the absence of, e.g., full-length β-actin mRNA. As expected, full-length β-actin mRNA is present in the IP that was not treated with RNase A. The two left-most lanes represent twofold dilutions of cellular RNA before IP.

3.7. Polymerase Chain Reaction

1. Using half of the reverse transcription reaction, add 1X thermophilic DNA polymerase buffer, 1.5 m*M* $MgCl_2$, 0.12 m*M* dNTP, 400 ng of each primer, 0.6 U of *Taq* polymerase, and 1 pmol of (α-^{32}P)-ATP (3000 Ci/mmol). Generally, 19 PCR cycles generate products in the linear range of amplification.
2. Electrophorese half of the PCR in a 5% polyacrylamide gel.
3. Dry the gel under vacuum at 80°C for 1 h and expose to X-ray film or a PhosphorImager screen.

4. Notes

1. Use of transiently transfected cells. If antibody to a particular mRNP protein of interest is not available, then this methodology can be adapted to analyze an epitope-tagged version of the protein that has been produced in cells from a transiently or stably introduced DNA expression vector. However, one notable advantage of analyzing endogenous cellular proteins relative to epitope-tagged versions of proteins is that a normal cellular abundance is more likely to ensure the presence of normal cellular mRNP. It is also possible to analyze mRNP that consists of mRNA deriving from transiently or stably introduced DNA expression vectors.
2. Nuclear and cytoplasmic fractionation. In cases where a comparison of mRNP composition between nuclear and cytoplasmic fractions is of interest, cells can be fractionated by numerous methods. One problem with many nuclear and cytoplasmic fractionation protocols is the requirement for a detergent concentration that may be sufficiently high to disrupt mRNP interactions. We have found that NE-PER kit (Pierce) preserves many mRNP interactions. However, we recommend resuspending the nuclear pellet in NET-2 buffer (rather than NER buffer) and proceeding as described in **step 6** of **Subheading 3.1.** Additionally, the purity of the nuclear fraction can be variable using the NE-PER kit.
   The purity of nuclear and cytoplasmic fractions can be assessed by Western blotting using antibodies that recognize exclusively nuclear or cytoplasmic proteins or RT-PCR to amplify primarily nuclear or cytoplasmic RNAs. Generally, contamination of the nuclear fraction with cytoplasm is a larger problem than contamination of the cytoplasmic fraction with nuclei ***(7)***. Proteins that should not be present in the nuclear fraction if nuclei are free of cytoplasm include Raf1, protein kinase Cα, and fodrin (Santa Cruz Biotechnology catalog; **refs. *8*** and ***9***). Additionally, U6 snRNA is mostly nuclear, whereas lys-tRNA is mostly cytoplasmic. It is often also desirable to check the integrity of the nuclear pore complex using the monoclonal antibody 414 (BAbCO), which recognizes a component of the nuclear basket (Nup153), a component of the nuclear pore within the nuclear envelope (p62), and two components of the cytoplasmic filaments (Nup214 and Nup358). Additionally, components of the endoplasmic reticulum, which is continuous with the nuclear envelope, can be assayed.
3. Cell growth and lysis. Exponentially growing cells are lysed using NET-2 buffer ***(10)*** rather than the commonly used radioimmune precipitation assay buffer,

which contains deoxycholic acid and SDS that may lead to the loss of some protein–protein interactions. NET-2 buffer contains 0.05% NP-40, which is sufficiently mild to preserve protein–protein and protein–RNA interactions.

4. Salt concentration for IP. If IP efficiencies prove to be too low, then the concentration of NaCl in the NET-2 buffer can be lowered to reduce the stringency of the IP. For example, if charged amino acids are critical for mRNP integrity, then a lower concentration of salt should increase the IP efficiency.
5. RNase treatment. It is possible to determine whether an mRNP protein that is recognized directly by the IP antibody maintains an interaction with other proteins that are present in the IP independently of RNA by treating the extract with RNase A before IP (**Figs. 1** and **2**). To this end, extract obtained after **step 8** in **Subheading 3.1.** is incubated for 30 min at 37°C in the presence of 10 µg of Ribonuclease A or, as a control, 10 µg of bovine serum albumin. The protocol is then continued starting with **step 1** in **Subheading 3.2.**
6. Analysis of proteins that comigrate with antibody light or heavy chain. The analysis of proteins migrating at approx 25 or 50 kDa is generally precluded by the presence of the light and heavy chain of the antibody used in the IP if antibodies used for Western blotting and the IP derive from the same species. To resolve this problem, the antibody can be covalently attached to Protein A or G agarose so that it will not be present in the eluate. However, because numerous antibody molecules will be inactivated once attached, larger amounts of antibody will be required. An alternative approach follows the steps outlined above but uses elution buffer that lacks β-mercaptoethanol so that antibody light and heavy chains maintain disulfide bonding and, therefore, comigrate at approx 150 kDa (which often manifests as a smear from approx 75 kDa toward the top of the gel; *see* **Fig. 3**). Additionally, **step 2** in **Subheading 3.3.** is performed on ice rather than at room temperature and samples are not heated immediately before electrophoresis.

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11

Gene Expression Analysis of Messenger RNP Complexes

Luiz O. F. Penalva, Scott A. Tenenbaum, and Jack D. Keene

Summary

RNA-binding proteins can organize messenger RNAs (mRNAs) into structurally and functionally related subsets, thus facilitating the coordinate production of gene classes necessary for complex cellular processes. Historically, in vitro methods primarily have been used to identify individual targets of mRNA-binding proteins. However, more direct methods are required for the identification of endogenously associated RNAs and their cognate proteins. To better understand posttranscriptional mRNA organization within the cell, we developed a systems biology approach to identify multiple-endogenous mRNA transcripts associated with RNA-binding proteins. This approach, termed ribonomics, takes advantage of high-throughput genomic array technologies that have greatly advanced the study of global gene expression changes. This chapter describes techniques for purifying mRNA–protein complexes (mRNPs) and identifying the associated mRNAs

Key Words

Genomics; microarrays; poly A binding protein (PABP); RNA-binding proteins; ribonomics; mRNA tagging; RNP; ribonucleoprotein; mRNA–protein complexes, posttranscriptional gene regulation.

1. Introduction

RNA-binding proteins play an important role in posttranscriptional gene expression and have the potential to generate genetic complexity through processes such as alternative splicing. RNA-binding proteins have also been shown to organize messenger RNAs (mRNAs) into subsets in a functionally relevant manner ***(1–4)***. Identifying the endogenous targets of RNA-binding proteins has been challenging and has primarily been limited to in vitro-based methods ***(5)***. In this chapter, we outline a strategy for the large-scale identification of mRNA subsets endogenously associated with mRNA-binding proteins. We

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have termed this approach to functional genomics, ribonomics, and have established four primary methodological steps: (1) isolation of targeted endogenous mRNA–protein complexes (mRNPs), (2) characterization of the protein and mRNA components associated with the targeted mRNP complexes, (3) identification of sequences or structural similarities among the detected mRNA targets, and (4) determination of functional relationships among the protein products encoded by the mRNA targets.

This chapter focuses on techniques applicable to the first two aspects of the ribonomic methodology, isolating mRNP complexes and identifying the associated mRNAs en masse using microarray analysis **Fig. 1**. Although the third and fourth features of ribonomic profiling have been explored in several studies ***(1–4)***, these issues are beyond the scope of this chapter.

2. Materials

2.1. Reagents

1. We recommend the use of distilled H_2O DNase and RNase free (Invitrogen, cat. no. 10977-015) to prepare the buffers and solutions described below. All tips and tubes must be RNase free. The use of RNaseZap (Ambion, cat. no. 9780.9782) is suggested to clean pipets and other materials.
2. Proteinase K. Prepare a solution at a concentration of 20 mg/mL. Store aliquots of 50 μL at –20°C and avoid freeze-thaw.
3. 1 *M* Dithiothreitol. Store aliquots of 20 μL at –20°C and avoid freeze-thaw.
4. 1X Phosphate-buffered saline (PBS).
5. Vanadyl ribonucleoside complexes (New England Biolabs, cat. no. 514029). Store aliquots of 50 μL at –20°C and avoid freeze-thaw.
6. Glycogen, molecular biology grade (La Roche, cat. no. 901-393). Store at –20°C.
7. Complete tablets, proteinase inhibitor (La Roche, cat. no. 1 697 498). Store at –20°C.
8. Bovine serum albumin (BSA)-fraction V proteinase free (La Roche, cat. no. 8 100-350). Store at 4°C.
9. RNase OUT 40 U/μL (Invitrogen, cat. no. 10777-019). Store at –20°C.
10. Protein A Sepharose 4 Fast Flow beads (Amersham Biosciences, cat. no. 17-0974-01). Store at 4°C.
11. Protein G Agarose beads (Sigma, cat. no. P4691). Store at 4°C.

2.2. Buffers

1. Polysome lysis buffer I: 10 m*M* HEPES, pH 7.0; 100 m*M* KCl; 5 m*M* $MgCl_2$; 25 m*M* ethylenediamine tetraacetic acid (EDTA); 0.5% Nonidet P-40. Add the additional components from **step 3** (below) before cell lysis.
2. Lysis buffer II: 10 m*M* HEPES, pH 7.0; 100 m*M* KCl; 5 m*M* $MgCl_2$; 25 m*M* EDTA; 0.5% Nonidet P-40; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 10% glycerol. Add the additional components from **step 3** (below) just before cell lysis.
3. Additional components for lysis buffers I and II: 2 m*M* dithiothreitol, 0.2% vanadyl ribonucleoside complex, one tablet of complete proteinase inhibitor/

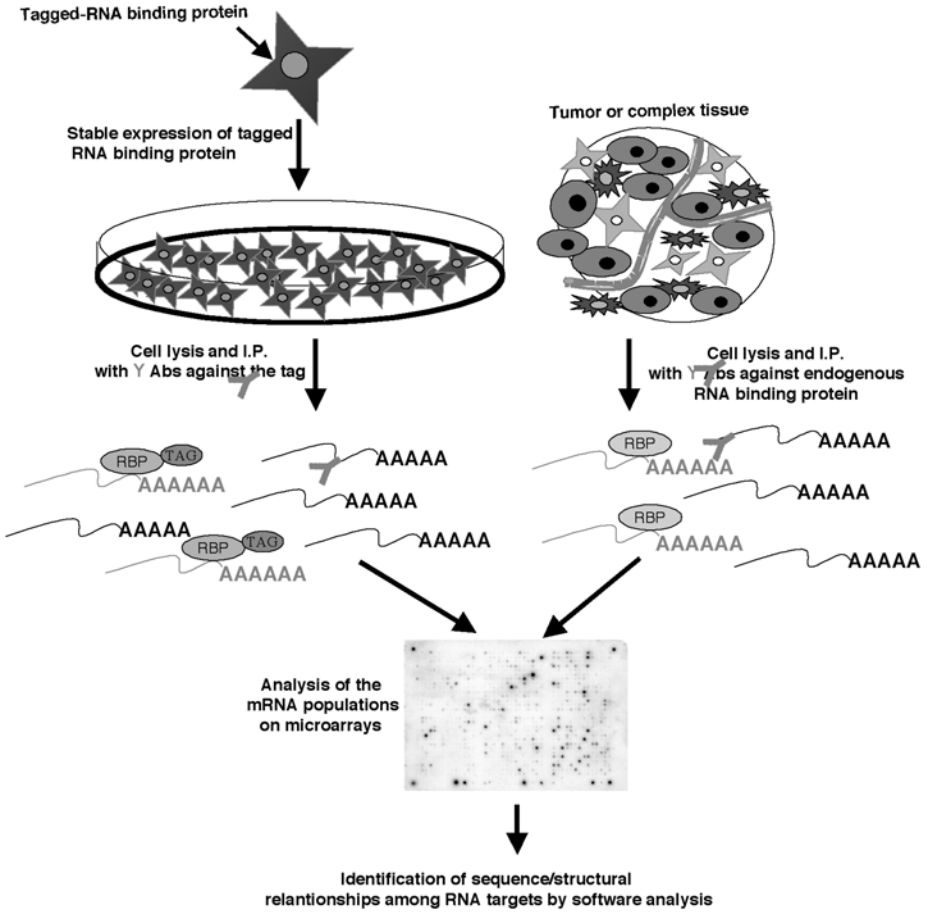

Fig. 1. Schematic of ribonomic analysis. Tagged or endogenous mRNA–protein complexes (mRNPs) are isolated from cell lines or a complex tissue such as a tumor. The mRNAs associated with the targeted mRNP complexes are then characterized by using an en masse assay, such as a cDNA microarray. Sequence and functional relationships amongst the detected mRNA targets and the protein products they encode can then be investigated.

50 mL of buffer, and 100 U/mL RNase OUT. Note that some of these components are added to the NT2 buffer before immunoprecipitation as noted in **step 3** of **Subheading 3.5.**

4. NT2 buffer: 50 m*M* Tris-HCl, pH 7.4; 150 m*M* NaCl; 1 m*M* $MgCl_2$; 0.05% Nonidet P-40.
5. Proteinease K digestion buffer: 100 m*M* Tris-HCl, pH 7.5; 10 m*M* EDTA; 50 m*M* NaCl; 1% sodium dodecyl sulfate.

3. Methods

3.1. Overview of Ribonomic Profiling

1. An overview of the methodology used to characterize targets of RNA-binding proteins is presented in **Fig. 1**. Monoclonal or polyclonal antibodies against endogenous or epitope-tagged RNA-binding proteins are used for immunoprecipitation reactions. (*See* **Fig. 2** for examples of gene expression profiles obtained using both endogenous and epitope tagged strategies.)
2. We have found that retroviral vectors work well for the production of stable cell lines expressing epitope-tagged RNA-binding proteins and suggest using the Pantropic Retroviral Expression System (Clontech, Palo Alto, CA).
3. Tagged-RNA binding proteins can also be expressed in transgenic animals. If a tissue-specific promoter drives the expression of the transgene, cell type-specific gene expression profiles can be obtained ***(6)***.

3.2. Preparation of RNP Lysates From Cultured Cells

1. Use 150-mm dishes to grow desired cell line (e.g., HeLa, 293, p19).
2. Wash twice with ice-cold PBS, harvest using a scraper, and pellet the cells by centrifugation at 4°C at 3000*g* for 5 min.
3. Generate the RNP cell extract by estimating the pellet volume and adding approx 1.5 vol of polysome lysis buffer I for isolating cytoplasmic RNPs or lysis buffer II for isolating both nuclear and cytoplasmic RNPs.
4. Pipet several times until the extract looks uniform, and spin in a microcentrifuge for 10 min (14,000*g*) at 4°C.
5. Remove the supernatant and save.
6. Resuspend the pellet in 1 vol of lysis buffer and repeat the pipetting and centrifugation and combine with the supernatant from **step 5** in **Subheading 3.2.**
7. Freeze the cell extracts in aliquots of 200–400 μL and stored at –80°C. Extracts typically range in concentration from 10–50 mg/mL of total protein, depending on the cytoplasmic volume of the cell type being used.

3.3. Preparation of Tissue or Tumor Cell RNP Lysates

1. Rinse freshly dissected material four to six times with ice-cold PBS.
2. Mince the tissue using a separation device such as a wire sieve, frosted glass slides or sharp tool while keeping the sample ice cold.
3. Homogenize the material in approx 2 tissue volumes of either lysis buffer I or II using a Dounce homogenizer.
4. Centrifuge the extract at slow speed to eliminate large cellular debris and then follow the steps as described in **Subheading 3.2.**

3.4. Antibody Coating of Bead Matrix

1. Swell or resuspend the desired amount of protein G agarose beads (for monoclonal antibodies) or protein A Sepharose beads (for rabbit serum–rabbit polyclonal) in 5–10 vol of NT2 buffer supplemented with 5% BSA.

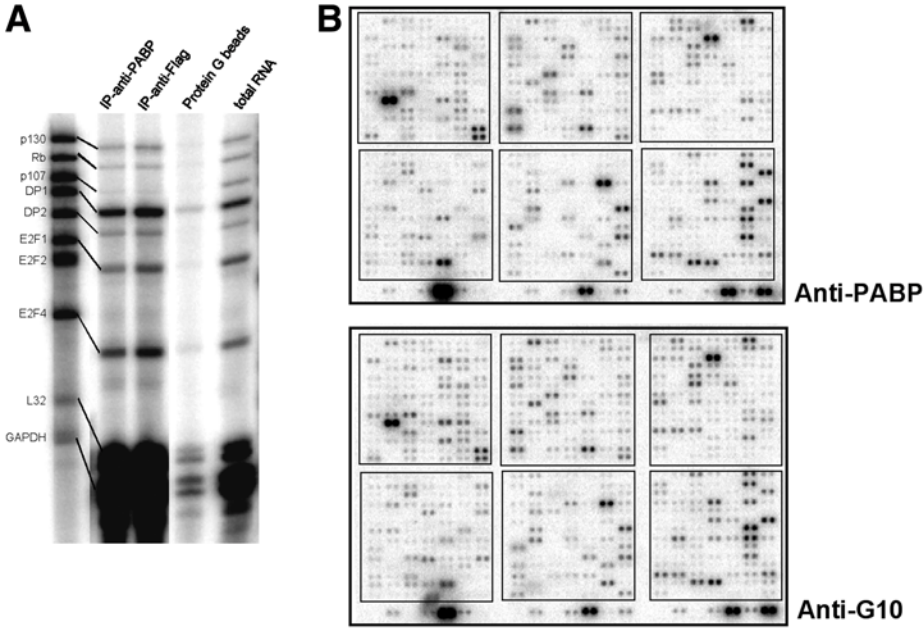

Fig. 2. Use of tagged-Poly A binding protein (PABP) to determine gene expression profiles. **(A)**, Human glioblastoma T98G cells, expressing a Flag-tagged PABP, were used to perform ribonomic profiling and a gene expression profile was obtained using an RNase protection assay (RPA) analysis. Immunoprecipitations were performed using anti-PABP serum, anti-Flag antibody, and nonantibody-coated protein-G beads as a control. Riboprobes were generated using the Pharmingen human tumor suppressor-1 template set (cat. no. 556161). **(B)**, Murine endothelial PY 4.1 cells expressing G10-tagged PABP were used to perform ribonomic profiling and a gene expression profile was obtained using microarray analysis. Immunoprecipitations were performed with anti-PABP rabbit serum and anti-G10 antibody. PABP associated mRNAs were detected using Atlas nylon mouse gene expression arrays (Clontech, cat. no. 7741–1).

2. Add the immunoprecipitating antibody or serum and incubate for at least 1 h at room temperature on a rotating device at room temperature or preferably overnight at 4°C (*see* **Notes 1–4**).
3. Beads coated with antibodies can be stored for several months at 4°C when supplemented with 0.02% sodium azide.

3.5. mRNP Immunoprecipitations

1. Centrifuge the RNP lysate in a Microfuge for 10 min (14,000*g*) at 4°C and transfer the supernatant to a new tube on ice.
2. Calculate the amount of antibody-coated beads necessary to perform the appropriate number of immunoprecipitations you are planning and rinse the beads several times with ice-cold NT2 buffer (*see* **Note 5**).

3. Resuspend the antibody-coated beads in NT2 buffer supplemented with 100 U/mL RNase OUT, 0.2% vanadyl ribonucleoside complexes, 1 m*M* dithiothreitol, and 20–30 m*M* EDTA.
4. The volume of resuspended beads in NT2 buffer should correspond to approx 10 times the volume of the RNP lysate being used (*see* **Note 6**).
5. Mix the resuspended antibody-coated beads several times by inversion, add the RNP lysate and tumble the immunoprecipitation reactions end-over-end at room temperature for 2–4 h or overnight at 4°C. A sample of the supernatant can be collected at the beginning of the incubation to serve as a total RNA control, which can assess RNase activity (*see* **Notes 7–9**).
6. After the incubation, spin the beads down and wash four to six times with approx 10–20 bed volumes of ice-cold NT2 buffer, vigorously mixing between each rinse.
7. Resuspend the washed beads in 600 µL of proteinase K digestion buffer plus 25 µL of the proteinase K stock solution and incubate for 30 min at 50°C, occasionally mixing.
8. Add 600 µL of phenol-chloroform to the bead suspension, vortex for 1 min, and centrifuge at 14,000*g* at 4°C for 10 min.
9. Remove the upper phase and repeat the extraction with 1 vol of chloroform.
10. Precipitate the RNA by adding 1 vol of isopropanol, 60 µL of 4 *M* ammonium acetate, 3 µL of 1 *M* $MgCl_2$, and 8 µL of glycogen.
11. Store samples at –80°C until ready for gene expression analysis.
12. To recover RNA, centrifuge samples for 30 min (14,000*g*) at 4°C and wash with 100 µL of 80% ethanol (*see* **Note 11**).

3.6. Options for Gene Expression Analysis

1. Targets of mRNA binding proteins can be identified and quantified by several methods. We prefer to use techniques in which mRNAs can be identified directly, without amplification by polymerase chain reaction or T7 RNA polymerase-based approaches.
2. Multiprobe-based RNase protection assays (PharMingen) are an ideal alternative for the optimization and high-throughput analysis of mRNP immunoprecipitations (*see* **Figs. 2A** and **3**).
3. We have identified many RNP-associated mRNAs using cDNA/genomic arrays and have found the CLONTECH Nylon Atlas cDNA Expression Array platform excellent for conducting ribonomic analysis (*see* **Figs. 2B**, **4**, and **5**). However, other array platforms have worked with varying success (*see* **Notes 12** and **13**; **refs. *3*, *4*,** and ***6***).
4. If gene expression analysis is performed using glass arrays that use Cy3 and Cy5 labeling or on Affymetrix arrays, we typically increase the amount of extract by three to five times that required for Atlas Nylon arrays.

4. Notes

1. We tested protein-A and protein-G beads from several different sources and tested the efficiency of RNP recovery. In **Fig. 3A**, we show a comparison of different

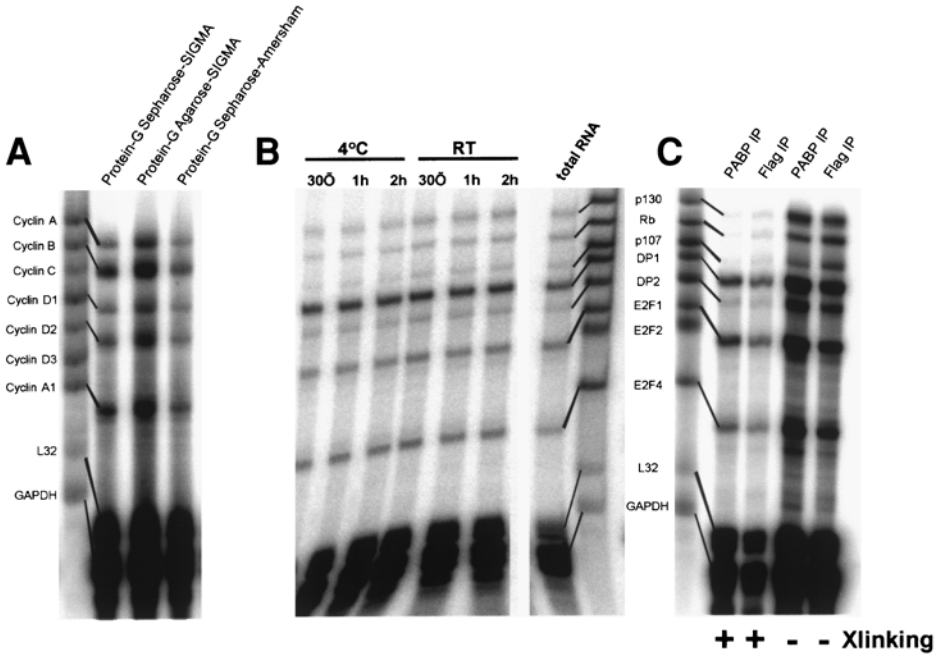

Fig. 3. Optimizing conditions for mRNP immunoprecipitations. RPA analysis of RNA from T98G cells expressing Flag-PABP was used to obtain gene expression profiles and quantify mRNA recovery. **(A)**, The efficiency of different brands of protein G beads in immunoprecipitating RNPs were compared. **(B)**, Immunopreciptitation reactions were performed at two temperatures and for different incubation periods. **(C),** The effect of formaldehyde crosslinking on immunoprecipitation efficiency was tested. Formaldehyde crosslinking conditions and preparation of cell extracts have been described previously ***(8)***. RPAs were performed with mRNA from T98G cells after immunoprecipitation using anti-Flag antibody **(A–C)** and anti-PABP serum **(C)**. Human cyclin-1 probe set (Pharmingen, cat. no. 556189; **A**) and Human tumor suppressor-1 probe set (Pharmingen, cat. no. 556161; **B** and **C**) were used to generate the radiolabeled riboprobes

Protein-G beads that were coated with anti-Flag antibody. A multiprobe RNase Protection Assay was used to quantify the amount of mRNA recovered in each case ***(2)***.

2. As a general rule, we recommend Protein-A Sepharose 4 Fast Flow beads (Amersham Biosciences) or Protein-A Sepharose CL-4B (Sigma) if you plan to use rabbit serum and Protein-G Agarose beads (Sigma) if you plan to use murine monoclonal antibodies.
3. Check the binding capacity of the beads and the antibody concentration and try to work in antibody excess to minimize background problems. Typically 2–20 μL

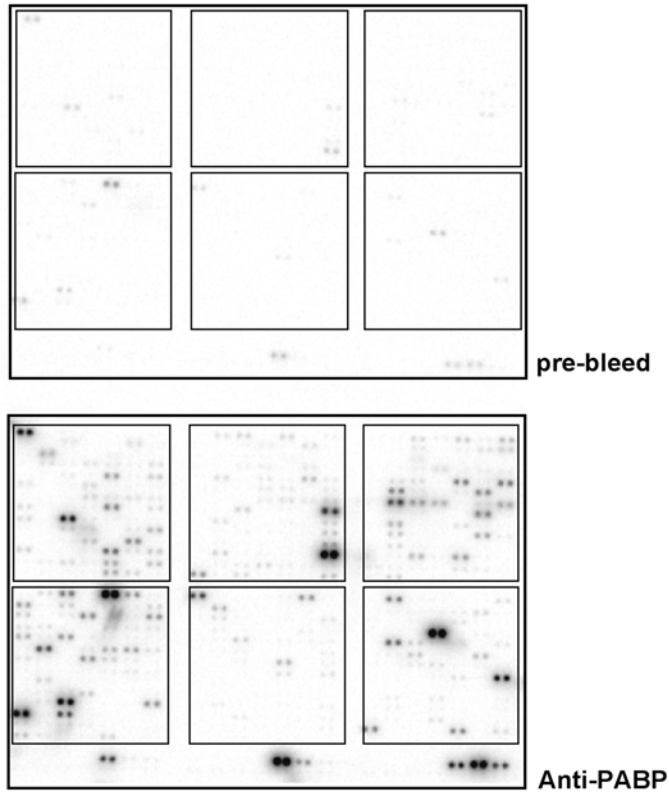

Fig. 4. Microarray analysis of the gene expression profiles obtained from PABP-associated mRNA populations. HeLa cell mRNA derived from immunoprecipitation with control prebleed serum (top) and anti-PABP rabbit serum (bottom) were radiolabeled and hybridized on human cDNA expression Atlas arrays (Clontech, cat. no. 7740-1).

of sera/immunoprecipitation reaction is used depending on the concentration of the antibody.

4. Antibody-coated beads can be prepared in bulk and stored at 4°C with 0.02% sodium azide.
5. Depending on antibody titer and RNP concentration, use 50–100 μL of packed antibody-coated beads and 100–400 μL of RNP lysate (approx 2–5 mg total protein) for each immunoprecipitation reaction.
6. Performing the immunoprecipitation reactions in larger volumes can decrease background problems.
7. We have noted that the temperature and length of incubation time can influence the efficacy of the immunoprecipitation reaction. Longer incubation times generally result in better RNP recovery (*see* **Fig. 3B**).

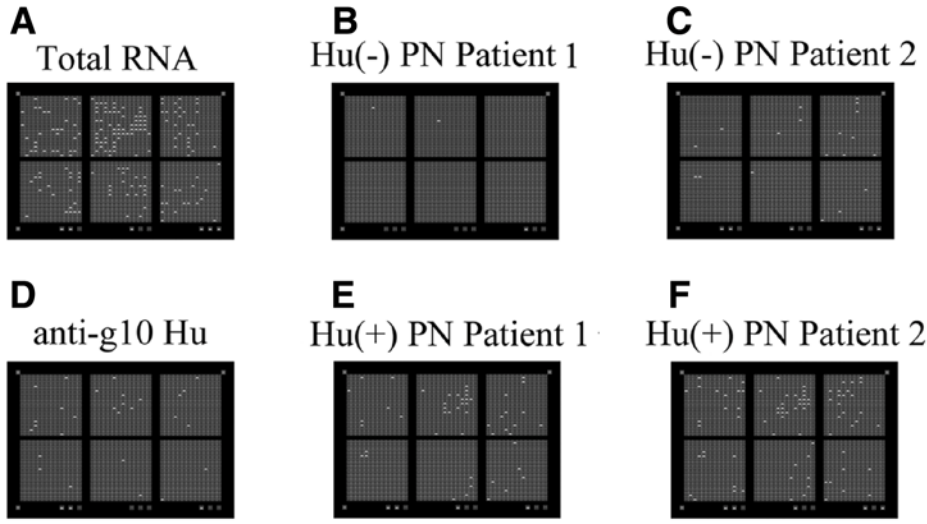

Fig. 5. Variation in the activity of human antibodies used to perform ribonomic profiling (*see* **Note 13**). Examples of several Hu-positive and Hu-negative paraneoplastic patient sera were used to immunoprecipitate mRNP complexes for ribonomic analysis. Precipitated mRNAs were analyzed on Nylon Clontech Atlas human 1.2 arrays using Clontech AtlasImage 2.02 and converted to computerized images. **(A)**, Total RNA; **(B)** and **(C)**, two negative Hu paraneoplastic patient sera; **(D)**, anti-g10 tagged Hu monoclonal antibody; **(E** and **F**), two positive Hu paraneoplastic patient sera.

8. Provided there is no RNA degradation or problems related to postlysis protein-mRNA exchange, immunoprecipitations should be performed for a minimum of 2–3 h at room temperature or overnight at 4°C. A low background is occasionally observed, which is presumably the result of nonspecific binding to the beads.
9. A concern when isolating mRNP complexes is the possibility of exchange of proteins and mRNAs. In principle, crosslinking agents, such as formaldehyde, could prevent this. However, we have found mRNA exchange to occur at a minimal level and crosslinking therefore to be unnecessary. In some cases, formaldehyde actually can interfere with subsequent mRNA detection methods (*see* **Fig. 3C**).
10. Several additional washes with NT2 buffer supplemented with 1–3 *M* urea can increase specificity and reduce background ***(5)***. However, it is important to first determine whether urea disrupts binding of the antibody to the target protein.
11. RNA pellets from isopropanol precipitations can detach from the centrifuge tube very easily. Extra care should be taken when resuspending the RNA pellet.
12. Depending on the quality of the antibody being used for ribonomic profiling, results and background can vary (*see* **Fig. 5**). Although nonspecific binding can occur, background also may arise from specific mRNA–antibody interactions ***(7)***.

13. Informative comparisons between total mRNA profiles and mRNP-associated mRNAs are frequently limited by the dramatic differences in signal intensity. There can be a large difference in the number of mRNA species detected in the total mRNA as compared with mRNP complexes, which makes most normalization approaches misleading. For this reason, we have typically not compared mRNP profiles with total RNA and suggest that totals be compared to other totals and mRNP immunoprecipitations compared with other mRNP immunoprecipitations.

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12

Using the λN Peptide to Tether Proteins to RNAs

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Summary

Proteins interacting with messenger RNAs (mRNAs) affect their nuclear processing, export, translation efficiency, stability, or cytoplasmic localization. Such RNA-binding proteins are often modular, containing RNA-binding domain(s) and other functional modules. To analyze the function of such proteins independent of their normal RNA-binding domains or to introduce effector modules to defined RNA-binding regions, a number of tethering approaches have been developed, often based on the use of large proteins and their specifically interacting RNA sequences. Here we report the use of a versatile system to tether proteins to mRNAs. The 22 amino acid RNA-binding domain of the λ bacteriophage antiterminator protein N (λN-(1-22) or λN peptide) is used to tag the protein of interest, and its specific 19 nt binding site (boxB) is inserted into the target RNA recruiting the properties of the fusion protein to the RNA. The major advantage of this system derives from the small size of the peptide and its target sequence, which facilitates cloning and its use for biochemical experiments and diminishes possible interferences with the fused protein. The chapter illustrates the use of this system to create dedicated mRNA-specific factors involved in processes, such as mRNA translation and nonsense-mediated mRNA decay.

Key Words

Bacteriophage λ; λN(1-22) peptide; nonsense-mediated mRNA decay; tethering approach; mRNA translation; RNA-binding domain; RNA-binding protein.

1. Introduction

A large number of proteins involved in messenger RNA (mRNA) processing, transport, translation, degradation, or localization interact with their target mRNAs either directly or join mRNP complexes to exert their respective biological activities. Such RNA-binding proteins often display a modular architecture that consists of RNA-binding domain(s) and effector sites. Several

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reports documented the functional properties of artificially tethered proteins to a reporter mRNA via a foreign RNA-binding domain ***(1–5)***. Tethered function analyses have been successfully used to investigate molecular aspects of protein function on target mRNAs ***(2)*** or to generate mRNA-specific "dedicated" versions of factors involved in general mRNA metabolism pathways ***(4,6)***. Here, two exemplary experimental setups for tethered function analyses using the N-peptide of bacteriophage λ are described. In contrast with larger RNA-binding proteins like the MS2-coat, IRP1, or U1A proteins, this small (22 amino acids) RNA-binding peptide potentially interferes less with the function and folding of the tagged protein. The assays presented in this chapter illustrate the method of λN-tethering, including: (1) tethering of eukaryotic translation initiation factors (eIFs) eIF4G, eIF4E, and eIF4A to a bicistronic reporter mRNA to analyze their function in mRNA translation initiation and (2) tethering of the hUpf3b protein to the 3'-untranslated region (UTR) of a reporter mRNA to assess its role in nonsense-mediated mRNA decay (NMD).

2. Materials

1. pSG-5 mammalian expression vector (Stratagene).
2. pCI-neo mammalian expression vector (Promega).
3. eIF4G, eIF4E, eIF4A, and hUpf3b cDNAs.
4. λN peptide (synthetic).
5. Oligonucleotide primers.
6. Cell culture medium and equipment.
7. Sodium dodecyl sulfate-polyacrylamide page electrophoresis (SDS-PAGE) equipment.
8. RNA-Gel equipment.
9. Hybridization oven.
10. Whatman 3MM chromatography paper.
11. Polyvinylidene difluoride membrane, for example, Immobilon P (Millipore, cat. no. IVPH00010).
12. Nylon membrane, for example, Nytran N (Schleicher & Schuell, cat no. 10416180).
13. Anti λN, antihuman eIF4A antibodies.
14. Anti rabbit IgG-horseradish peroxidase conjugated antibody.
15. Skimmed milk powder.
16. General materials for cloning of reporter and effector plasmids.
17. Material for calcium phosphate transfection.
18. TRIzol and TRIzol LS reagent (Invitrogen, cat. nos. 15596-026 and 10296-010).
19. Material and equipment for radiolabeled probe preparation.
20. Material and equipment for metabolic labeling ([^{35}S] methionine).
21. CAT ELISA kit (Roche Applied Science, cat. no. E1960).
22. Renaissance Western blotting reagent (NEN, cat. nos. NEL107, NEL105).
23. Protein A Sepharose (Amersham, cat. no. 17-0780-01).

24. Church buffer: 7% SDS, 0.5 *M* sodium phosphate, pH 7.2; 1 m*M* ethylenediamine tetraacetic acid.
25. Northern washing buffer: 1% SDS, 50 m*M* sodium phosphate, pH 7.2.
26. Western transfer buffer: 48 m*M* Tris-HCl, pH 8.3; 39 m*M* glycine, 0.04% SDS.
27. TTBS: 20 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl; 0.1% Tween-20.
28. Buffer Z: 100 m*M* Na_2HPO_4, pH 7.2; 10 m*M* KCl; 1 m*M* $MgSO_4$; 0.36% β-mercaptoethanol.
29. Lysis buffer I: Tris-HCl, pH 7.4; 300 m*M* NaCl; 1% Triton.
30. 2X BBS: 50 m*M* BES, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, pH 6.96.
31. Lysis buffer II: 50 m*M* Tris-Cl, pH 7.2; 150 m*M* NaCl; 0.5% NP-40; 5 m*M* vanadyl–ribosyl complex; 1 m*M* phenylmethyl sulfonyl fluoride.

3. Methods

This chapter is divided into two parts describing the use of tethering approaches to study, respectively, mRNA translation initiation factors functions and proteins potentially involved in human NMD.

3.1. Tethering Translation Initiation Factors to Bicistronic RNA Constructs

In eukaryotes, the heterotrimeric cap-binding complex eIF4F plays a critical role in recruiting ribosomes to mRNAs ***(7–9)***. Each of its three essential subunits plays a specific role: the eIF4E subunit binds the m7GpppN cap structure present at the 5' end of mRNAs; the scaffolding protein eIF4G has a central role, it interacts with multiple-binding partners bringing together the mRNA ends, initiation factors, and the small subunit of the ribosome; the ATP-dependent RNA-helicase eIF4A unwinds the secondary structure of the mRNA. Altogether, the binding of eIF4F to a capped mRNA permits the recruitment of ribosomes and allows translation initiation to occur. The tethering approach described here has been developed in human cell lines to analyze the ability of these three proteins to initiate translation independent of their natural recruitment to the mRNA in vivo. We are confident that it can be adapted to other cellular and cell-free systems.

3.1.1. Effector Plasmids

Mammalian expression vectors were designed to fuse the λN peptide to mutated forms of human eIF4E, eIF4G, or eIF4A. A mutant version of human eIF4E was used, with a tryptophan-to-leucine substitution at position 102 (W102L) that has been shown to abolish cap-binding activity ***(10)***. The second mutation used introduces a tryptophan-to-alanine substitution at position 73 (W73A) and abolishes eIF4E binding to eIF4G, another essential translation initiation factor ***(11)***. These proteins were fused to an aminoterminal λN peptide to generate the proteins λ4E-102 and λ4E-73-102 encoded, respectively,

by the plasmids pSG-λ4E-102 and pSG-λ4E-73-102. Finally, the sequence of mouse eIF4AI (**ref. *12***; which is identical to human eIF4AI at the amino acid level; **ref. *13***) was fused to the λN peptide to generate the chimeric protein λ4A (plasmid pSG-λ4A). The plasmid pSG-λ4G encodes the chimeric protein λN peptide, human eIF4GI. The effector plasmids were constructed on the basis of the pSG5 vector (Stratagene) allowing fusion protein expression under the control of the CMV promoter. The cloning strategies are described in greater detail elsewhere ***(2,5)***. The N-terminal sequence of all fusion proteins is: MDAQTRRRERRAEKQAQWKAANGGS, where the underlined amino acids represent the λN-(1-22) RNA-binding domain and three additional amino acids (two glycine and a serine) serve as a linker between the λN peptide and the fusion partner.

3.1.2. Reporter Vectors

The reporter mRNA, also expressed from the pSG5 vector, is a bicistronic transcript encoding the firefly luciferase (LUC) as an upstream cistron, and the open reading frame encoding the chloramphenicol acetyl transferase (CAT) downstream. The binding site of the λN-peptide, referred to as boxB, was cloned in the intercistronic region. The boxB is located 69 nt downstream of the LUC stop codon and 46 nt upstream of the CAT initiator codon (**Fig. 1**). Alternatively, the binding site for the MS2 coat protein (MSC) was inserted at the same position as a negative control. A mammalian expression vector (pCMVβ) encoding β-galactosidase (β-gal) is also included in the experiment, serving as a control for transfection efficiency. The cloning strategies for the used plasmids were described previously ***(2)***.

3.1.3. HeLa Cell Transfection

Transfection of HeLa cells is performed by calcium phosphate precipitation using 0.5 μg of the effector plasmids pSGλ4G, pSGλ4E-102, pSGλ4E-73-102, or pSGλ4A; 9 μg of reporter vectors pSGMSC or pSGboxB; and 3 μg of pCMVβ expressing β-gal (*see* **Note 1**).

3.1.4. Controls for Reporter mRNA and Fusion Protein Expression

3.1.4.1. Reporter mRNA Expression: Northern Blot Analysis

The integrity of the reporter mRNA and its level of expression were assessed by northern blot analysis.

1. Isolate total RNA from transfected cells using the Trizol reagent (Invitrogen) following the manufacturer's instructions.
2. Resolve 10 μg of total cellular RNA on a 1% formaldehyde-containing agarose gel and transfer onto a Nylon membrane (e.g., Nytran N, Schleicher & Schuell).

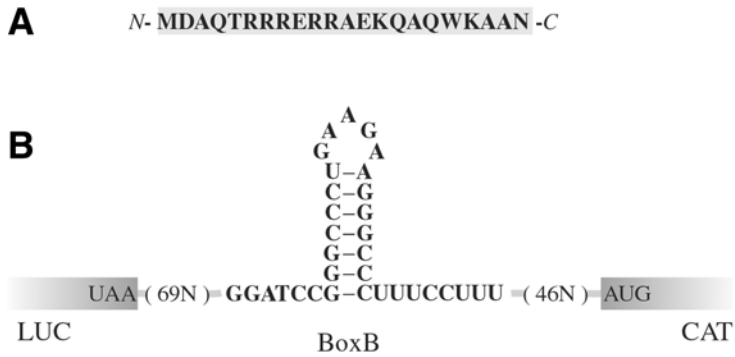

Fig. 1. Sequences of the λN peptide **(A)** and the boxB **(B)**.

3. Prepare radioactive probes specific for the reporter mRNA from linearized plasmids by random-primed labeling with α-^{32}P-dCTP using the Oligolabeling kit (Pharmacia) according to the manufacturer's instructions.
4. Hybridize the blots with the probe at 65°C overnight in Church buffer.
5. Wash the membrane three times with Northern washing buffer for 5 min at 65°C.

Figure 2A shows an autoradiography obtained after exposure of the membrane hybridized with different probes, for cells transfected with various amounts of pSGλ4E-102. The expression of λ4E-102 does not systematically affect the level of the bicistronic boxB mRNA (compare lanes 2–4 with lane 1 and the quantitative data stated below). Hence, the fusion protein λ4E-102 does not stimulate CAT expression by affecting the transcription or the stability of the reporter mRNA. Theoretically, the generation of uncapped monocistronic cleavage products of the bicistronic mRNA could also involve an λ4E-102-mediated activation of CAT expression. However, such shorter mRNAs of ≥1 kb were not detectable (**Fig. 2A**) even after longer exposures.

3.1.4.2. Effector Protein Expression: Western Blot Analysis

To assess the expression of the λ4G and λ4E-102, λ4E-73-102, and λ4A fusion proteins with a single antibody, antisera were raised against the λN-(1-22) peptide (*see* **ref. *5***). Western analysis of extracts from cells transfected with the different effector plasmids is performed as detailed below:

1. Lyse transfected cells using the CAT-ELISA kit (Roche Applied Science) following the manufacturer's instructions (*see* **Note 2**).
2. Resolve equal amounts of protein lysates on an SDS-containing polyacrylamide gel. Blot the gel onto a polyvinylidene fluoride microporous membrane, for example, Immobilon P (Millipore) by transfer in Western transfer buffer for 4 h at 350 mA using a Trans Blot SD (Bio-Rad).

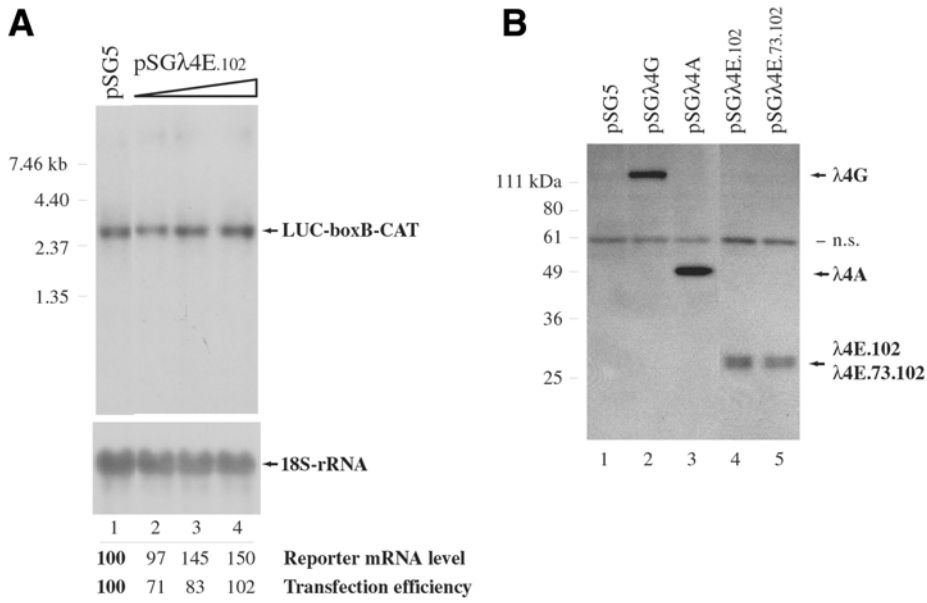

Fig. 2. Expression of the fusion proteins and of the reporter mRNA. **(A)**, The expression of λ4E-102 does not affect the levels and the integrity of the reporter mRNA. Total mRNA was extracted from cells cotransfected with pCMVβ, the bicistronic vector pSGboxB, and the empty vector pSG5 (lane 1) or 0.25 μg (lane 2), 0.5 μg (lane 3), or 1 μg (lane 4) of pSGλ4E-102 and subjected to Northern analysis. Consecutive hybridizations were performed with a probe, which recognizes both LUC- and CAT-coding regions of the bicistronic mRNA (upper panel), and with a probe for 18S ribosomal RNA (lower panel). The positions of RNA markers (sizes in kb) are shown on the left. The numbers below the autoradiograph indicates the amount of bicistronic mRNA relative to the control in lane 1 (set to 100%), after normalization for the 18S rRNA signal. The transfection efficiency data, measured by β-gal assay and processed in the same fashion, are stated below. **(B)**, Cells were cotransfected with pCMVβ and 0.5 μg of pSG5 (lane 1), pSGλ4G (lane 2), pSGλ4E-102 (lane 3), or pSGλ4E-73-102 (lane 4). Twenty micrograms of cell lysate was subjected to Western analysis. Molecular mass markers (in kDa) are indicated on the left, arrows indicate the positions of the fusion proteins. A nonspecific protein (n.s.) that crossreacts with the α-λN antibodies is indicated on the right. Transfection efficiency in lanes 2–4 varied no more than twofold.

3. Block the membrane with 5% nonfat skimmed milk in TTBS for 1 h at room temperature.
4. Incubate with α-λ antibodies (1/250 dilution) in TTBS for 1 h at room temperature.
5. Wash four times for 5 min in TTBS.

6. Incubate with secondary antibodies: anti-rabbit-IgG coupled to horseradish peroxidase (Amersham; 1/5000 dilution), wash and subject to *Renaissance* detection (NEN).

Figure 2B shows a representative immunoblot analysis of the expression of the studied fusion proteins and demonstrates their correct expression.

3.1.5. Functional Assays

The three assays described herein allow one to measure the expression of the downstream CAT cistron, which is normally translated very poorly. Determination of luciferase activity identifies potential effects of the tethering on the cap-dependent translation of the upstream LUC cistron and/or stability of the mRNA. The expression of the β-gal serves as a control for the transfection efficiency. This approach permits to assess rapidly and in a sensitive manner the ability of the tethered factors to specifically initiate the translation of the downstream cistron in vivo.

3.1.5.1. CAT Assay

Lyse-transfected cells using the CAT-ELISA kit (Roche Applied Science) and determine the CAT protein level following the manufacturer's instructions. Establish a CAT calibration curve using a CAT standard (Roche Applied Science).

3.1.5.2. Luciferase Assay

Use 0.5-μg protein extracts to measure the luciferase enzymatic activity using the Luciferase Assay System (Promega) following the manufacturer's instructions.

3.1.5.3. β-Gal Assay

Use 10 μg of the protein extract to determine the β-gal activity as follows:

1. Dilute 10 μg of extracts into 500 μL of buffer Z prewarmed at 37°C.
2. Add 100 μL of ortho-nitrophenyl-D-galactopyranoside (4 mg/mL in buffer Z) and incubate at 37°C.
3. When the reaction turns yellow, stop by the addition of 250 μL of Na_2CO_3 (1 *M*).
4. Measure the OD at 420 nm in a spectrophotometer.
5. Calculate arbitrary units of β-gal activity after normalization of the OD420 nm measurement by the time of the reaction.

After correction for transfection efficiency (β-gal activity), the results are expressed in relation to a cotransfection with the "empty" fusion protein expression vector pSG5 (termed "relative CAT and LUC expression"). **Figure 3** shows that the protein λ4E-102 mediates CAT expression at a level that is nearly identical to that of λ4G (approximately eightfold stimulation). The activation of CAT expression is specific because λ4E-102, like λ4G, fails to activate CAT

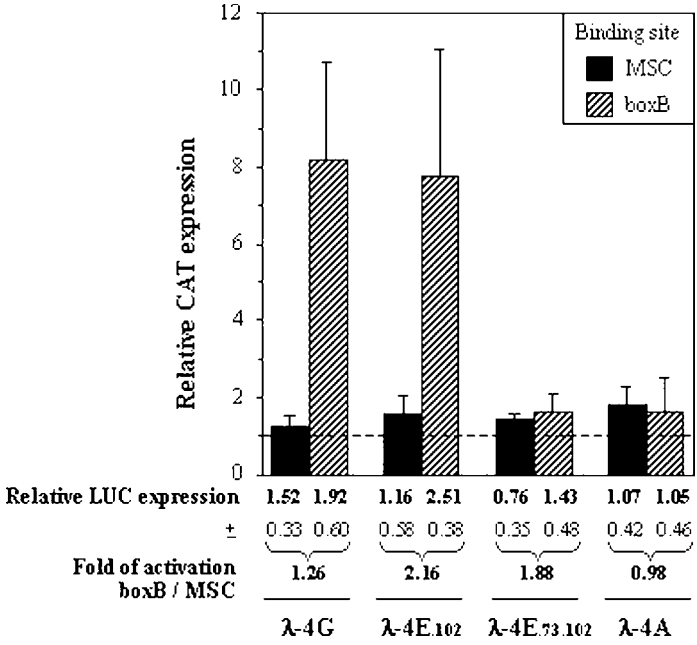

Fig. 3. The fusion protein λ4E-102 but notλ4E-73-102 or λ4A activates the expression of a downstream cistron in a binding site-specific manner. Effects of tethering the fusion proteins λ4G, λ4E-102, λ4E-73-102, and λ4A (respectively, from left to right) to the bicistronic reporter mRNA on CAT and LUC expression. Cells were cotransfected with 9 μg of the bicistronic vectors containing the specific boxB binding site (black bars) or MSC (dashed bars) as a control and 0.5 μg of the effector plasmids expressing λ4G, λ4E-102, λ4E-73-102, or λ4A. In all cases, 3 μg of a plasmid expressing β-gal is cotransfected to allow correction for transfection efficiency. Each bar represents the average of 4–7 independent experiments (with standard deviation) and gives CAT expression relative to the corresponding control transfection using the empty vector pSG5 (set to 1, dashed line). LUC expression data were equally processed and are given below the corresponding bars.

expression when the boxB is replaced by the binding site for the MSC as a negative control. To test whether the translational activation λ4E-102 involves eIF4G binding, we used the mutant λ4E-73-102. Expression of λ4E-73-102 fails to activate CAT expression (**Fig. 3**), demonstrating a requirement for eIF4G recruitment in λ4E-mediated translational activation. Finally, when λ4A is expressed, no binding site-specific stimulatory effect on the expression of CAT and LUC is detected. In particular, λ4A fails to activate CAT expression even when the specific BoxB site is present in the intercistronic space of the reporter mRNA (for a discussion of the results, *see* **ref. *5***).

3.1.6. Interaction of the Fusion Proteins with Cofactors

To investigate the mechanisms of action of the tethered fusion proteins, the ability of different λN-fusion proteins to interact with other endogenous eIF4F subunits in vivo was investigated. In addition, this experiment further confirms that the λN peptide does not perturb the folding of the studied proteins. For this purpose, cotransfected cells were metabolically labeled with [^{35}S] methionine, followed by immunoprecipitation analysis using anti λN antisera. The factors that coimmunoprecipitate with the fusion proteins are further analyzed by autoradiography or Western blot.

1. Wash the transfected cells with methionine-free RPMI.
2. Add 2.5 mL of methionine-free RPMI containing 5 μL [^{35}S] methionine (20 μCi/mL; Amersham) and incubate for 2 h at 37°C.
3. Wash cells with cold phosphate-buffered saline, collect, and lyse them by incubation on ice for 45 min in 300 μL of lysis buffer.
4. Eliminate cellular debris by centrifugation.
5. Use 100 μL of the lysate and 20 μL of a λN antibodies for immunoprecipitation in 1 mL of lysis buffer; incubate for 1 h at 4°C.
6. Add 80 μL of protein A Sepharose and incubate 1 h at 4°C.
7. Wash three times with lysis buffer I and elute by boiling in 15 μL of Laemmli buffer.
8. Process the samples for SDS-PAGE, followed either by autoradiography or immunoblot analysis.

The resulting autoradiography (**Fig. 4A**) shows the recovery of all λN-fusion proteins by the antisera. Importantly, a doublet of bands migrating at around 220 kDa is specifically coimmunoprecipitated with λ4E-102 (lane 2) and λ4A (lane 4) but not with λ4E-73-102 (lane 3) or λ4G (lane 5). These bands represent eIF4G and are indeed selectively observed only when the expressed fusion proteins retain an ability to bind eIF4G. A faint band, which is recovered with λ4E-102 and λ4G (lanes 2 and 5) but not with λ4E-73-102 and λ4A (lanes 3 and 4) can also be discerned at 45 kDa, suggesting that it may represent endogenous eIF4A. This was directly supported by western analysis of the same samples using antisera against eIF4A (**Fig. 4B**). These data show that λ4E-102, but not λ4E-73-102 can assemble into heterotrimeric eIF4F complexes. λ4A can form a complex with endogenous eIF4G, arguing against the possibility that the expressed fusion protein is grossly misfolded or somehow sequestered from the endogenous translation initiation factors.

The data presented in this chapter demonstrate the utility of the "dedicated initiation factor" approach as an in vivo strategy to study the translation initiation pathway and to delineate functionally important domains of and interactions between initiation factors. The "dedicated initiation factor" assay also

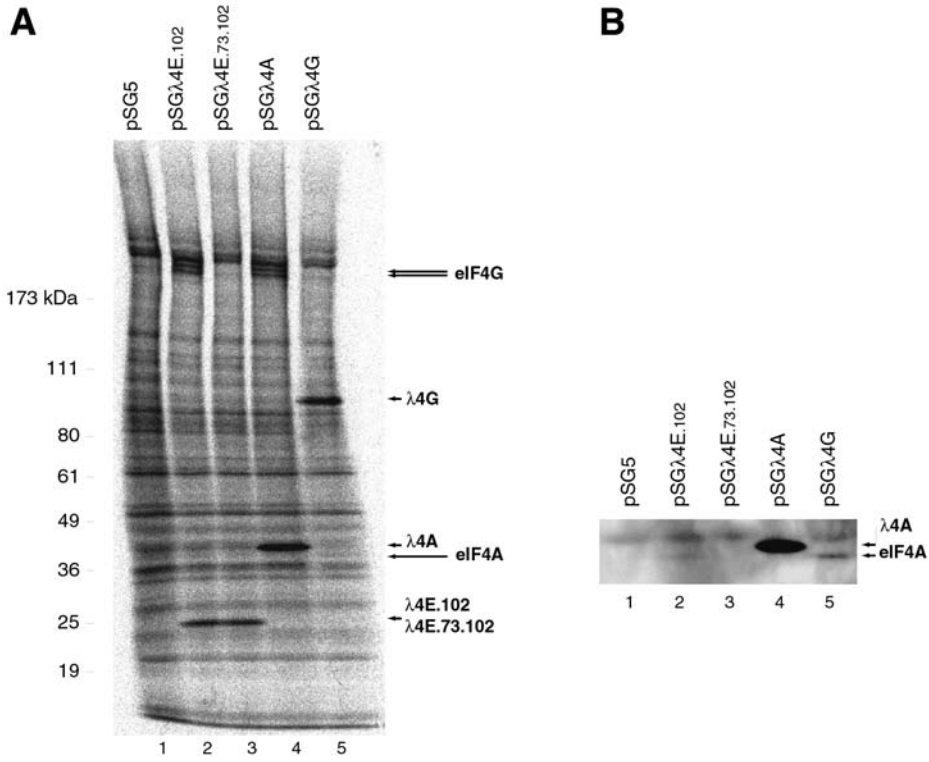

Fig. 4. Interaction of the fusion proteins with endogenous eIF4F subunits. Cells were transfected and protein extracts were prepared from metabolically labeled cells for coimmunoprecipitation. The samples were then resolved on a linear SDS-PAGE gradient gel (5% to 15%). **(A)**, The labeled proteins were detected by autoradiography. The position of the different fusion proteins, as well as the proteins which coimmunoprecipitate with them, are indicated. **(B)**, Equal aliquots of the same samples were subjected to Western analysis with α-4A antibodies. The positions of the transfected λ4A protein as well as the endogenous eIF4A are indicated by arrows on the right.

provides a versatile tool for detailed mutational analyses of initiation factor function in vivo without perturbing general cellular translation.

3.2. Tethering Putative NMD Factors to Assay NMD

In eukaryotes, messenger RNAs containing premature termination codons are degraded by a mechanism referred to as NMD. It has been shown that translation termination codons, which are located more than 50–55 nt upstream of the 3' most exon–exon junction, are defined as premature and that mRNAs harboring such premature termination codons are committed to rapid degrada-

tion ***(14,15)***. Components of the exon junction complex ***(6,16–18)***, as well as additional *trans*-acting factors, are thought to be involved in this discrimination process ***(4,19,20)***.

In this section, we outline the functional analysis of proteins potentially involved in NMD. A tethering approach allows to demonstrate the ability of proteins such as the established NMD factor hUpf3b to trigger NMD when recruited to a reporter mRNA in human cell lines. We describe (1) the construction of the vectors for the expression of the λN-effector proteins, (2) the construction and function of the reporter plasmid used for NMD analysis, (3) the transfection of mammalian cells with reporter and effector plasmids, and (4) analysis of mRNA, and (5) analysis of protein expression.

3.2.1. Effector Plasmids

The effector plasmids for the expression of putative NMD factors are comparable with the vectors described in **Subheading 3.1.1.** The sequence coding for the λN-peptide was amplified by polymerase chain reaction (PCR) from the pλ-4G plasmid and inserted into the *Nhe*I and *Xho*I sites of the pCI-neo mammalian expression vector (Promega). The GGS linker (glycine–glycine-serine) of the pSG5-derived sequence was simultaneously substituted by a PPL linker (proline–proline–leucine). The cDNA encoding the hUpf3b protein was PCR amplified from HeLa cDNA using the following primer pair (5'-TTTT-CTCGAGATGAAGGAAGAGAAGGAGCACAG-3';5'-TTTTTCTAGA-TCACTCCTCTCCTCCTTCTTTTCTATGGC-3') and inserted in frame into the *Xho*I and *Xba*I sites of the pCI-λN plasmid to generate the plasmid pCI-λN-hUpf3b.

3.2.2. Reporter Plasmids

3.2.2.1. General Considerations

The characteristics of the reporter mRNA are critical for the experimental setup and should be chosen depending on the function of the tethered protein that one wants to analyze. If mRNA degradation is assayed, then the reporter mRNA should preferentially have a long half-life time in the absence of the tethered factor. This increases the detectable differences between the negative controls and the functional proteins. Ideally, the mRNA used for the construction of the reporter is not expressed in the cells used for the assays (e.g., HeLa cells) to avoid interference (e.g., crosshybridization) of endogenous transcripts with the transfected reporter mRNAs. Taking these points into consideration, the β-globin mRNA represents a suitable reporter mRNA because β-globin is not expressed in most human cells used for transfection experiments (e.g., HeLa, HEK 293), has a long half-life and a high expression level and possesses

an appropriate length for Northern blot analysis. Additionally, the β-globin mRNA represents an extensively characterized model system for the study of NMD, and therefore, this choice facilitates the comparison with other experimental results.

3.2.2.2. Insertion of BoxB Sites into the Reporter mRNA

The number of binding sites inserted into the 3'-UTR of the reporter mRNA has to be considered because increasing the number of binding sites can increase the sensitivity of the assay ***(4)***. The position of the sites within the 3'-UTR is a second variable. If the wild-type 3'-UTR might contain regulatory sequences, it is important that the tethering sites do not interfere with the normal expression of the transcript. The spacing of the boxB sequences is an additional point that needs to be addressed. Too little spacing between the binding sites might result in a decreased accessibility of individual sites. Conversely, large sequence stretches between two boxB sequences might reduce the cooperation of adjacent tethered proteins. Furthermore, a higher local concentration of the effector on the targeted mRNA might support its efficient function.

Five boxB sites were inserted into the 3'-UTR of a β-globin expression vector as outlined below. A plasmid for the expression of the β-globin mRNA has been described previously ***(14)***. In this plasmid, an *Xho*I restriction site was introduced by site-directed mutagenesis at a position approx 55 nt downstream of the termination codon. An intron at this position has previously been shown to activate NMD, thus supporting the notion that this is an NMD-competent position within the β-globin 3'-UTR. In the *Xho*I site, a single boxB-sequence was introduced using annealed oligonucleotides with appropriate overhangs. Additional boxB sequences were inserted by PCR amplification of two boxB-sequences with suitable primers contributing a 5' *Sal*I and a 3' *Xho*I site. The PCR product was then ligated into the *Xho*I site, which destroys the *Xho*I site in the 5' position. Thus, with a second round of PCR fragment cloning, additional boxB sequences were introduced (*see* **Note 3**). The successive insertion of a single and two double boxB sequences using the procedure described previously created five boxB sites in the 3'-UTR of the β-globin mRNA. The structure of the 3'-UTR of the 5boxB reporter construct is schematically shown in **Fig. 5**.

3.2.2.3. Design of a Control Plasmid

The effect of the tethered protein on the reporter mRNA needs to be compared with an mRNA that is not affected by the coexpression of the λN-fusion protein. Ideally, this control mRNA can be detected with the reporter-specific probe in the Northern blot and differs in size from the reporter mRNA to allow for the efficient separation of reporter and control mRNAs on the same gel. A

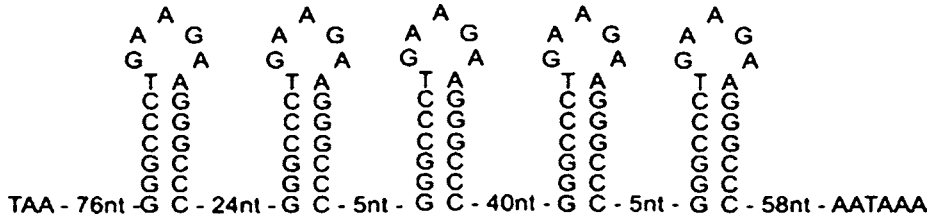

Fig. 5. Spacing of boxB sites for tethered function analysis. Schematic representation of the five boxB sites in the reporter construct for NMD assays.

control plasmid that fulfills these criteria was constructed by inserting additional β-globin sequences into the second and third exons of a β-globin expression vector. Note that the introduction of more than 300 nt into the internal exon (exon 2) can activate cryptic splice sites ***(21)***. To avoid this problem, exogenous sequences should preferentially be introduced into either the first or the last exons.

3.2.3. Assaying the Effects of the Tethered Protein

3.2.3.1. Transfection of HeLa Cells

Titration experiments established an optimal ratio of 1:5:8 (control plasmid:5boxB reporter plasmid:λN-hUpf3b expression plasmid) for our experiments. Depending on experimental circumstances, the optimal ratio should be determined individually. For all experiments, this ratio needs to be optimized again. The plasmids were transfected into HeLa cells using BBS-mediated calcium phosphate precipitation ***(22)***, but other transfection methods also can be used (*see* **Note 4**).

1. Twenty-four hours before transfection, seed HeLa cells at a density of 1.5–2 × 10^5 cells per well in six-well plates (*see* **Note 5**).
2. The next day, cotransfect the cells with 0.5 μg of control plasmid, 2.5 μg of 5boxB reporter plasmid, and 4 μg of pCI-λN-hUpf3b expression vector. The total amount of plasmid DNA per transfected well should not exceed 8 μg for the BBS/calcium chloride method described here. Adjust to a total volume of 90 μL with sterile H_2O and then add 10 μL of 2.5 *M* $CaCl_2$. Add 100 μL of 2X BBS and mix well. Incubate for 20 min at room temperature, and then add the mixture to the cell culture medium and incubate in an incubator with 3% CO_2.
3. Twenty hours later, wash cells twice with 1 mL of Tris-buffered saline and add fresh cell culture medium. Cultivate the cells in an incubator with 5% CO_2 to allow for mRNA and protein expression of the transfected vectors.

Twenty-four hours after washing, the cells can be harvested to analyze mRNA and protein expression.

3.2.3.2. Preparation of Protein Lysates and Total Cytoplasmic RNA

1. Remove cell culture medium from the transfected cells, wash twice with 1 mL of phosphate-buffered saline, and then keep the plates on ice.
2. Add 280 μL of ice-cold lysis buffer II to the cells and incubate on ice for 5 min (*see* **Note 6**).
3. Transfer the lysates to precooled microcentrifuge tubes.
4. Spin the lysate in a refrigerated microcentrifuge (10 min, 10,000*g*, 4°C) to pellet nuclei and other insoluble components.
5. Transfer 30–50 μL of the supernatant to fresh microcentrifuge tubes for later analysis of protein expression. Store at –80°C.
6. Transfer the remaining supernatant to microcentrifuge tubes containing 750 μL of TRIzol reagent and isolate the RNA according to the manufacturers recommendations (*see* **Note 7**).
7. Resuspend the RNA pellet in an appropriate volume of sterile water. Determine the RNA concentration of the samples at 260 nm in a spectrophotometer. Store the RNA at –80°C.

3.2.4. Analysis of mRNA and Protein Expression

3.2.4.1. Analysis of Reporter mRNA Expression

The ability of a tethered protein to act as a *bona fide* NMD factor is assessed by its ability to decrease the abundance of the reporter mRNA. The expression of both control and reporter mRNAs are determined simultaneously by Northern blotting with a β-globin-specific probe. The experimental procedure is outlined in **Subheading 3.1.4.1.** A Northern blot autoradiography showing the effect of tethered hUpf3b on reporter mRNA abundance is shown in **Fig. 6A**.

3.2.4.2. Analysis of Protein Expression

The expression analysis of the λN-tagged effector protein are performed on aliquots of the same extracts that were used for the RNA analysis. This is essential to directly correlate the observed effects on the reporter with the expression of the effector protein. Especially when mutated proteins are analyzed, the equal expression of all mutants has to be determined. The procedure described in **Subheading 3.1.4.2.** can be used for the immunoblot analysis of λN-tagged proteins. A representative immunoblot of the λN-hUpf3b expression is shown in **Fig. 6B**.

3.2.4.3. Control Experiments

When the tethering of a putative NMD protein significantly affects reporter mRNA levels, we suggest performing numerous control experiments to check the specificity of the observed effect. First, the reporter mRNA without any or with mutated binding sites should be cotransfected with the λN-fusion protein

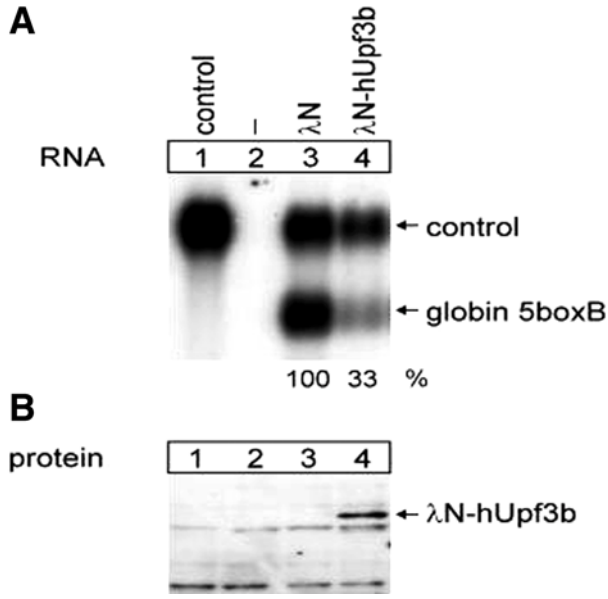

Fig. 6. Tethered function analysis of hUpf3b. HeLa cells were cotransfected with the 5boxB reporter plasmid, the control plasmid, and either the pCI-λN expression vector (lane 3) or the λN-hUpf3b expression vector (lane 4). Untransfected HeLa cells or cells transfected with the control plasmid alone are shown in lanes 2 and 1, respectively. **(A)**, Northern blot analysis of the 5boxB reporter mRNA and control mRNA expression using a β-globin-specific probe. Expression levels were determined with a phosphoimager and represent the mean values of several independent experiments. **(B)**, Immunoblot analysis of the λN-hUpf3b expression of aliquots from the same experiment as in **(A)**. λN-tagged hUpf3b was detected using the anti-λN-specific antibody (lane 4). The λN peptide without a fused protein partner is too small to be detected on a Western blot of this type (lane 3).

to exclude nonspecific degradation that is not mediated by the direct λN/boxB interaction. Second, the physiological relevance of the observed effect has to be addressed. In the case of tethered NMD factors, the decreased abundance of the reporter mRNA that is caused by the NMD effectors must recapitulate established criteria of NMD. Several different control experiments are conceivable to address this point. It has been demonstrated that the degradation of NMD substrates depends on the translation of the respective mRNA ***(14,23)***. Thus, blocking the translation of the reporter mRNA should abolish the destabilizing effect of the tethered protein. Furthermore, dominant-negative inhibitors of the cellular NMD process are expected to inhibit the function of a tethered NMD-factor. A dominant-negative mutant of hUpf1 that negatively influences the

degradation of TPI and β-globin NMD substrates was identified previously *(24)* and was also shown to interfere with the function of tethered RNPS1, hUpf2, hUpf3a, and hUpf3b *(4,6)*. Similarly, RNAi approaches can be employed as additional controls.

3.3. Perspectives

The functional tethering of a protein via a foreign RNA-binding domain represents a versatile tool to assess the function and the domain architecture of proteins involved in diverse steps of mRNA metabolism. The use of the very small λN-peptide for this approach offers several advantages over the other RNA-binding proteins used so far for tethered function analyses. Because of its small size, the peptide can be easily fused to the desired protein. Alternatively, the λN-peptide might be synthesized by chemical peptide synthesis and further modified (e.g., biotinylation, etc.) for in vitro approaches. Over and above that, the λN-peptide seems to represent a most suitable RNA-binding domain for the tethering of small proteins (<40 kDa) to reporter mRNAs because it potentially interferes less with the function and the folding of the fusion partner than larger RNA-binding proteins may.

4. Notes

1. A detailed protocol for the high efficiency transfection of HeLa cells is described in **Subheading 3.2.3.1.** but alternative protocols may be used as well. If the transfection conditions are changed, the amounts of plasmid DNA for optimal transfection efficiencies and good assay readouts must be determined in advance.
2. For immunoblot analysis, any kind of lysis buffer is suitable for the preparation of protein extracts. Nonetheless, the usage of the CAT-ELISA kit is recommended, because lysates have to be prepared for subsequent analysis of CAT activity (*see* **Subheading 3.1.5.1.**).
3. The introduction of boxB sites into the desired position can be achieved by various methods. Especially site-directed mutagenesis protocols offer a fast method to construct several plasmids with varying boxB numbers in parallel. The drawback of this procedure is that each boxB site to be introduced requires a different oligonucleotide.
4. Different transfection methods exist for the efficient delivery of expression plasmids into mammalian cells. The time points for the washing and harvesting steps, as well as total amounts of plasmid DNA that can be transfected, differ between different protocol and must therefore be optimized in advance.
5. Transfection in six-well plates generally yields sufficient RNA and protein for three to four Northern and immunoblots, respectively (20–30 μg of total cytoplasmic RNA and 30–50 μg of protein lysates). If more material is required for additional analyses, the protocol can be scaled-up for the transfection of HeLa cells in 60-mm or 94-mm cell culture dishes.

6. When larger cell culture vessels are used, cells should be transferred into microcentrifuge tubes with a sterile cell scraper before lysis. Large amounts of cells can be passed through a syringe after adding the lysis buffer. This can significantly increase the yields of total cytoplasmic RNA and protein.
7. Total cytoplasmic RNA can be isolated by various protocols. Most commonly a modified phenol/guanidinium isothiocyanate/chloroform protocol is used that produces high yields of pure RNA. The isolation step is outlined here with the TRIzol LS reagent, but all comparable reagents from other suppliers can be used. Alternatively, RNA can be isolated by other methods (e.g., spin columns).

Note Added in Proof

Austin et al. ***(25)*** reported mutants of the λN peptide with increased affinity for boxB. These mutants may be useful for some of the applications described in this chapter.

Acknowledgments

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13

An Efficient System for Cap- and Poly(A)-Dependent Translation In Vitro

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Summary

The 3' poly(A) tail of eukaryotic messenger RNAs (mRNAs) acts synergistically with the 5' cap structure to enhance translation. This phenomenon has been explained by the simultaneous binding of poly(A)-binding protein (PABP) and a cap-binding protein (eIF4E) to eIF4G that results in the circularization of the mRNA (closed-loop model). We developed a robust cell-free protein synthesis system to study poly(A)-dependent translation. In nuclease-treated extracts of Krebs-2 ascites cells, the mRNA poly(A) tail and the cap structure synergistically stimulate translation. We also describe an efficient procedure for depleting PABP from translation extracts. Greater than 98% of PABP can be depleted from extracts by preincubation with either of the PABP-interacting proteins (Paip2 or Paip1) coupled to beads, and these depleted extracts fail to support efficient translation of poly(A)$^+$ mRNAs. Translation activity is restored to depleted extracts by the addition of recombinant PABP.

Key Words

mRNA poly(A) tail; mRNA cap structure; translational synergy; in vitro translation; Krebs-2 cell-free system; poly(A)-binding protein (PABP); PABP-interacting proteins (Paip1 and Paip2); PABP depletion.

1. Introduction

The initiation of translation in eukaryotic cells is modulated by physical interactions between the 3' poly(A) tail and the 5' cap, m^7GpppN (where N is any nucleotide) structures of the messenger RNAs (mRNAs) ***(1,2)***. In the closed loop model ***(3)***, the eIF4G subunit of the cap-binding protein complex eIF4F (eIF4E·eIF4G·eIF4A) simultaneously interacts with poly(A)-binding protein (PABP) and the cap-binding protein eIF4E, leading to circularization of the mRNA. In conjunction with these interactions, eIF4G also binds eIF4A, an

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RNA-dependent ATPase/ATP-dependent RNA helicase; eIF3, a 40S ribosome-associated initiation factor as well as other regulatory proteins (for a review, *see* **ref. *4***). However, the role of the poly(A) ribonucleoprotein in translation initiation is controversial. Other than stabilizing eIF4F-mRNA interactions and promoting the recruitment of mRNAs to the 40S ribosomal subunit ***(5)***, PABP may facilitate ribosomal subunit joining ***(6,7)*** or increase the rate of ribosome recycling after translational termination ***(8)***. PABP interacting proteins 1 and 2 (Paip1 and Paip2) interact with PABP to regulate poly(A)-dependent translation. Paip1 (54 kDa) stimulates translation when it is overexpressed in COS-7 cells ***(9)***. In contrast, Paip2 (14 kDa) strongly inhibits translation of poly(A)$^+$ mRNAs, apparently by destabilizing the PABP-poly(A) complex ***(10)***.

The study of translational regulation by the PABP-poly(A) tail complex has benefited greatly from the development of in vitro systems that reproduce cap-poly(A) synergy ***(10–14)***. One of these systems ***(10,14)***, a nuclease-treated Krebs-2 cell extract, is described in this chapter. We also describe an efficient procedure for depleting PABP from this extract.

2. Materials

All solutions should be prepared using analytical grade reagents and glass-distilled deionized H_2O. Other general technical aspects and experimental precautions are described elsewhere ***(15–17)***.

1. Krebs-2 ascites carcinoma cells are available from several laboratories ***(10,15,18–22)***.
2. Earle's balanced salt solution (EBSS; Invitrogen Life Technologies, Gaithersburg, MD).
3. Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L D-glucose and L-glutamine (Invitrogen Life Technologies). To 500 mL of the medium, add 2 mL of 7.5% sodium bicarbonate and 1 mL penicillin (10,000 U/mL)-streptomycin (10,000 µg/mL) solution (*see* **Note 1**).
4. 40-mL Dounce glass homogenizer with tight-fitting pestle (Kontes, Vineland, NJ).
5. Micrococcal nuclease (nuclease S7; Roche Diagnostics, Laval, QC, Canada): 15,000 U/mL. Dissolve the contents of the bottle (15,000 U) in 1 mL of H_2O; aliquot, and store at –20°C.
6. 75 m*M* $CaCl_2$.
7. Ethylene glycol-*bis*(2-aminoethyl ether)-*N*,*N*'-tetraacetic acid: 0.2 *M*. Use KOH to neutralize a suspension of the free acid in water to a pH of approx 7.3.
8. ATP: 100 m*M*. Dissolve ATP in water, then adjust the pH to 6.0–7.0 with 50% (w/v) KOH; store at –20°C.
9. Guanosine triphosphate (GTP): 100 m*M*. Dissolve GTP in water and then adjust the pH to 6.0–7.0 with 50% (w/v) KOH; store at –20°C.
10. 1 *M* Creatine phosphate (dipotassium salt, Calbiochem, San Diego, CA; *see* **Note 2**).

11. Creatine phosphokinase (rabbit skeletal muscle, Calbiochem): 20 mg/mL. Dissolve the contents of the bottle (20 mg) in 1 mL of 25 m*M* HEPES-KOH, pH 7.3; 1 m*M* dithiothreitol (DTT), and 10% (w/v) glycerol; store at –70°C in 100-µL aliquots (*see* **Note 3**).
12. Total L-amino acid mix (lacking L-methionine): 1 m*M* of each amino acid. The mixture is commercially available (Promega, Madison, WI) or may be prepared using an amino acid kit (Sigma, St. Louis, MO; this prepared mixture should be filter-sterilized). Store at –20°C in 1-mL aliquots.
13. L-methionine (Sigma): 0.4 m*M* in water; store at –20°C.
14. [^{35}S]Methionine, translational grade: 1200 Ci/mmol, 10 mCi/mL.
15. Sigmacote (Sigma).
16. 1 *M* HEPES-KOH, pH 7.7 (*see* **Note 4**).
17. 1 *M* DTT; store at –20°C.
18. Buffer A (10X stock): 350 m*M* HEPES-KOH, pH 7.3; 1.46 *M* NaCl; 110 m*M* D-glucose. Filter-sterilize through a 0.22-µm filter under vacuum and store at 4°C. Dilute to 1X with water as required.
19. Buffer B: 25 m*M* HEPES-KOH, pH 7.3; 50 m*M* KCl; 1.5 m*M* $MgCl_2$; 1 m*M* DTT. Prepare fresh as required.
20. Buffer C: 25 m*M* HEPES-KOH, pH 7.3; 1 *M* potassium acetate; 30 m*M* $MgCl_2$; 30 m*M* DTT. Prepare fresh as required.
21. Buffer D: 25 m*M* HEPES-KOH, pH 7.3; 50 m*M* KCl; 75 m*M* potassium acetate; 2 m*M* $MgCl_2$.
22. Master mix: 10 m*M* ATP, 2 m*M* GTP, 100 m*M* creatine phosphate, 1 mg/mL creatine phosphokinase, 19 unlabeled L-amino acids (lacking L-methionine, 0.2 m*M* each), 125 m*M* HEPES-KOH, pH 7.3. Prepare using the stock solutions above and store at –70°C in 50-µL aliquots.
23. 2.5 m*M* Spermidine (trihydrochloride); store at –20°C.
24. Trichloroacetic acid (TCA): 10% and 5% (w/v) aqueous solutions containing 0.1% DL-methionine; store at 4°C.
25. Plasmids encoding luciferase: T3luc and T3luc(A)$^+$ ***(23)***.
26. Plasmids for bacterial expression of GST-Paip2 (pGEX-6P-2-Paip2) ***(10)***, GST-Paip1 (pGEXHMK-Paip1) ***(9)***, and GST (pGEX-6P-2; Amersham Biosciences Corp., Baie d'Urfé, QC, Canada).
27. Glutathione Sepharose 4B (Amersham Biosciences Corp).
28. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 1.8 m*M* KH_2PO_4, pH 7.3.
29. Plasmid encoding PABP (His_6-tagged): pET3b-PABP(RRMs 1-4)-His ***(24)***.
30. Ni-NTA agarose (Qiagen Inc., Mississauga, ON, Canada).
31. Luciferase Assay System (Promega).
32. Bioluminometer (e.g., Lumat LB 9507; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).
33. 1.5X Sodium dodecyl sulfate (SDS) sample buffer : 75 m*M* Tris-HCl, pH 6.8; 7.5% (v/v) 2-mercaptoethanol; 15% (v/v) glycerol; 0.15% (w/v) bromophenol blue.
34. Phosphorescence imaging system (e.g., BAS-2000 analyzer, FUJI Medical Systems USA, Inc., Stamford, CT).

3. Methods

3.1. Preparing Ascites Cell Extract

Krebs-2 ascites tumor cells are propagated within the peritoneal cavity of approx 20-g female BALB/c mice with 7- to 8-d intervals between fluid collection ***(15)***. One mouse provides 4–8 mL fluid containing approx 10^8 cells/mL. The cells may be used directly to prepare the extract. However, we found that by preincubating the cells in a nutritionally rich medium (e.g., DMEM) the translational capacity of the extract could be enhanced by three to fivefold. Therefore, we recommend that the extract be prepared in the following manner.

A flow chart for the preparation of the extract is given in **Fig. 1**.

1. Harvest the ascites fluid from 10–15 mice (avoid collecting bloody tumors) and dispense equal volumes into two 250-mL conical Corning tubes containing ice-cold EBSS (approx 200 mL per 20–40 mL of fluid). To prevent cell clotting, mix the cell suspension after each transfer of ascites to the tubes.
2. Collect the cells by centrifugation (120*g* for 8 min at 4°C) and resuspend them with ice-cold EBSS.
3. Collect the cells by centrifugation as above. Note the cell volume after the second centrifugation to estimate cell number (the pellet contains approx 3×10^8 cells/mL).
4. Suspend the pellet in DMEM at 10^7 cells/mL and dispense 200–250 mL into each of two to four 1-L Erlenmeyer flasks (treated with Sigmacote according to the manufacturer's instructions; *see* **Note 1**). Seal the flasks with rubber stoppers.
5. Incubate the cells for 2 h at 37°C under gentle (100 rpm) agitation on a rotary shaker, then chill the cell suspension on ice and filter it through two layers of cheesecloth. Carry out all subsequent procedures at 0–4°C unless otherwise specified.
6. Collect the cells by centrifugation as above and wash them with buffer A (twice as above, and once with centrifugation at 750*g* for 8 min to obtain a compact pellet). Thoroughly remove the supernatant by aspiration.
7. Resuspend the cell pellet in 2 vol of buffer B and allow the cells to swell for 20 min.
8. Break the cells with approx 30 strokes of a tight-fitting Dounce homogenizer. An abrupt decrease in viscosity and frothing of the suspension are indicative of cell lysis (*see* **Note 5**).
9. Confirm cell lysis by staining an aliquot of the homogenate with 0.04% trypan blue and inspecting it under a microscope (compare the aliquots that were withdrawn before and after homogenization).
10. After cell lysis, increase the osmotic strength of the homogenate by adding a one-ninth volume of buffer C. Pour the homogenate into 30-mL Corex tubes and centrifuge at 18,000*g* (e.g., Sorvall SS-34 rotor, 12,000 rpm) for 20 min at 4°C.
11. Collect the supernatant and dispense it into 200-μL aliquots (avoid collecting the upper lipid layer). Extracts for which the OD_{260} ranges from 35 to 50 U/mL produce high translation activity.

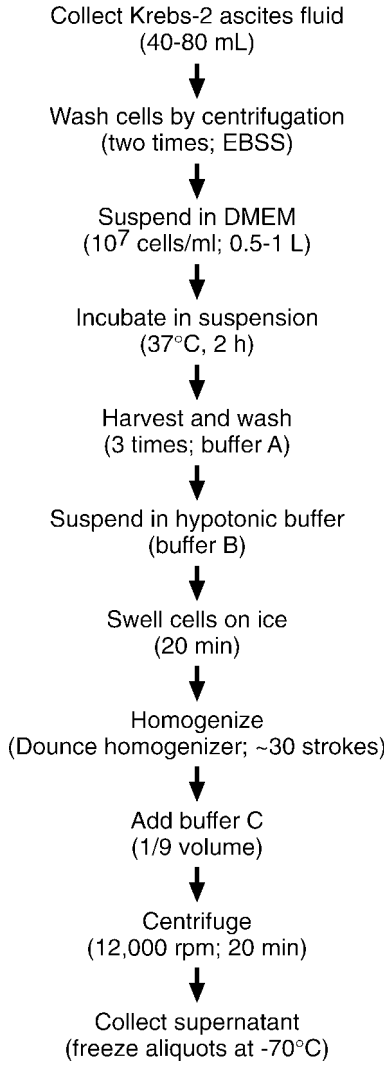

Fig. 1. Flow chart for the preparation of the Krebs-2 cell extract. *See* text for details.

12. Flash-freeze the aliquots on dry ice or liquid nitrogen and store at –70°C. If not thawed and refrozen, the extract remains active for several years. Repeating freezing and thawing of the extract decreases its activity and, therefore, should be avoided.

3.2. Nuclease Treatment of the Extract

To destroy endogenous mRNAs, the extract is treated with micrococcal nuclease in the presence of $CaCl_2$ ***(16,18)***.

1. Thaw the extract in a 20°C water bath and quickly chill it on ice.
2. For 200 µL of extract, add 2 µL of micrococcal nuclease (15,000 U/mL) and 2 µL of 75 m*M* $CaCl_2$.
3. Mix and incubate at 20°C for 20 min (*see* **Note 6**). Stop the reaction by adding 3 µL of 0.2 *M* ethylene glycol-*bis*(2-aminoethyl ether)-*N*,*N*'-tetraacetic acid (3 m*M* final concentration).
4. Chill the extract on ice and clarify it by centrifugation in a 1.7-mL microcentrifuge tube (2 min, 14,000*g* and 4°C). Hereafter, the nuclease-treated Krebs-2 cell extract is referred to as S10.

3.3. Translation Conditions

1. Translation reactions contain, by volume, 50% S10, 10% master mix, 10% 2.5 m*M* spermidine, 5% 0.4 m*M* L-methionine or [^{35}S]methionine (if labeled proteins are desired), and 25% mRNA solution and water (*see* **Notes 7** and **8**). Before dispensing specific reagents to individual reactions, combine ingredients that are common to all samples. For example, to 200 µL S10, add 40 µL of master mix, 40 µL of 2.5 m*M* spermidine, and 20 µL of 0.4 m*M* L-methionine (or substitute [^{35}S]methionine for the L-methionine).
2. Dispense 7.5-µL aliquots of this mixture to precooled plastic tubes containing 2.5 µL (2.5 µg/mL final concentration) luciferase mRNA (a control tube contains no mRNA).
3. Mix each reaction with gentle agitation so as to avoid frothing and incubate the tubes at 30°C for 1–2 h (*see* **Note 9**).
4. Chill tubes on ice and withdraw two aliquots of 3 µL from each translation reaction to measure luciferase activity using an appropriate assay system and equipment (e.g., the Promega luciferase assay system and the Berthold Technologies Lumat LB 9507 bioluminometer). If luminescence is higher than 10^7 (i.e., out of the linear range of detection) then dilute the remainder of the samples 10- to 100-fold with PBS and repeat the assay. Subtract the background luminescence measured for the –mRNA control and calculate data averages for replicate samples.
5. For analysis of [^{35}S]methionine incorporation into proteins, stop the reactions by adding 2 vol of 1.5X SDS sample buffer. Withdraw a 3-µL aliquot from each reaction and determine TCA-insoluble radioactivity (*see* **Subheading 3.8.**).
6. To approximately determine the amount of radioactivity incorporated into any particular protein relative to all proteins, subject the samples to SDS-polyacrylamide gel electrophoresis (PAGE) and detect the labeled proteins by autoradiography of the dried gel ***(25)***.
7. To precisely quantify radioactivity in individual protein bands, use the BAS-2000 analyzer (FUJI Medical systems USA, Inc.) or similar instrument.

3.4. Estimating Translational Synergy Between the Cap and Poly(A) Tail

In the system described, the mRNA 5' cap and 3' poly(A) structures are important determinants of translation efficiency (*see* **ref. *14*** and **Note 10**). The

stimulation of translation by mRNA capping (10- to 15-fold) is significantly greater than by polyadenylation (approximately twofold). Importantly, these structures exert a synergistic effect on translation rates ***(14)***.

A comprehensive assessment of translational synergy between the cap and poly(A) tail requires that the activities of four mRNA templates be compared, that is, cap$^-$/poly(A)$^-$, cap$^-$/poly(A)$^+$, cap$^+$/poly(A)$^-$, and cap$^+$/poly(A)$^+$ (*see* **refs. *12–14***; **Fig. 2A**). Synergy is defined as the ratio of translational stimulation in the presence of both the cap and poly(A) relative to the sum of the stimulation measured individually for the cap or poly(A) alone. The relative translation efficiencies of the four variants of luciferase mRNAs in a typical S10 preparation are shown in **Fig. 2B**. In this example, the magnitude of synergy is approx 5.1 [i.e., (1286 – 20)/(34 – 20)+(253 – 20)]. This value is greater than one, implying that the cap and poly(A) exert a synergistic rather than an additive effect on translation. It is noteworthy that the magnitude of synergy depends on many factors including the nature and concentration of the mRNA being translated. We recommend using low mRNA concentrations (1–3 μg/mL) to study cap-poly(A) tail interactions because high concentrations decrease synergy as well as the dependency of translation on the cap and poly(A) tail ***(14)***. Synergy also varies with different S10 preparations but a range of 4.5–6.0 is typical (at 2.5 μg/mL luciferase mRNA).

3.5. Determining PABP Dependency of Translation

Paip2 exhibits the unique ability to inactivate PABP by displacing it from the poly(A) tail ***(10)***. Consequently, this Paip2 activity may be exploited to determine the level of PABP dependence of translation for any particular mRNA. In S10 preparations, Paip2 preferentially inhibits the translation of poly(A)$^+$ luciferase mRNA (**Fig. 3A**). At a saturating Paip2 concentration of approx 10 μg/mL (corresponding to 0.1 μg in the 10-μL assay) the poly(A)-dependent stimulation of translation is essentially abrogated. Recombinant PABP rescues Paip2-mediated inhibition of translation in a dose-dependent manner, thus demonstrating that PABP is required for efficient translation of poly(A)$^+$ mRNA (**Fig. 3B**). For poly(A)$^+$ mRNA, the contribution of PABP-dependent translation to overall translation is likely to be as high as 94% (given that Paip2-insensitive activity is approx 6%; *see* **Fig. 3A**). Paip2 is not absolutely specific in that it inhibits poly(A)$^-$ mRNA translation at a low level and thus the 94% inhibition value is higher than the degree of inhibition caused by the removal of poly(A) [i.e., 85% (162 – 24)/162; **Fig. 3A**]. However, it is convenient to use 94% value as a rough measure of the dependency of translation on poly(A) because this approach obviates the need to generate mRNA variants that lack the poly(A) tail.

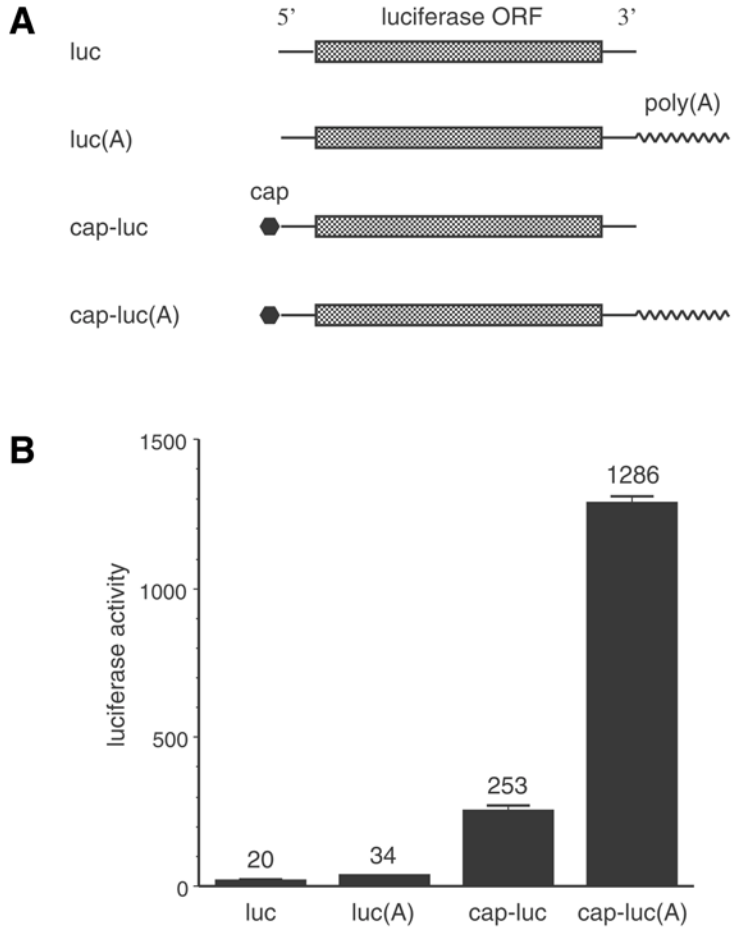

Fig. 2. **(A)**, Schematic representation of the mRNAs used to estimate translational synergism between the 5' cap structure and the 3' poly(A) tail. ORF and poly(A) denote open reading frame and the polyA) tail of 98 adenosines, respectively. **(B)**, Synergistic stimulation of translation by the 5' cap and 3' poly(A) tail of the mRNA. S10 was supplemented with 2 μg/mL uncapped or capped luciferase mRNA, each either containing or lacking a poly(A) tail, and incubated at 30°C for 1 h. Luciferase activity measured in 3-μL aliquots of translation reactions is given in arbitrary units (one unit corresponds to 1000 relative light units).

Another approach to determine the dependency of translation on PABP is to compare translation in PABP-depleted and mock-depleted extracts (*see* **Subheadings 3.6.** and **3.7.**).

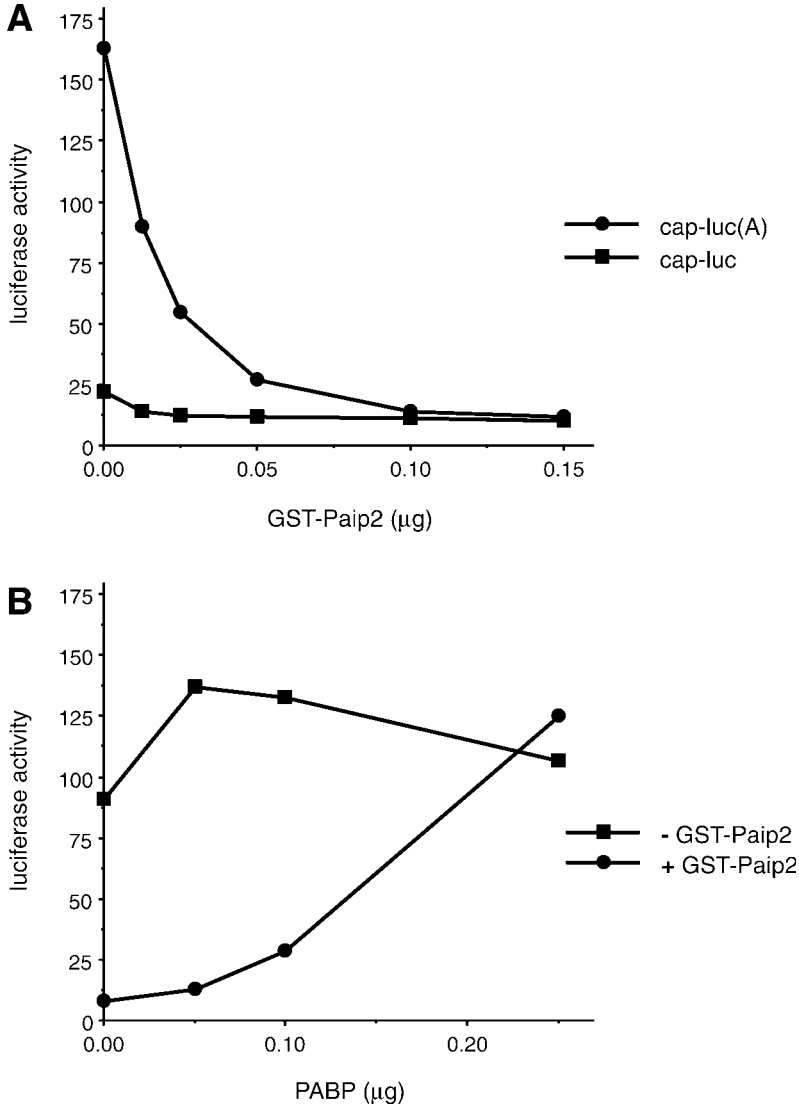

Fig. 3. **(A)**, Paip2 preferentially inhibits the translation of polyadenylated mRNAs. S10 reactions (10 μL) were incubated at 30°C for 1 h with capped polyadenylated or capped nonpolyadenylated luciferase mRNA (2 μg/mL) in the presence of increasing amounts of GST-Paip2. **(B)**, Restoration of Paip2-inhibited translation activity by PABP. Increasing amounts of His-PABP were added to translation reactions containing capped polyadenylated luciferase mRNA (as in **A**) in the absence or presence of 75 ng of GST-Paip2. Luciferase activity was measured as described in **Fig. 2**.

3.6. Affinity Depletion of PABP From Nuclease-Treated Extracts

PABP is an abundant cellular protein, the great majority of which is associated with polysomes and other high-molecular-weight complexes *(26)*. Therefore, it is difficult to deplete PABP from translational extracts. Two techniques for PABP depletion, namely chromatography with poly(A)-Sepharose and precipitation with immobilized antibodies to PABP, have also been attempted in this laboratory but have proven inadequate (Y. V. Svitkin and H. Imataka, unpublished observations). Surprisingly, sufficient depletion of PABP from the nuclease-treated extracts of Krebs-2 or HeLa cells was achieved by using precipitation with GST-Paip2 (or GST-Paip1) coupled beads. Below, we present a protocol for PABP depletion (adapted for 450 μL of S10).

1. Withdraw approx 0.8 mL of the 75% glutathione Sepharose 4B slurry from the stock bottle and wash it with 10 mL of cold PBS. Centrifuge the slurry at 500*g* for 5 min at 4°C. Repeat the wash two more times. Decant the supernatant and resuspend the beads in an equal volume of PBS.
2. Dispense 320 μL of the 50% glutathione Sepharose 4B slurry into each of two 1.7-mL microcentrifuge tubes. Add 320 μL of PBS and 30 μg of GST-Paip2 (or 60 μg GST-Paip1). To a control tube, add 20 μg GST instead of the GST-Paip fusion proteins. Allow proteins to adsorb at 4°C overnight while mixing with end-to-end rotation. Pellet the beads using a microcentrifuge at 3000*g* for 2 min at 4°C and then decant the supernatant. Wash the beads three more times as above with 1 mL buffer D. Remove as much of the wash buffer as possible after the final spin.
3. Add S10 to the beads containing immobilized proteins (450 μL S10 per 150 μL pelleted beads). Allow PABP to adsorb for 2 h at 4°C with rotation. Pellet the beads in a microcentrifuge as above. Collect the supernatant and centrifuge it again to remove any residual beads (14,000*g*, 1 min, and 4°C). This supernatant constitutes the PABP-depleted extract that may be utilized in translation assays or frozen at –70°C in small aliquots.
4. Subject 3–6 μL (30–60 μg of protein) of each extract to SDS-PAGE through a 10% mini-gel and determine the degree of PABP depletion by western blotting using anti-PABP (*see* **Subheading 3.8.**).

Invariably, near-complete (>98%) depletion of PABP can be achieved by this procedure (**Fig. 4A**; *see* **Note 11**). Considering that PABP interacts with eIF4GI *(24)*, some loss of eIF4GI in PABP-depleted extracts could be expected. However, we have found that eIF4GI loss is negligible (**Fig. 4A**).

3.7. Characterization of the PABP-Depleted System

The ability of S10 to translate poly(A)$^+$ mRNA decreases approx 16-fold after PABP depletion regardless of whether GST-Paip2 or GST-Paip1 is used for PABP adsorption (**Fig. 4B**). Addition of recombinant PABP rescues trans-

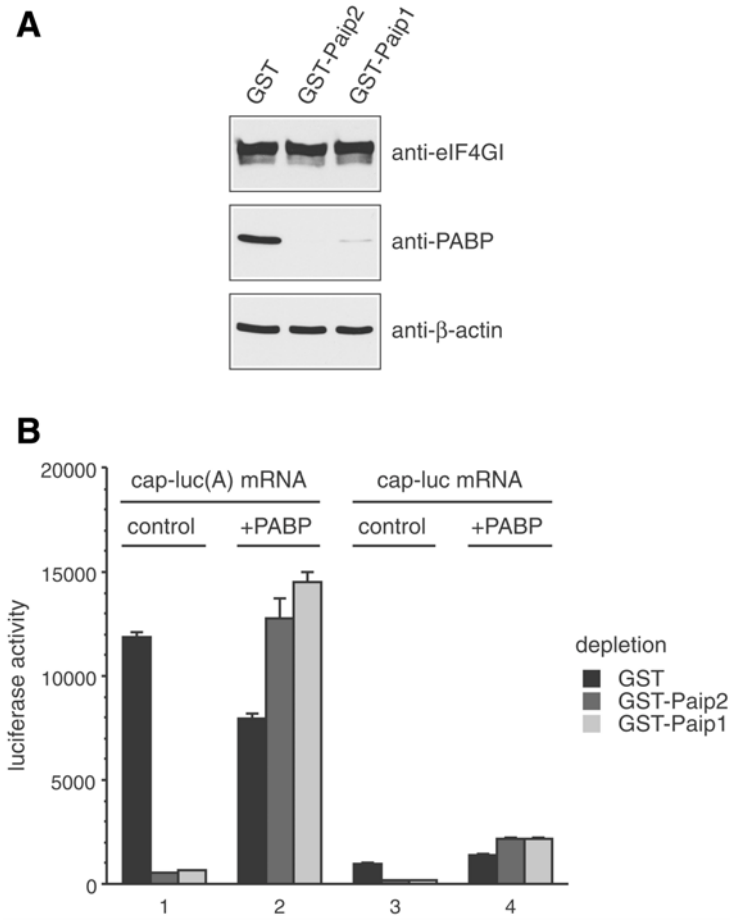

Fig. 4. Affinity depletion of PABP from Krebs-2 cell extracts and its effect on translation. **(A)**, S10 extracts (50 μg) treated with glutathione-Sepharose-bound GST, GST-Paip2, or GST-Paip1 were resolved via SDS-PAGE (7.5% gel) and analyzed by Western blotting using anti-eIF4GI, anti-PABP, or anti-β-actin as indicated (*see* **Subheading 3.8.**). The β-actin blot served as an internal control for loading. Immunopositive protein bands were detected by chemiluminescence and quantified using a LAS-1000 Luminescent Image Analyzer (FUJI Medical Systems USA, Inc.). PABP depletion using GST-Paip2 and GST-Paip1 columns was 99.5% and 98%, respectively (these values were obtained after normalization of the amounts of PABP to those of β-actin). **(B)**, Capped poly(A)$^+$ (1 and 2) or capped poly(A)$^-$ (3 and 4) luciferase mRNAs (2 μg/mL) were translated in S10 extracts that were either depleted of PABP (adsorbed to GST-Paip2 or GST-Paip1) or mock depleted (adsorbed to GST). The reactions were supplemented with either human recombinant PABP (10 μg/mL; 2 and 4) or control buffer (1 and 3) as indicated. Conditions for the incubation and luciferase assay are described in **Fig. 2**.

lation, suggesting that the process of PABP depletion does not remove or inactivate other critical components of the translational machinery. PABP depletion also inhibits translation of poly(A)$^-$ mRNA by three- to fourfold, and this inhibition is reversed by PABP (**Fig. 4B**). These data are consistent with the two to threefold inhibition of poly(A)$^-$ mRNA translation by Paip2 (**Fig. 3A**). However, this inhibition of poly(A)$^-$ mRNA translation cannot be easily explained at present.

3.8. Supporting Protocols

1. DNA manipulations were performed using standard methods ***(25)***. Luciferase-encoding plasmids ***(23)*** were linearized with *Bam*HI and transcribed with T3 RNA polymerase ***(14)***.
2. Syntheses of uncapped or capped RNA transcripts were performed with the RiboMAX system (Promega) according to the manufacturer's instructions. The integrity of in vitro synthesized RNA was verified by agarose gel electrophoresis in the presence of formaldehyde ***(25)***.
3. The GST fusion proteins GST-Paip1 and GST-Paip2 were expressed in *Escherichia coli* BL21 (DE3) cells and purified on glutathione-Sepharose resin (Amersham Biosciences Corp.) according to the manufacturer's recommendations.
4. Human recombinant PABP containing a C-terminal polyhistidine (His_6) tag was expressed in *E. coli* and purified on a Ni-NTA agarose column (Qiagen Inc.) ***(24)***. SDS-PAGE and Western blot analysis of eIF4G and PABP were described previously ***(10,14,25)***.
5. For Western blot analysis of β-actin, a mouse monoclonal antibody against β-actin (Sigma) was used at 1:5000 dilution. Immunopositive protein bands were detected on X-ray film using Western Lightning chemiluminescence kit (Perkin-Elmer Life Sciences, Inc., Boston, MA).
6. TCA-insoluble radioactivity was assayed in 3-μL aliquots of translation samples that had been transferred onto 1.5 × 1.5-cm squares of no. 1 Whatman filter paper (Whatman International Ltd, Maidstone, UK; the paper squares were marked with a pencil before use). The samples were fixed with cold 10% TCA containing 0.1% methionine. The filters were washed successively on a rotary shaker with 5% TCA containing 0.1% methionine (two times at room temperature and once at 90°C) and 98% ethanol at room temperature. Incubations were performed for 5 min with shaking. The filters were dried, and radioactivity was determined by liquid scintillation counting.

4. Notes

1. An alternate methionine-free DMEM (ICN Biomedicals Inc., Orangeburg, NY) may be used. Importantly, methionine-free DMEM is the medium of choice if [^{35}S]methionine labeling of proteins is intended. Incubation of cells in this medium depletes the endogenous methionine pool and, as a result, increases the specific activity of the radiolabeled products in vitro.

2. Sodium ions at high concentration inhibit translational initiation of capped mRNAs ***(27)***. Therefore, it is critical that the potassium salt of creatine phosphate be used rather than the sodium salt.
3. Some creatine phosphokinase preparations are contaminated with RNase ***(28)***. We have never encountered this problem while using the creatine phosphokinase manufactured by Calbiochem.
4. 1 *M* HEPES-KOH, pH 7.7, is used to prepare working solutions; it results in pH 7.3 after dilution. The stock solution should be sterilized by filtration through a 0.22-μm filter.
5. The cell lysis process is critical. Limiting the number of strokes of the Dounce homogenizer and using a moderately hypotonic buffer (50 m*M* KCl) prevents damage to nuclei and leakage of components that inhibit the activity of the extract ***(29)***.
6. We recommend that a titration experiment be performed with each batch of the nuclease to determine the amount that produces the highest signal to noise ratio in the translation assay.
7. It has been reported that Krebs-2 cell extracts are deficient in tRNA ***(19)***. However, we have not observed any significant tRNA dependence of S10 prepared by the method described here. Therefore, it is not necessary to include tRNA in the reaction cocktail.
8. mRNAs containing viral internal ribosome entry sites require a higher concentration of salts for optimum translation. Translational activities using encephalomyocarditis virus, poliovirus, or hepatitis C virus internal ribosome entry sites are 5- to 10-fold higher in reactions that are supplemented with a mixture containing 2.5 m*M* spermidine, 750 m*M* potassium acetate, and 10 m*M* $MgCl_2$ than in those supplemented with 2.5 m*M* spermidine alone.
9. Translation reactions may partially evaporate during incubation. Therefore, always perform incubations in a covered water bath (not in a metal heating block) to minimize evaporation and condensation of water on the lids of the plastic tubes as well as temperature fluctuations.
10. Increasing the length of the poly(A) tail from 15 to 98 Å residues significantly enhances cap-dependent translation in a HeLa cell-free system ***(12)***. Therefore, luciferase mRNA bearing a tail of 98 Å-residues ***(14,23)*** was used in these studies.
11. Trace amounts of GST-Paip2 (and GST-Paip1) may sometimes be detected in PABP-depleted S10 preparations (as revealed by western blot analysis using anti-GST antibodies). This leaching is most likely because of the presence of the reduced glutathione in the extract. Using sufficient amounts of glutathione-Sepharose beads (i.e., more than 125 μL per 450 μL of S10) prevents or significantly reduces such leaching. In addition, overloading the beads with the GST fusion proteins should be avoided (*see* text for the proper protein-to-beads ratio). Importantly, our method of PABP depletion applies only to micrococcal nuclease-treated extracts. When an untreated extract is used, less than 70% of the PABP is removed using beads containing GST-Paip2. This phenomenon may a consequence of the fact that a significant fraction of PABP is tightly associated with mRNA, which remains intact in the untreated extract.

Acknowledgments

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14

A Poly(A) Tail-Responsive In Vitro System for Cap- or IRES-Driven Translation From HeLa Cells

Christian Thoma, Antje Ostareck-Lederer, and Matthias W. Hentze

Summary

In cells, the poly(A) tail stimulates translation from messenger RNAs bearing a cap structure or viral IRES elements. This 3' end-mediated stimulation of translation is not reflected in commonly used commercial cell-free translation systems prepared from rabbit reticulocytes or wheat germ. We describe a simple procedure to generate poly(A) tail-responsive translation extracts from HeLa cells. We suggest that this procedure should be adaptable to many animal cell lines.

Key Words

Poly(A) tail; cap structure; internal ribosome entry site (IRES); translation; RNA stability.

1. Introduction

Translation initiation of cellular messenger RNAs (mRNAs) bearing a 5' ^{7}mGpppG cap structure is greatly stimulated by the 3' poly(A) tail ***(1–3)***. In eukaryotic cells, the cap structure and the poly(A) tail synergize to drive translation initiation ***(1***,***2***,***4)***. This synergism involves the cap-binding protein eIF4E, besides eIF4G a component of the eIF4F translation initiation complex, and the poly(A)-binding protein Pabp1/PABP. A simultaneous interaction of eIF4G with eIF4E and Pabp1/PABP was first described in yeast ***(5)*** and has subsequently been reported for plant and mammalian cells ***(6***,***7)***. As an alternative to cap-driven translation initiation, protein synthesis can also initiate in a 5' end-independent way, mediated by internal ribosome entry sequences (IRES; **refs. *8–12***). IRESes are commonly located within long 5'-untranslated regions and

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can occur in (picorna-) viral as well as cellular mRNAs. It has been reported that the poly(A) tail can also stimulate translation driven by viral IRESes ***(13–15)***, although the exact mechanism underlying this effect of the poly(A) tail is not yet fully understood.

In vitro translation systems have been used for decades to enable cell-free protein synthesis and to investigate the mechanisms of translation. Initial in vitro studies of translation were performed in extracts derived from rabbit reticulocytes and wheat germ ***(16,17)***. However, the contribution of the poly(A) tail to cap-dependent translation could not be recapitulated in these extracts. The first in vitro translation system that could reflect the synergism between the cap structure and the poly(A) tail was derived from *Saccharomyces cerevisiae* ***(18)***. To date, several in vitro translation systems that recapitulate this synergy have been reported, including extracts derived from *Drosophila melanogaster* embryos ***(19)***, HeLa cells ***(14)***, rabbit reticulocytes ***(13)***, and Krebs II ascites cells ***(15)***. In this chapter, we describe a cell-free translation system based on HeLa cell extracts, which is simple to prepare and which displays a strong poly(A) tail contribution to the cap-dependent and IRES-mediated initiation of translation ***(14)***.

2. Materials

2.1. HeLa Extracts

1. HeLa cells S3 (ATCC, Rockville, MD).
2. Joklik Media (Biochrom, Berlin, Germany).
3. Fetal bovine serum, penicillin/streptomycin, glutamine (Gibco, Karlsruhe, Germany).
4. Lysis buffer: 10 m*M* HEPES, pH 7.6 (Biomol, Hamburg, Germany); 10 m*M* potassium acetate (Merck, Darmstadt, Germany); 0.5 m*M* magnesium acetate (Merck, Darmstadt, Germany); 5 m*M* dithiothreitol (Biomol, Hamburg, Germany), two tablets Protease inhibitor (ethylenediamine tetraacetic acid free, Roche, Mannheim, Germany; *see* **Note 1**).
5. Phosphate-buffered saline: 140 m*M* NaCl; 2.7 m*M* KCL; 10 m*M* Na_2HPO_4; 1.8 m*M* KH_2PO_4, pH 7.3.
6. Dounce homogenizer (Landgraf, Langenhagen, Germany).

2.2. Transcription

1. ^{7}mGpppG (Kedar; Warsaw, Poland).
2. ApppG (NEB; Frankfurt, Germany).
3. RNAsin, 5X transcription buffer, T3/T7 RNA polymerase (Stratagene, La Jolla, CA).
4. Dithiothreitol (Biomol, Hamburg, Germany).
5. Cytidine triphosphate (CTP), adenosine triphosphate (ATP), uridine triphosphate (UTP), guanosine triphosphate (GTP), [^{32}P]UTP (PB10163, 400 Ci/mmol, 10 mCi/mL, Amersham, Freiburg, Germany).

6. Linearized plasmid.
7. RQ DNAse (Promega, Mannheim, Germany).
8. Phenol, phenol:chloroform:isoamyl alcohol (25:24:1) (Amresco, Solon, OH).
9. Chromaspin 100 columns (Clontech, Heidelberg, Germany).
10. Sodium acetate, ethanol (Merck, Darmstadt, Germany).
11. Tris-borate/ethylenediamine tetraacetic acid electrophoresis buffer.
12. Agarose.

2.3. Translation

1. HeLa extracts.
2. 2 m*M* Amino acids (Sigma, Taufkirchen, Germany).
3. 5 m*M* Spermidine (Sigma, Taufkirchen, Germany).
4. 100 m*M* solution ATP, 100 m*M* solution GTP (Amersham, Freiburg, Germany).
5. HEPES (Biomol, Hamburg, Germany).
6. RNAse Inhibitor (Eppendorf, Hamburg, Germany).
7. Creatine phosphate, creatine kinase, micrococcal nuclease (Roche, Mannheim, Germany).
8. Magnesium acetate, potassium acetate (Merck, Darmstadt, Germany).
9. H_2O.
10. Luciferase Assay System (Promega, Mannheim, Germany).

3. Methods

The methods described below outline the extract preparation ***(14)***, the in vitro transcription ***(20)***, and the in vitro translation (***14***; Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation) procedures.

3.1. Preparation of HeLa Cell Extract

For large-scale production of translation extracts, HeLa cells (S3) growing in suspension are used. The total procedure requires approx 6 d. The starting culture is transferred into complete Joklik's medium and grown in suspension at 37°C on a magnetic stirrer. The culture is expanded to maintain a cell density between 2.5 and 5×10^5 cells/mL. Eight liters of suspension culture are collected by centrifugation at 700*g* for 15 min at 4°C and washed three times with ice-cold phosphate-buffered saline before disruption. The cells are disrupted in a hypotonic HEPES-based lysis buffer. The cell pellet is resuspended in an equal volume of ice cold lysis buffer. After 5-min incubation on ice, the lysate is transferred into a Dounce homogenizer (working capacity: 15 mL) and homogenized by applying 18 strokes. The lysed extracts are centrifuged at 13,000*g* for 5 min at 4°C. The supernatants are pooled, snap frozen in liquid nitrogen and stored at –80°C. These extracts usually have a protein concentra-

tion of 15–20 mg/mL. In our experience, frozen extracts remain active for several months, and can be freeze-thawed up to two times without profound loss of activity.

3.2. In Vitro Transcription Protocol

^{7}mGpppG-capped mRNAs for in vitro translation are transcribed from *BamH*I-linearized LUC template plasmids. A-capped mRNAs are transcribed from Not-1 linearized-EMCV-LUC or -Ecl136II-linearized BiP-LUC templates in 25-μL reactions (*see* **Note 2**). The RNA is purified by phenol and chloroform:isoamylalcohol (24:1) extractions after DNAse treatment with 1 U/μg plasmid DNA and RQ DNAse for 15 min at 37°C. To remove the nonincorporated ^{7}mGpppG or ApppG, respectively, the transcripts are centrifuged twice through two Chromaspin-100 columns (Clontech), followed by ethanol precipitation. The pellet is washed in 70% ethanol and resuspended in water. mRNA concentration and integrity are assessed by trace labeling and agarose gel electrophoresis.

3.3. In Vitro Translation Protocols

3.3.1. Cap-Dependent Translation

The poly(A) tail contribution to cap-dependent translation is an important feature of translation in vivo and can be recapitulated in the HeLa cell-derived translation system. **Figure 1** illustrates the known interaction between the poly (A) tail and the cap structure.

In the in vitro translation assay, the mRNA is added to the HeLa cell extract mix and incubated for 30 min at 37°C. It is optional to remove endogenous mRNAs by previous treatment of the extracts with micrococcal-nuclease

(Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation). If desired, a micrococcal–nuclease treatment of the extracts prior to the translation reaction is performed by incubation of 4 μL of HeLa extracts with 0.04 U of micrococcal-nuclease and 1 m*M* calcium acetate per reaction for 6 min at 26°C. The reaction is stopped by adding ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA) to a final concentration of 2 m*M*. **Table 1** shows the detailed protocol of the in vitro translation assay of cap-dependent mRNAs. These values are optimal for a 10-μL translation reaction. The reaction mix is incubated at 37°C for 30 min and stopped by snap-freezing in liquid nitrogen. In **Fig. 2**, the contribution of the poly(A) tail to cap-driven translation is shown. Cap-driven translation is stimulated approximately fivefold by the poly(A) tail in micrococcal-nuclease treated extracts. We commonly observe some batch to batch variation ranging from a four- to sixfold effect of the poly(A) tail. Luciferase expression was determined using the luciferase assay system (Promega).

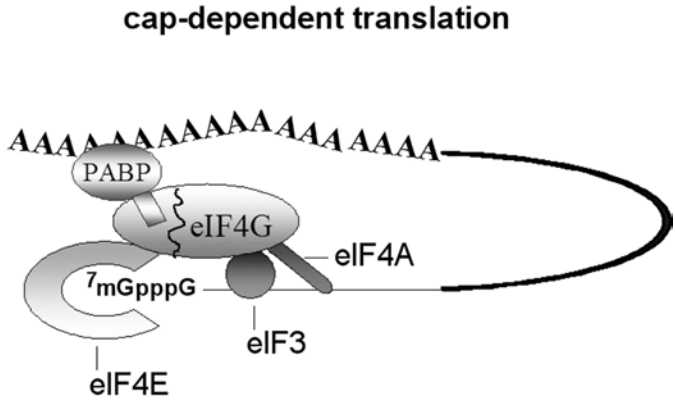

Fig. 1. Scheme representing the poly(A) tail contribution to cap-dependent initiation of translation.

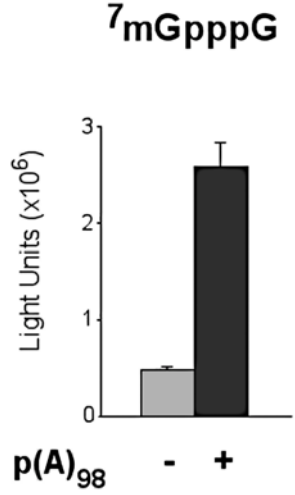

Fig. 2. Stimulative effect of the poly(A) tail on cap-driven translation in micrococcal nuclease-treated HeLa cell extracts. Translation assay of Luc mRNAs modified with either a ^{7}mGpppG cap alone or a ^{7}mGpppG cap and a 98-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). For additional information, *see also* **ref. *14*** and Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation.

Table 1
Components of Translation Reactions

Reagent	Volume (μL per reaction)
Cell extract	4
2 m*M* Amino acids	0.3
100 m*M* ATP	0.08
10 m*M* GTP	0.1
1 *M* HEPES, pH 7.6	0.16
Prime RNAse inhibitor	0.2
1 *M* Creatine phosphate	0.2
10 mg/mL Creatine phosphokinase	0.04
5 m*M* Spermidine	0.1
125 m*M* Magnesium acetate	0.2
2 *M* Potassium acetate	0.2
10 ng mRNA template	1.0
H_20 added to 10 μL	

3.3.2. Viral IRES-Mediated Translation

IRES-driven initiation of translation is best understood for the EMCV-IRES (**Fig. 3**). The HeLa cell-derived in vitro system is a useful tool to investigate the effect of the poly(A) tail on IRES-mediated translation. Using the HeLa cell system, a stimulatory effect of the poly(A) tail on the EMCV IRES-mediated translation can be observed (**Fig. 4**; ***14***). The poly(A) tail contribution enhances translation approx threefold in untreated extracts. For the EMCV-IRES, the optimal concentration of magnesium and potassium is slightly different from cap-dependent translation: 4 m*M* magnesium acetate and 80 m*M* potassium acetate (final concentration). EMCV-IRES (25 ng) bearing mRNA is used. For the other components, *see* **Table 1**.

3.3.3. Cellular IRES-Mediated Translation

The HeLa cell-derived translation system also recapitulates the function of the IRES of the BiP mRNA. **Figure 5** demonstrates the stimulatory effect of the poly(A) tail on BiP-IRES mediated translation. Translation is approx sixfold stimulated by the poly(A) tail for the BiP IRES in micrococcal-nuclease treated extracts. Magnesium and potassium acetate are used as described for the EMCV-IRES. The optimal amount of BiP mRNA is 50 ng in a 10-μL translation reaction. For the other components of the translation mix, *see* **Table 1**.

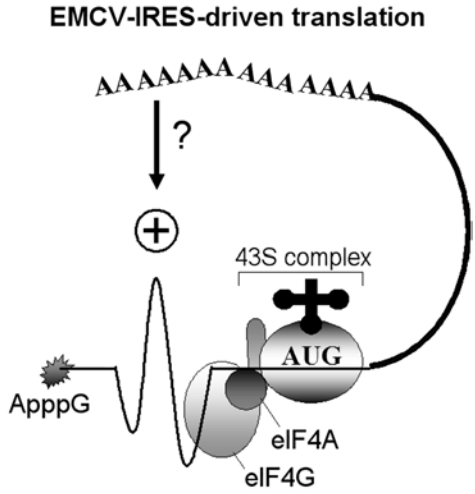

Fig. 3. Schematic representation of the poly(A) tail contribution to EMCV IRES-mediated initiation of translation.

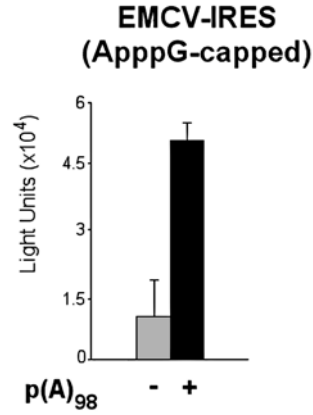

Fig. 4. Translation driven by the EMCV IRES is stimulated by the poly(A) tail in HeLa cell extracts. Translation assay of ApppG-capped, monocistronic EMCV IRES containing Luc mRNAs either with or without 98-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). *See also* **ref. *14***.

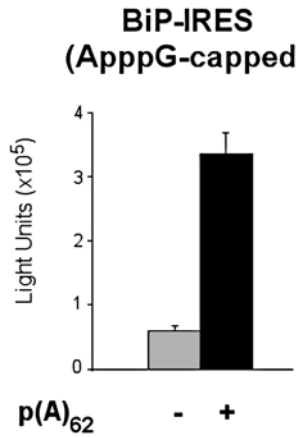

Fig. 5. Stimulative effect of the poly(A) tail on cellular IRES-driven translation in HeLa cell extracts. Quantitative analysis of luciferase expression from an ApppG-capped, monocistronic BiP IRES containing Luc mRNA either with or without a 62-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). For additional information, *see also* Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation.

The cell-free translation system described here appears to reflect important features of mRNA translation in vivo. In particular, it displays stimulation of cap-and IRES-driven translation by the poly(A) tail. We suggest that the procedure described here can be adapted to other cell lines growing in suspension, and modified to allow the preparation of extracts from cells that grow adhesively.

4. Notes

1. Filter and store on ice.
2. The transcription reactions contain 1 m*M* ATP, CTP, UTP; 10 m*M* DTT; 1 U Inhibit Ace (5Prime-3Prime, PA); 7 m*M* ^{7}mGpppG (Kedar, Warsaw, Poland) or 7 m*M* ApppG (NEB), 1 µL 1:50 α-[^{32}P]UTP (Amersham, PB10163, 10 mCi/mL) for trace-labeling, 1X transcription buffer with 60 U of T3 RNA-Polymerase (Stratagene, La Jolla, CA). After 5 min preincubation at 37°C, GTP is added to a final concentration of 1 m*M* for additional 60 min. For the transcription of "uncapped" mRNAs (e.g., for IRES-driven translation), we strongly recommend to replace the ^{7}mGpppG analog by ApppG, rather than simply omitting ^{7}mGpppG. "A-capped" transcripts are stable but cannot bind eIF4E.

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15

Assessing Messenger RNA Decapping in Cellular Extracts

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Summary

Removal of the 5' cap from a messenger RNA (mRNA) is an integral part of all mRNA decay pathways and can be a highly regulated event. Assays designed to assess decapping in vitro need to effectively resolve four products of mRNA decay: ^{7me}GpppG produced by 3'–5' shortening of the transcript by the exosome, ^{7me}GMP produced by the scavenger decapping enzyme DcpS acting on the product of exosomal decay, ^{7me}GDP produced by the Dcp1/2 decapping enzyme, and free phosphate, which can be generated by phosphatases in the extract acting upon either of the two products of decapping noted above. We have outlined both thin-layer chromatography and acrylamide-gel based approaches that can be used to assess decapping activities.

Key Words

Decapping; mRNA decay; mRNA turnover; thin-layer chromatography; mRNA capping; exosome; scavenger decapping; Dcp1/2; cytoplasmic extracts.

1. Introduction

Removal of the 5' cap from messenger RNAs (mRNAs) is an important part of mRNA turnover. After deadenylation, most mRNAs in yeast get decapped by the Dcp1/2 enzyme, leading to the degradation of the body of the transcript by the 5'-3' exonuclease Xrn1p ***(1,2)***. Dcp1/2-mediated decapping generates ^{7me}GDP and is regulated by several factors, including the poly(A) tail ***(3)***, Edc1p, Edc2p ***(4)***, Lsm proteins ***(5)***, and eIF4E ***(6)***. Decapping also plays a major role in mRNA surveillance/nonsense-mediated decay ***(7)***. An alternative 3'–5' decay pathway exists as well in yeast that appears to play a more prominent role in mRNA decay in mammalian cells ***(8–10)***. A complex called the

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exosome, which is largely composed of 3'–5' exonucleases, degrades deadenylated transcripts in this pathway to oligonucleotides. A scavenger decapping activity, called DcpS, then acts on these short transcripts, releasing ^{7me}GMP. Both major pathways of mRNA decay, therefore, involve a decapping event.

Regulated mRNA decapping mediated by Dcp1/2 can be effectively reproduced in vitro in yeast, trypanosome, and mammalian extracts ***(3,11–13)***. Furthermore, DcpS activity can also be readily observed acting at the terminal step of the exosome-mediated decay pathway ***(10)***. Measuring mRNA decapping, therefore, can be a powerful tool for those interested in assessing post-transcriptional regulation at the level of RNA turnover. Assays designed to assess decapping in vitro need to effectively resolve four products of mRNA decay: ^{7me}GpppG (produced by 3'–5' shortening of the transcript by the exosome), ^{7me}GMP (produced by the scavenger decapping enzyme DcpS acting on the oligomeric products of exosomal decay), ^{7me}GDP (produced by the regulated Dcp1/2 decapping enzyme), and free phosphate (which can be generated by phosphatases in the extract acting upon either ^{7me}GMP or ^{7me}GDP).

In this chapter, we describe how to effectively produce cap-labeled RNA substrates for in vitro decapping reactions as well as three assays designed to identify reaction products. Although a one-dimensional (1D) thin-layer chromatography (TLC) assay has been used most extensively in the field to date, it has both resolution and cost issues that can reduce its effectiveness. As alternatives, we present both a two-dimensional (2D) TLC approach and an acrylamide gel-based system. The acrylamide gel-based system offers several benefits over the TLC-based approaches that, in our opinion, make it the assay of choice.

2. Materials

2.1. Reagents for the Preparation of Cap-Labeled RNA Substrates

1. 10X RNA polymerase buffer: 400 m*M* Tris-HCl, pH 7.9; 60 m*M* $MgCl_2$; 20 m*M* spermidine; 100 m*M* dithiothreitol; store at –20°C.
2. 15X Capping buffer: 750 m*M* Tris-HCl, pH 7.9; 90 m*M* KCl; 37.5 m*M* dithiothreitol; 18.8 m*M* $MgCl_2$; 1.5 mg/mL iodoacetylated bovine serum albumin (BSA); store at –20°C.
3. RNA gel loading buffer: 20 m*M* Tris-HCl, pH 7.6; 8 *M* urea; 1 m*M* ethylenediamine tetraacetic acid (EDTA); 0.02% w/v xylene cyanol; 0.02% w/v bromophenol blue; store at room temperature.
4. RNase free ddH_2O.
5. 10X rNTP mixture: 5 m*M* rNTPs (5 m*M* each rATP, rGTP, rUTP, rCTP); prepare in 10 m*M* Tris-HCl, pH 8.0, rather than ddH_20 to ensure stability; store at –20°C.
6. Phenol saturated with 10 m*M* Tris-HCl, pH 8.0.
7. Phenol:chloroform:isoamyl alcohol 25:24:1, prepare with Tris-saturated phenol.

8. 10 *M* Ammonium acetate.
9. 100% Ethanol, store at –20°C.
10. 80% Ethanol, store –20°C.
11. 10 m*M S*-adenosyl methionine, store at –20°C.
12. α-[^{32}P]GTP (800 Ci/mmol).
13. 30% Acrylamide (19:1 acrylamide:*bis*-acrylamide).
14. 10X Tris-borate-EDTA.
15. Urea.
16. Ammonium persulfate.
17. TEMED.
18. Restriction enzyme.
19. Phage RNA polymerase (e.g., SP6, T3, T7).
20. Vaccinia virus capping enzyme (Ambion).
21. RNase inhibitor, for example, RNasin (Roche).
22. 10 mg/mL Proteinase K (dissolve in RNase free ddH_2O and store in small aliquots at –20°C, repeated freeze-thaw should be avoided).

2.2. Reagents for the Decapping Reaction

1. 10X CE buffer: 500 m*M* Tris-HCl, pH 7.9; 300 m*M* $(NH_4)2SO_4$, 10 m*M* $MgCl_2$; store at –20°C.
2. Phenol saturated with 10 m*M* Tris-HCl, pH 8.0.
3. HeLa cytoplasmic S100 extract ***(14–16)***.
4. 200 μ*M* ^{7me}GpppG (optional), store at –20°C.

2.3. Reagents and Supplies for One-Dimensional TLC

1. PEI-F TLC Plates (Baker Corp., Phillipsburg, NJ).
2. 12" × 3" × 14" (or equivalent) glass TLC chamber with cover (Fisher Scientific).
3. 500 m*M* EDTA, pH 8.0.
4. 450 m*M* $(NH_4)_2SO_4$.

2.4. Reagents and Supplies for 2D TLC

1. PEI-F TLC plate (Baker Corp., Phillipsburg, NJ).
2. Approx 12" × 3" × 14" (or equivalent) glass TLC chamber with cover (Fisher Scientific).
3. Solvent Aa: 1 *M* acetic acid adjusted to pH 3.5 with ammonium hydroxide.
4. Solvent Sa: prepared by adding 222 g of ammonium sulfate to 300 mL of deionized H_2O. Adjust the final pH to 3.5 by adding concentrated sulfuric acid.
5. Guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), ^{7me}GMP, ^{7me}GDP, ^{7me}GTP markers (Sigma, St. Louis, MO): Resuspend solids to 25 m*M* in 10 m*M* Tris-HCl, pH 8.0, and store at –20°C.

2.5. 20% Acrylamide Gel-Decapping Assay

1. 40% Acrylamide/*bis*-acrylamide (37.5:1).
2. Urea.

3. 10% w/v Ammonium persulfate.
4. TEMED.
5. 10X Tris-borate-EDTA.
6. RNase-free ddH_20.
7. Tris-saturated phenol.
8. RNA gel loading buffer.

3. Methods

3.1. Preparation of Cap-Labeled mRNA Substrates

To detect the removal of the mRNA cap, it is necessary to generate mRNA substrates that are specifically and uniquely radiolabeled at the cap structure. This is achieved in two steps. First, an RNA is transcribed from a DNA template using a bacteriophage RNA polymerase (e.g., SP6, T7). The transcript is then capped and radiolabeled at the γ-phosphate of the ^{7m}GpppG moiety using vaccinia virus capping enzyme in the presence of α-[^{32}P]GTP and *S*-adenosyl methionine (SAM). The transcript is then purified by polyacrylamide gel electrophoresis and eluted from an excised band. This not only serves to separate the transcript from unincorporated radionucleotides but also allows the overall quality of the transcript to be assessed and ensures that only full-length transcripts are selected for use.

1. Assemble the following transcription reaction in a 1.5-mL centrifuge tube.
   1 μL linearized DNA template (1 μg/μL; approx 0.5 pmol; *see* **Note 1).**
   1 μL 10X rNTP mixture
   1 μL 10X RNA polymerase buffer
   1 μL phage-derived RNA polymerase
   0.5 μL RNase inhibitor (40 U/μL)
   5.5 μL Rnase-free ddH_2O
2. Incubate the reaction mixture at 37°C for 1 h.
3. Add 150 μL dH_2O to the reaction mixture.
4. Add 200 μL phenol:chloroform:isoamyl alcohol (25:24:1), vortex briefly, and centrifuge at 16,000*g* for 2 min. Remove the aqueous phase to a clean 1.5-mL tube.
5. Add 40 μL 10 *M* ammonium acetate and 500 μL ice cold 100% ethanol to the aqueous phase, mix by inversion and place tube on dry ice for 15 min.
6. Centrifuge at 16,000*g* for 10 min.
7. Remove supernatant carefully so as not to disturb the pellet (*see* **Note 2**).
8. Wash the pellet with 200 μL of ice cold 80% ethanol. Centrifuge briefly and remove as much of the supernatant as possible.
9. Dry the pellet thoroughly via lyophilization.
10. Resuspend the pellet by vortexing in the following reaction mixture:
    4.5 μL α–[^{32}P]GTP (800 Ci/mmol)
    0.5 μL 15X capping buffer
    0.5 μL 10 m*M* SAM (*see* **Note 3**)

11. Use a brief spin in a microcentifuge to bring the contents down to the bottom of the tube.
12. Add 1 μL of RNase inhibitor (*see* **Note 4**) and 1 μL of capping enzyme.
13. Incubate at 37°C for 30 min.
14. Add 152.5 μL of dH_2O.
15. Repeat **steps 4–7**.
16. Resupend the pellet in 5 μL of RNA gel loading buffer. Vortex vigorously to ensure the pellet is completely resuspended. Centrifuge briefly (3 s) to concentrate the suspension to the bottom of the tube.
17. Heat the mixture to 90°C for 30 s, then quickly cool on ice.
18. Gel purify the RNA as described elsewhere in this book (*see* **Note 5**).

3.2. Decapping Reaction

1. Assemble the following reaction (*see* **Note 6**).

   1μL 10X CE buffer
   1μL Cap-labeled RNA (100,000 cpm/μL)
   4μL Rnase-free ddH_2O
   4μL HeLa S100 extract (*see* **Note 7**; add this last and mix gently)

2. Incubate at 30°C for 30 min.
3. Proceed to one of the three analysis steps in the following section.

3.3. 1D TLC Analysis of Decapping

The 1D TLC assay has been adapted to study decapping in mammalian cells. This assay allows analysis of the products of decapping by following the migration of the decay products as spots on thin layer chromatography sheets alongside standard markers. The advantage of this assay is that one can quantitatively analyze multiple decay reactions at one time and compare the products of Dcp1/2-mediated decapping as well as the decay products of the exosome and DcpS scavenger activity. There is one important disadvantage of this approach. Free phosphate, which may be generated from Dcp1/2 or DcpS decapping products by the action of phosphatases in extracts, comigrates with ^{7me}GDP. This does not allow accurate quantitation of decapping and makes definitive identification of Dcp1/2 products impossible without the inclusion of a confirmatory assay (*see* **Note 8**). Also, note that the quality of the RNA substrate and nonspecific RNA degradation cannot be assessed using this assay. Because of their large size, RNA degradation products generated by random nucleases will not migrate from the origin. **Figure 1** shows a typical analysis of mRNA decapping by 1D TLC.

1. Prerun a PEI-F cellulose sheet in a TLC chamber with RNase-free water (approx 500 mL of water in a standard TLC chamber). Incubate the sheets for approx 1 h or until the water reaches the top of the sheet. Dry the cellulose sheets either at

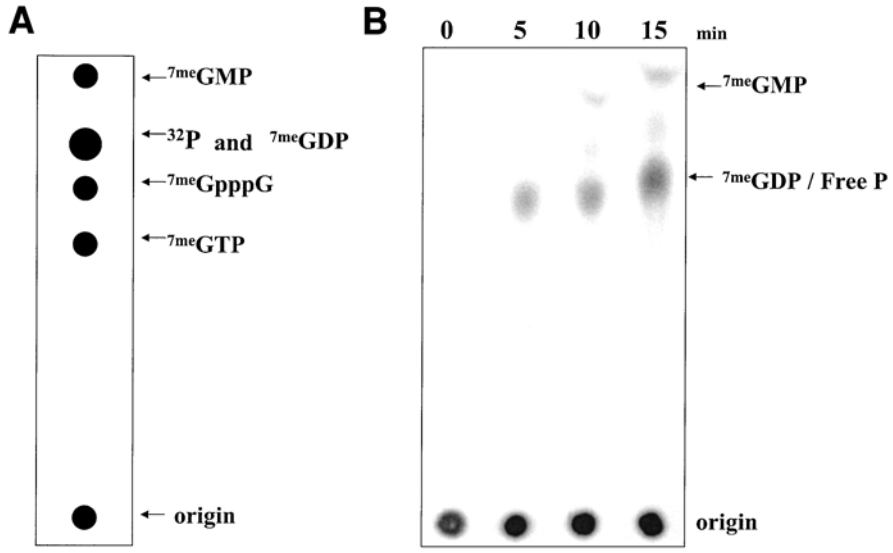

Fig. 1. 1D TLC analysis of mRNA decapping products. **(A)**, Diagrammatic representation of the migration of relevant decapping products by 1D TLC. **(B)**, Cap-labeled GemARE-A0 RNA was incubated for the indicated time in HeLa cytoplasmic S100 extract. The reaction mixture was then spotted at the origin and the products resolved by 1D TLC.

room temperature or by placing them in a 37°C incubator (*see* **Note 9**). Add approx 500 mL of a 450 m*M* $(NH_4)_2SO_4$ solution to the TLC chamber. Close the chamber and allow it to equilibrate for approx 15 min.

2. Prepare a 10 μL decapping reaction with 100,000 cpm of cap-labeled RNA as described above (*see* **Note 10**). Incubate the decapping reaction at 30°C for the desired times.
3. Stop the reaction at each time point by the addition of 1 μL of 500 m*M* EDTA. Keep the stopped reaction tubes on ice until all the reactions are complete (*see* **Note 10**).
4. Using a pencil (*see* **Note 11**), draw a line 2.5 cm from the bottom of the PEI-F cellulose sheet. Using this line as a guide, mark the place where the reactions will be spotted on the cellulose sheet by drawing dots starting 1.5–2 cm from the edge and continuing 1.5–2 cm apart (*see* **Note 12**).
5. Spot the decapping reaction (no more than 10 μL) on the prerun cellulose sheets with a pipet. Wait until the spots have dried before placing the TLC sheet in the chamber (*see* **Note 13**). Leave the TLC sheet in the chamber until the solvent reaches the top of the sheet (approx 2 h).
6. Remove the TLC sheet from the chamber, wrap it in clear plastic wrap, and place in a phosphorimager cassette or in a cassette with X-ray film for imaging.

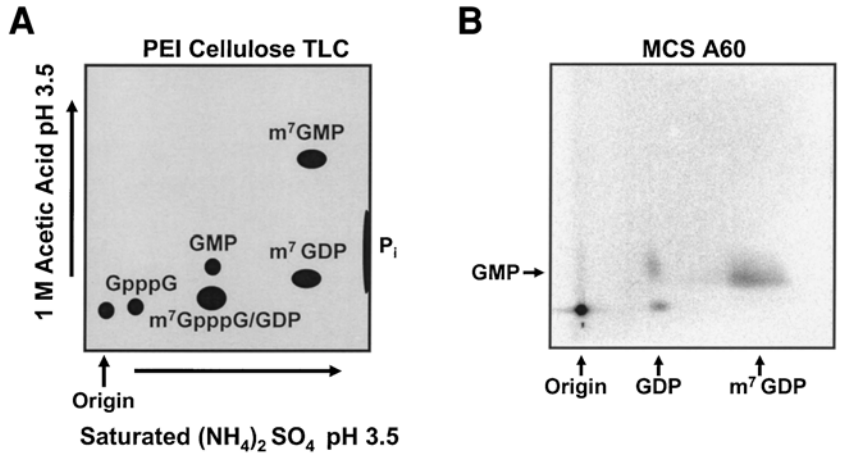

Fig. 2. 2D analysis of mRNA decapping products. **(A)**, Diagrammatic representation of the migration of relevant decapping products by 2D TLC. **(B)**, Cap-labeled MCS-A60 RNA was incubated in cytoplasmic extract from *Leptomonas seymouri* and the products of decapping were resolved by 2D TLC.

3.4. 2D TLC Analysis of Decapping

The protocol for 2D TLC for resolution of decapping reaction products has been largely adapted from methodologies previously described by Bochner and Ames *(17)*. 2D TLC analysis allows for the clear separation of decapping reaction products. **Figure 2A** shows that ^{7me}GDP, produced by Dcp1/2-dependent cleavage of capped RNA is separated from both ^{7me}GMP and Pi. GDP and GMP can also be resolved from their methylated counterparts using this assay system as well (**Fig. 2B**). The drawbacks for this technique are the time involved to perform two TLC runs and the fact that only one sample can be analyzed per chromatography sheet.

1. Perform a decapping reaction as described above (*see* **Notes 14** and **15**). Equilibrate the glass chromatography chamber with solvent Aa (approx 500 mL) for 15 min before addition of plates.
2. Apply the entire sample in 2- to 3-μL aliquots to the PEI-F TLC plate, 3 cm from the bottom and 1.5 cm from the left edge of the plate. Dry each aliquot onto the plate (either by air-drying or by using a handheld dryer) before subsequent aliquots are applied.
3. After the entire 10 μL decapping reaction is spotted, wash the plate for 5 min in methanol, and let it air-dry for approx 30 min.
4. Roll the plate into a tube (keep the cellulose layer on the inside) and fasten it with a rubber band at each end. This allows several plates to be run in a single TLC chamber.

5. Chromatograph the TLC sheet in the first dimension using approx 500 mL of solvent Aa (**Fig. 2**). The solvent will reach the top of the plate in approx 1.5 h.
6. After developing in the first dimension, unroll the plate, wash it for 20 min in methanol and let it air-dry.
7. Rotate the plate 90° relative to the original direction of chromatography, roll it up again, fasten the ends with rubber bands, and resolve in the second dimension using approx 500 mL of solvent Sa in the TLC chamber.
8. Let the Sa solvent reach the top of the plate (approx 3–4 h).
9. After chromatography in the second dimension, wrap the plate in plastic wrap, and expose it to film or to a phosphorimager screen.

3.5. Acrylamide Gel-Based Analysis of Decapping

RNA substrates used in the in vitro decapping assay are radiolabeled at the γ phosphate of the cap ($^{7me}G^{32}$pppN). Therefore, the only possible end products of the decay reactions are ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, ^{7me}GpppG, and Pi. The 20% denaturing gel assay is able to clearly resolve those products, making it an ideal tool for analyzing the decapping reactions. The use of the gel assay has an added advantage of allowing assessment of the quality of the input RNA and any nonspecific RNA degradation that might have occurred during the reactions. These advantages over other methods make the acrylamide gel assay our method of choice when analyzing mRNA decapping. **Figure 3** shows a typical acrylamide-gel based analysis of mRNA decapping products.

1. Prepare a 20% acrylamide gel (37.5:1 acrylamide to *bis*-acrylamide) containing 7 *M* urea.
2. Prerun the gel to remove the salt front from the gel (*see* **Note 16**).
3. After the decapping reaction is finished add 10 µL Rnase-free ddH_2O and 20 µL Tris-saturated phenol to the reaction. Vortex the reaction briefly and centrifuge for 2 min at 13,000*g*. Transfer 10 µL of the aqueous phase (top) to a new tube (*see* **Note 17**).
4. Add 4 µL of RNA gel loading buffer to the sample.
5. Denature the reaction contents by heating at 90°C for 30 s. Quick cool the sample on ice.
6. Load the reaction onto the prerun 20% gel.
7. Allow the bromophenol blue to electrophorese approximately one-third of the way down the gel.
8. Visualize the gel by autoradiography or by phosphorimager analysis (*see* **Note 18**).

4. Notes

1. The DNA template may be produced by cloning a sequence of interest into a vector downstream of a bacteriophage promoter. The vector may then be linearized at a suitable restriction enzyme site to produce the template. Alternatively, the template may be produced by annealing synthesized DNA oligomers containing the sequence of interest downstream of a bacteriophage promoter ***(18)*** or

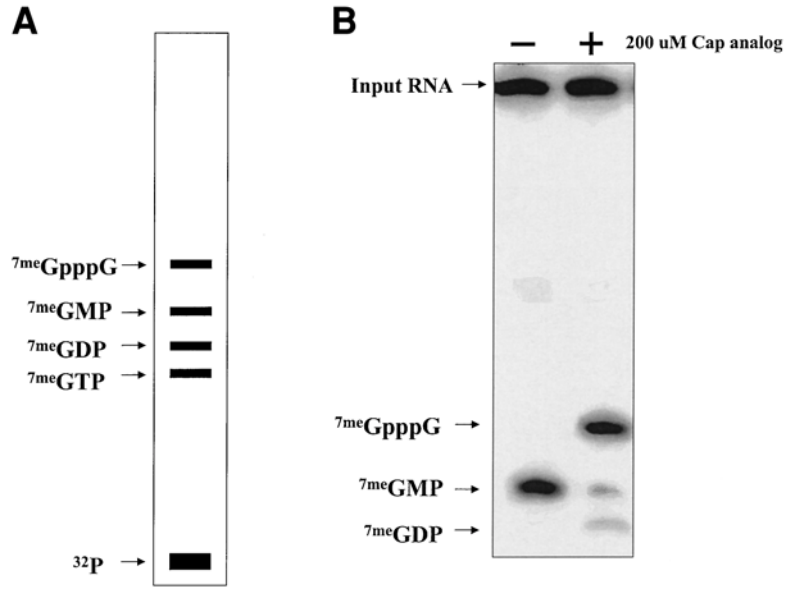

Fig. 3. Acrylamide gel-based analysis of mRNA decapping products. **(A)**, Diagrammatic representation of the migration of relevant decapping products by 20% acylamide gel electrophoresis. **(B)**, Cap-labeled GemARE-A0 RNA was incubated for 30 min in HeLa cytoplasmic S100 extract in the presence or absence of 200 μ*M* ^{7me}GpppG cap analog. Reaction products were resolved on a 20% acrylamide gel. The region of the gel that would have contained free phosphate has been cropped from the picture because only trace amounts of [^{32}P] were detected.

polymerase chain reaction products ***(19)***. The quality of the template is a primary determinate of the quality of the transcript and the efficiency of cap labeling. Care should be taken to generate templates that are free from ribonucleases. This is particularly important if the template has been generated by processing with enzymes. The quality of the template can be ensured by incubating with 20–30 μg of proteinase K in 400 μL stop buffer for 15 min at 37°C followed by phenol/chloroform extraction, ethanol precipitation, and resuspension in a suitable volume of Rnase-free water.

2. Removal of as much of the ethanol supernatant as possible is important to ensure that no significant traces of ammonium salt are carried over to the next step of the reaction. Salt contamination may inhibit the capping reaction. Also, note that ammonium acetate is used rather than sodium acetate to ensure that unincorporated rNTPs fail to precipitate.
3. SAM is necessary to ensure efficient labeling by the capping enzyme. This enzyme comprises guanyltransferase and methyltransferase subunits and thus is able to transfer radiolabeled GMP to the 5' triphosphate of the RNA substrate and methy-

late that product in a single incubation. Reactions performed in the absence of SAM (or in SAM of poor quality) are very inefficient.

4. The addition of RNase inhibitors is not absolutely necessary to the transcription reaction (**step 1**). It has been our experience, however, that some preparations of capping enzyme can contain significant levels of RNase. Therefore, addition of an RNase inhibitor is highly recommended at this step.
5. Using this approach, we routinely obtain 3–5 million cpm of cap-labeled, gel purified RNA. If yields are lower, the probable cause is a low-yield transcription reaction. Troubleshooting transcription reactions can be found in Chapter 16 of this volume.
6. Cap analog (^{7me}GpppG) can also be added. The addition of this analog will increase the efficiency of Dcp1/2-mediated decapping by sequestering cap-binding proteins. Note that it will also inhibit the activity of the scavenger DcpS decapping activity.
7. Although we routinely use HeLa S100 cytoplasmic extract, similar results have been obtained using S100 extracts from several other mammalian cell types. Yeast extracts ***(11)*** and trypanosome extracts ***(12)*** have also been used to study decapping by these methods.
8. One of the major limitations of this assay is the comigration of ^{7me}GDP, the major product of Dcp1/2 mediated decapping, and free phosphate, which is a byproduct of exosome-mediated decay. Two approaches can help alleviate this problem in the context of the 1D TLC assay. First, the identity of the spot can be confirmed as ^{7me}GDP by the preparation of a separate reaction. Following decapping, the addition of NDP kinase (Sigma) will convert ^{7me}GDP to ^{7me}GTP, which will migrate distinctly from orthophosphate. Alternatively, the use of 0.75 *M* LiCl as the mobile phase solvent for 1D TLC can provide an improved separation of free phosphate and ^{7me}GDP.
9. The PEI-F cellulose sheets can be prerun in water and then stored at 4°C.
10. Varying quantities of RNA can be used in the decapping reactions (ranging from 50,000 cpm to 200,000 cpm of cap-labeled RNA.)
11. When conducting an experiment with numerous time points, it is preferable to make a cocktail with all the reactions and remove 10 μL per reaction at each time point.
12. Do not use ink to write on the cellulose sheets as it will smear. In addition, do not attempt to write on a wet TLC plate. Wait until it is thoroughly dry to avoid damaging the surface.
13. A hair dryer can be used to completely dry the reaction spots on the PEI-F cellulose sheets. The spots have to be completely dried to achieve optimal resolution of the decay products.
14. To remove salts that affect migration on the TLC plate, reaction volumes are increased to 100 μL with deionized H_2O and the aqueous phase is extracted twice with an equal volume of phenol/chloroform and once with chloroform. The aqueous phase is then reduced to approx 10–20 μL by lyophilization.
15. To aid in the identification of radiolabeled decapping reaction products, unlabeled, commercially available methylated and nonmethylated nucleotides can be

spotted onto the TLC plates as markers. At least 25 μ*M* of each nucleotide is necessary to produce a nonfluorescent spot that is illuminated on the plate using a 254-nm UV light. Unlabeled and labeled nucleosides and nucleotides should be run together on a single plate to ensure correct molecular assignments.

16. Apply 3 μL of loading buffer at the beginning of the prerun step. The bromophenol blue and xylene cyanol can then be used as tracking dye to follow the salt front to determine the time necessary for its removal. For 20 × 19 cm and 0.75-mm thick gels, the time needed is about 1 h when the gel is run at 800 V. Failure to sufficiently prerun the gel will result in two or more of the products of the decapping reaction running together at the salt front. This can cause significant misinterpretation of data.
17. Proteins can impede the clear resolution of the products. Therefore, if problems arise, not loading the whole reaction mixture might be advisable. Because of the small volume of the reaction, diluting it with water will make the extraction more manageable. Only half (10 μL) of the aqueous phase is transferred to the new tube to ensure that no proteins from the interface are in the sample.
18. As an option, one may wish to prepare radiolabeled markers to assist in the identification of decapping products. Radiolabeled ^{7me}G*pppGOH or G*pppGOH can be prepared by digestion of methylated or nonmethylated ^{32}P-cap-labeled RNA with nuclease P1 (Roche, Indianapolis, IN) in CE buffer at 37°C for 30 min following manufacturer's recommendations. Radiolabeled ^{7me}G*pppGp or G*pppGp can be prepared by digestion of methylated or nonmethylated ^{32}P cap-labeled RNA with nuclease T1 (Roche) in CE buffer at 37°C for 30 min following the manufacturer's recommendations

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16

Analysis of RNA Exonucleolytic Activities in Cellular Extracts

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Summary

Three types of exonucleases contribute to the turnover of messenger RNAs in eukaryotic cells: (1) general 3'-to-5' exonucleases, (2) poly(A)-specific 3'-to-5' exonucleases, and (3) 5'-to-3' exonucleases. All three of these activities can be detected in cytoplasmic extracts from a variety of eukaryotic cells. In this chapter, we describe the preparation and use of HeLa cytoplasmic S100 extracts to study these three distinct exonuclease activities. Also included is an immunodepletion protocol that can be used to identify the enzyme responsible for a given activity. These protocols can be easily expanded to the study of *trans*-acting factors, *cis*-acting RNA sequence elements, and the interplay of components involved in RNA turnover in various mammalian cell types.

Key Words

Deadenylation; mRNA turnover; in vitro assay; cytoplasmic extracts; exonuclease; exosome; phosphothioates; poly(A); mRNA stability.

1. Introduction

The study of deadenylation-dependent messenger RNA (mRNA) turnover has expanded dramatically in the last several years with the advent of successful in vitro-based assay systems ***(1–6)***. The turnover of the vast majority of mammalian mRNAs is initiated by deadenylation or shortening of the poly (A) tail ***(7,8)***. After deadenylation, the body of the transcript is degraded in a highly processive fashion by exonucleolytic activities in both yeast and mammalian cells. The identification and characterization of these enzymatic activities is therefore important to gain an understanding of mechanisms of transcript-specific regulation at the level of mRNA stability.

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Several poly(A)-specific 3'-to-5' exonucleolytic activities have been implicated in the initial step of mRNA decay. PARN mediates efficient deadenylation in mammalian cytoplasmic extracts through an interaction with the 5' cap structure ***(9–11)***. Two additional deadenylase activities have been identified in yeast, CCR4/Pop2 and Pan2/3 ***(12,13)***. Homologs of these factors exist in mammalian cells as well. Therefore, it is likely, that multiple, independently regulated deadenylases play a role in mRNA decay.

Both 5'-to-3' and 3'-to-5' exonuclease activities can play a role in degrading the body of deadenylated mRNAs. After removal of the cap structure and generation of a 5' monophosphate by the Dcp1/2 decapping enzyme, the conserved exonuclease Xrn1p rapidly degrades the mRNA in a 5'-to-3' direction ***(14,15)***. Alternatively, the deadenylated transcript may be rapidly degraded in a 3'-to-5' direction by the exosome, a conserved complex composed largely of exonucleases ***(16)***. Interestingly, AU-rich elements that mediate the rapid turnover of many short-lived mRNAs in mammalian cells have been shown to facilitate loading of the exosome and stimulate 3'-to-5' exonucleolytic decay in vitro ***(17,18)***. The contribution of exonucleolytic enzymes and pathways to the full complement of elements that influence mRNA stability remains to be established.

This chapter describes effective assays to study deadenylation and exonuclease activity in cytoplasmic extracts derived from mammalian cells. All of these assays contain two key components: high-quality RNA substrates and active cellular extracts. Detailed protocols are provided to generate a variety of intact RNA substrates that are free of contaminating nonspecific ribonucleases that could confound interpretation of the assays. To provide as much versatility as possible, both large- and small-scale approaches to generate cytoplasmic S100 extracts are presented. It should be noted that many of the methods presented here have also been successfully adapted to yeast and trypanosome extracts ***(3,4)***. Finally, we present an immunodepletion protocol that can be used to identify responsible enzymes or regulatory factors. In total, these techniques should provide a researcher with a comprehensive set of tools to assess riboexonuclease activities in cell-free systems.

2. Materials

2.1. Preparation of Cytoplasmic Extracts From Mammalian Cells

1. HeLa S3 spinner cells (ATCC).
2. Phosphate-buffered saline (PBS): 137 m*M* NaCl , 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.4.
3. JMEM (Sigma).
4. Horse serum (Sigma).
5. 2 m*M* Glutamine (Invitrogen).

6. Phenylmethyl sulfonyl fluoride (PMSF; Roche Molecular Biochemicals); Prepare 100 m*M* stock in isopropanol.
7. Dithiothreitol (DTT; Roche Molecular Biochemicals).
8. 40-mL and 1-mL Dounce homogenizers (Kontes) with B-type pestle.
9. Bradford Reagent (Bio-Rad).
10. Glycerol.
11. Dialysis membranes (Spectrum Laboratories).
12. Buffer D: 20 m*M* HEPES-KOH, pH 7.9; 20% (v/v) glycerol, 100 m*M* KCl; 0.2 m*M* ethylenediamine tetraacetic acid (EDTA); 1 m*M* PMSF (add fresh before use), 1 m*M* DTT (add fresh before use).
13. Buffer A (10 m*M* HEPES-KOH, pH 7.9; 1.5 m*M* $MgCl_2$; 10 m*M* KCl; 1 m*M* DTT (add fresh before use); 1 m*M* PMSF (add fresh before use). Store at +4°C.
14. Buffer B (0.3 *M* HEPES-KOH, pH 7.9; 1.4 *M* KCl, 30 m*M* $MgCl_2$).

2.2. Preparation of RNA Substrates: Nonpolyadenylated DNA Template

1. Restriction enzyme(s), various (New England Biolabs).
2. Restriction enzyme buffers (New England Biolabs).
3. Proteinase K.
4. RNase-free dH_2O (*see* **Note 1**).

2.3. Preparation of RNA Substrates: Polyadenylated DNA Template

1. T4 DNA Ligase Buffer: 50 m*M* Tris-HCl, pH 7.5; 10 m*M* $MgCl_2$; 10 m*M* DTT; 1 m*M* ATP, 25 μg/mL bovine serum albumin (BSA).
2. T4 DNA Ligase (New England Biolabs).
3. Restriction enzyme(s); various (New England Biolabs).
4. *Taq* polymerase (Invitrogen).
5. *Taq* polymerase buffer: 20 m*M* Tris-HCl, pH 8.4; 50 m*M* KCl; 1.5 m*M* $MgCl_2$.
6. 10 m*M* dNTP mix.
7. Polymerase chain reaction (PCR) primers, various (Operon).
8. RNase-free dH_2O (*see* **Note 1**).

2.4. Production and Purification of Radiolabeled RNA Transcripts

1. 10X RNA polymerase buffer: 40 m*M* Tris-HCl, pH 7.9; 6 m*M* $MgCl_2$; 2 m*M* spermidine; 10 m*M* DTT. Store at –20°C.
2. Unpolyadenylated or polyadenylated DNA template.
3. 10X rNTP mix (5 m*M* adenosine triphosphate [ATP], 5 m*M* cytidine 5' triphosphate [CTP], 0.5 m*M* guanosine triphosphate [GTP], 0.5 m*M* uridine 5' triphosphate [UTP]; prepare in Tris-buffered solution; store in freezer).
4. Cap analog (^{7me}GpppG; Amersham Pharmacia).
5. SP6 RNA polymerase (New England Biolabs).
6. RNase inhibitor (Roche Molecular Biochemicals).
7. α[^{32}P]UTP, 800 Ci/mmol (Perkin-Elmer).
8. Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1).

9. Ethanol (100% and 70% and 80% solutions).
10. 10 *M* Ammonium acetate.
11. tRNA (Sigma).
12. Dry ice.
13. Proteinase K.
14. HSCB: 25 m*M* Tris-Cl, pH 7.6; 400 m*M* NaCl; 0.1% sodium dodecyl sulfate.

2.5. Gel Purification of RNA Substrates

1. RNA loading buffer: 25 m*M* Tris-HCl, pH 7.6; 8 *M* urea; 1 m*M* EDTA; 0.02% bromophenol blue; 0.02% xylene cyanol.
2. 30% Acrylamide-*bis*-acrylamide (19:1).
3. Urea.
4. Ammonium persulfate (10% w/v solution).
5. TEMED.
6. 10X TBE: 0.89 *M* Tris, 0.89 *M* boric acid, and 0.02 *M* EDTA.
7. 1X TBE.

2.6. In Vitro mRNA Turnover Assays

1. Radiolabeled RNA transcript.
2. Polyadenlyate homopolymer (heterogeneous sized poly(A), Sigma).
3. 500 m*M* Phosphocreatine stock (Sigma).
4. 100 m*M* ATP stock; make a 10X phosphocreatine/ATP mix: 250 m*M* phosphocreatine and 15 m*M* ATP; store at –80°C.
5. 5' rGMP (Sigma).
6. 10% Polyvinyl alcohol (30K-70K LALLS, Sigma).
7. HeLa cytoplasmic extracts.
8. Tris-saturated phenol, pH 8.0.
9. Ethanol (100% and 80% solutions).
10. SP6 and T7 RNA polymerases (New England Biolabs).
11. SP6 and T7 RNA polymerase buffer: 40 m*M* Tris-HCl, pH 7.9; 6 m*M* $MgCl_2$; 2 m*M* spermidine; 10 m*M* DTT.
12. T3 RNA polymerase (Promega).
13. T3 RNA polymerase buffer: 40 m*M* Tris-HCl, pH 7.9; 10 m*M* NaCl; 6 m*M* $MgCl_2$; 10 m*M* DTT; 2 m*M* spermidine; 0.05% Tween-20.
14. tRNA (Sigma).
15. Dry ice.
16. HSCB: *see* **Subheading 2.4.**, **step 14**.
17. Materials for running an 8 *M* urea gel (*see* **Subheading 2.5.**).

2.7. Assessing the Direction of Exonucleolytic Decay: Making the 5' RNA Fragment

1. DNA template (i.e., linearized pBluescript II KS digested by *Eco*RV).
2. All materials required for production of a radiolabeled RNA transcript.

2.8. Assessing the Direction of Exonucleolytic Decay: Making the 3' RNA Fragment

1. DNA template.
2. 5 m*M* 5' GMP (Sigma).
3. All other materials (except 5-m*M* cap analog) required for production of a radiolabeled RNA transcript.

2.9. Assessing the Direction of Exonucleolytic Decay: Making the Bridge-Ligated RNA

1. RNA oligomers with and without site-specific phosphothioate substitutions (Dharmacon).
2. Radiolabeled and capped RNA (T3 produced RNA; 5' fragment, described in **Subheading 2.7.**).
3. Radiolabeled, polyadenylated, uncapped RNA (+/– ARE; 3' fragment, described in **Subheading 2.8.**).
4. T4 DNA ligase buffer: 50 m*M* Tris-HCl, pH 7.5; 10 m*M* $MgCl_2$; 10 m*M* DTT; 1 m*M* ATP; 25 μg/mL BSA.
5. DNA 5' bridge oligomer (1 pm/μL).
6. DNA 3' bridge oligomer (1 pm/μL).
7. RNase-free dH_2O.
8. RNase inhibitor.
9. 25 m*M* ATP.
10. T4 DNA ligase (New England Biolabs).
11. Phenol:chloroform:isoamyl alcohol (25:24:1).
12. Ethanol (100% and 80% solutions).
13. 10 *M* Ammonium acetate.
14. tRNA (Sigma).
15. Dry ice.
16. Materials for running an 8 *M* urea gel (*see* **Subheading 2.5.**).
17. Proteinase K.
18. HSCB: *see* **Subheading 2.4.**, **step 14**.

2.10. Immunodepletion Assays

1. HeLa S100 cytoplasmic extracts.
2. Antibodies (preimmune and specific).
3. RNase inhibitor.
4. Protein A Sepharose (Sigma).
5. Buffer D: *see* **Subheading 2.1.**, **step 12**.

3. Methods

3.1. Preparation of Cytoplasmic S100 Extracts From Mammalian Cells: Cell Growth and Maintenance

HeLa cells are grown in JMEM containing 10% horse serum and 2 m*M* glutamine. Cell density is kept below approx 5×10^5 cells/mL in spinner cul-

tures at 37°C by splitting the culture 1:1 approx every 24 h. For optimal extracts, it is best to keep the cell density low to ensure uniform growth properties across the entire population. We typically harvest 6 L of cells per preparation of cell lysate for convenience as determined by the largest size rotor available.

3.2. S100 Extraction Protocol: Large-Scale Preparation (see Note 2)

1. Pour the 6 L of HeLa cells into 1-L centrifuge bottles and using the Beckman JLA 8.1000 rotor (or equivalent) centrifuge at 3500*g* for 10 min at 4°C.
2. Discard media and wash each pellet by resuspending it in cold PBS. Use 10 mL per pellet and transfer to two 50-mL conical centrifuge tubes (30 mL each).
3. Pellet cells in tabletop centrifuge (IEC) at 1500*g* for 2 min at 4°C.
4. Pour off PBS/media supernatant and wash pellet 1X with cold PBS. Pellet cells as in **step 3**.
5. Note packed cell volume (PCV; typically approx 5 mL per 3 L of HeLa cells).
6. Add 5X PCV of cold buffer A.
7. Incubate resuspended cells on ice for 10 min to allow cells to swell.
8. Recover the cells by centrifuging at 1500*g* for 2 min at 4°C (*see* **Note 3**).
9. Decant supernatant carefully and resuspend pellets in 2 times the original PCV of buffer A.
10. Transfer suspension to a prechilled 40-mL Dounce homogenizer (Kontes or Wheaton) and lyse cells with 10 gentle strokes using a B-type pestle.
11. Transfer the lysed cells to two new 50-mL conical tubes and centrifuge in tabletop centrifuge at 1500*g* for 4 min at 4°C.
12. Pipet supernatant into a single new 50-mL conical tube, being careful not to transfer any pelleted nuclei (*see* **Note 4**).
13. Add 0.11 vol of buffer B to cytoplasmic fraction and mix gently.
14. Distribute lysate to appropriate sized tubes for a swinging bucket rotor and centrifuge at 100,000*g* for 1 h at 4°C in an ultracentrifuge.
15. Pool the supernatant from each centrifuge tube and add 100% glycerol to a final concentration of 10%.
16. Dialyze the supernatant against approx 100X volumes of buffer D for 30 min at 4°C (*see* **Note 5**).
17. Transfer supernatant to a centrifuge tube and centrifuge at 27,000*g* in an SS34 rotor (or equivalent) for 10 min at 4°C.
18. Aliquot dialyzed S100 lysate into microcentrifuge tubes and store at –80°C (*see* **Note 6**).

3.3. Small-Scale Preparation: Isolation of Cytoplasm From Attached Cells

This procedure is for preparing cell lysates from cells on plates or low-density cultures. Although this will not be an S100 fraction, it works quite well for fibroblastic cells and other cells that cannot be grown in spinner cultures.

1. Wash attached cells twice using ice-cold PBS.

2. Scrape the cells using a rubber policeman into 1 mL of cold PBS. It is best to have the plates on ice at this point to keep everything as cold as possible.
3. Pipet cells into 1.5-mL centrifuge tubes and pellet at approx 4500*g* for 2 min at 4°C. Remove the PBS and start the lysis protocol.
4. Resuspend the cell pellet in 5 PCV of cold buffer A.
5. Allow the cells to swell on ice for 10 min.
6. Pellet the cells in the tabletop centrifuge as before (4500*g*, 2 min, 4°C).
7. Carefully pour off the supernatant making sure to not lose any cells. This pellet is not compact and it is often best to pipet off the residual amount of buffer A so as not to disturb the cell pellet (*see* **Note 3**).
8. Resuspend the pellet in two original PCV of buffer A and transfer the suspension into a 1-mL Dounce homogenizer (Kontes) that has been precooled on ice.
9. Lyse the cells with 10 strokes of a B-type pestle. Gentle strokes are all that are necessary and will avoid loss of lysate.
10. Transfer the lysed cells to a new 1.5-mL centrifuge tube and pellet the nuclei in the microcentrifuge (4500*g*, 2 min, 4°C).
11. Transfer the supernatant to a new tube. This is the cytoplasmic fraction. Be careful not to contaminate this fraction with nuclei (*see* **Note 4**).
12. Add 0.11 vol of ice-cold buffer B to the cytoplasmic fraction.
13. Centrifuge the cytoplasmic fraction at 16,000*g* for 15 min at 4°C. This is at full speed for most microcentrifuges.
14. Transfer the supernatant to a new 1.5-mL centrifuge tube. If there is a white layer on the top of your fractions, carefully remove it with a pipet and discard. Although we have not performed a comprehensive check of this layer, it is most likely caused by contaminated membrane lipids and is dispensable.
15. Add 100% glycerol to a final concentration of 10%.
16. Dialyze the supernatant against 100 vol of cold buffer D for 30 min (*see* **Note 5**).
17. After dialysis, aliquot the cytoplasmic extract and store at –80°C. Store in useful sized aliquots to avoid repetitive freeze-thaws (*see* **Note 6**).

3.4. Preparation of RNA Substrates for Exonuclease Assays: Nonpolyadenylated DNA Template

The general idea behind the preparation of RNA substrates rests in the knowledge of the characteristics of cellular mRNAs. If you wish to test certain elements of an RNA molecule for activity in RNA turnover, then it is best to start with a transcript that contains the global characteristics of all mRNAs: most notably a 5' cap structure and a 3' poly (A) tail. Subsequent assays can be performed with transcripts lacking these terminal modifications to directly assess exonuclease properties. Here, we describe a method of obtaining both polyadenylated and nonpolyadenylated substrates. Note that any sequence can be cloned into an expression vector containing any one of the three common phage RNA polymerase promoter sites (SP6, T3, or T7) for generating an RNA substrate to be used in this assay.

The key to making quality RNA is having clean DNA as a template. Maxiprep DNA from a Qiagen filter based kit or a standard alkaline lysis/PEG precipitation maxiprep protocol ***(19)*** is optimal. We use DNA generated by both methods. It is essential to maintain RNase-free conditions of all solutions, reagents, and labware.

1. Digest the DNA template of interest for 2 h at 37°C with the desired restriction enzyme to ensure efficient and complete digestion. We routinely use a 100-µL reaction volume to digest 20 µg of DNA vector template.
2. Add 300 µL of HSCB buffer and 3 µL of a 10 mg/mL proteinase K solution to the digestion and incubate at 37°C for 15 min to remove protein contaminants.
3. Add 400 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and vortex for several seconds.
4. Centrifuge in a microcentrifuge for 3 min at full speed.
5. Transfer top aqueous layer to a new microcentrifuge tube being extremely careful not to get any of the interface as a contaminant.
6. Add 1 mL of 100% ethanol and place on dry ice for 10 min.
7. Pellet the precipitated DNA at full speed in a microcentrifuge for 10 min.
8. Remove the supernatant and wash the DNA pellet with 70% ethanol.
9. Dry the pellet by lyophilization in a Speed-vac if available.
10. Resuspend the DNA pellet in RNase-free dH_2O for a final concentration of 1 µg/µL.

This DNA can now be used to produce RNA without a poly(A) tail for exosome assays or can be used in the following procedure to generate a DNA template with a polyadenylated tail of 60 nt.

3.5. Preparation of RNA Substrates for Exonuclease Assays: Polyadenylated DNA Template

This procedure generates a DNA template that produces an RNA with a polyadenylate tail of 60 nt ***(19)***.

1. We use the *Hin*dIII digested DNA prepared above to ligate to an annealed pair of oligos that have a 5' overhang complementary to the *Hin*dIII sequence, 60 A/T residues, an *Nsi*I restriction site, and a downstream primer sequence (DPS):
   5' AGCTAAAA(50)AAAAATGCATTACCTCGAGCACTC
   ATTTT(50)TTTTTACGTAATGGAGCTCGTGAG
2. Ligation reaction: Mix 1 µg of DNA, 1 pmol annealed oligos, 2 µL of 10X T4 DNA ligase buffer (NEB), 1 µL of T4 DNA ligase, and add dH_2O to a final volume of 20 µL. Incubate the reaction at 16°C for 2 h.
3. The ligation mix is then subjected to PCR amplification using the following conditions: 1 µL of the ligation, 10 µL 10X PCR buffer (Promega) containing 1.5 m*M* $MgCl_2$, 50 pmol downstream primer (DPS) (5'TACCTCGAGCACTC), 50 pmol of an SP6 primer not containing the complete core sequence (5'-CATACGATTTAGGTGAC), 1 U *Taq* polymerase, and dH_2O to 100 µL. The reaction times are as follows: denature at 94°C for 30 s, anneal at 55°C for 30 s,

and polymerize at 72°C for 1 min, repeating for 30 cycles.

4. The PCR product is then purified by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation as described previously. The DNA is resuspended in a digestion reaction for *Nsi*I to remove the DPS: 10 μL of 10X *Nsi*I buffer (NEB), 20 U of *Nsi*I enzyme, and dH_2O to 100 μL. Incubate the reaction for 1 h at 37°C to ensure complete digestion of template. This will generate a template which terminates precisely at the final A of the poly(A) tail.
5. Add 10 μL 10% SDS and 3 μL of 10 mg/mL proteinase K. Incubate for 15 min at 37°C.
6. Repeat **steps 3–10** in **Subheading 3.4.** to finalize the DNA template preparation.

3.6. Production and Purification of Radiolabeled RNA Transcripts

1. Add the following reagents to a microcentrifuge tube:
   1 μg of DNA template (prepared as described in **Subheadings 3.4.** or **3.5.**)
   1 μL of 5 m*M* cap analog (^{7me}GpppG)
   1 μL of 10X rNTP mix (5 m*M* ATP, 5 m*M* CTP, 0.5 m*M* GTP, 0.5 m*M* UTP)
   1 μL of 10X RNA polymerase buffer (NEB)
   0.5 μL of RNase inhibitor (Roche)
   4.5 μL of [α^{32}P]-UTP (Perkin-Elmer 800 Ci/mmol)
   1 μL of SP6 RNA polymerase (NEB)
2. Incubate the 10-μL reaction at 37°C for 1 h.
3. Add 150 μL of dH_2O to the reaction for a final volume of 160 μL.
4. Add 160 μL of phenol:chloroform:isoamyl alcohol and vortex for several seconds.
5. Centrifuge at full speed in a microcentrifuge for 3 min and transfer the top aqueous phase to a new microcentrifuge tube.
6. Add 40 μL of 10 *M* NH_4OAc and 500 μL of 100% ethanol. Incubate on dry ice for 10 min (*see* **Note 7**).
7. Pellet RNA at full speed in a microcentrifuge for 10 min. Wash with 80% ethanol and vacuum-dry the pellet.
8. Resuspend the labeled RNA pellet in 5 μL of RNA loading buffer by vigorously vortexing.
9. Heat sample at 90°C for 30 s and load onto a 5% denaturing polyacrylamide gel containing 8 *M* urea. It is advisable to prerun the gel for approx 30 min before loading the RNA to ensure that migration of the transcript will not be impeded by the salt front.
10. The labeled RNA is recovered via gel purification. Stop the gel when appropriate for the size of the RNA expected based on the movement of the bromophenol blue and xylene cyanol dyes.
11. Expose the gel to film and visualize the transcribed RNA band(s).
12. Align the film to the gel and excise the appropriate RNA band(s) with a sterile razor blade.
13. Place the excised RNA gel slab into 400 μL of HSCB and 20 μg of proteinase K. Passively elute the RNA overnight at room temperature.

14. Pipet the HSCB (which now contains the eluted RNA) into a new tube. Clean the transcript with phenol:chloroform extraction (using 400 μL and performed as outlined in **steps 4** and **5** above) and precipitate the RNA by adding 2.5 vol of 100% ethanol (place on dry ice for 10 min and proceed as described above in **step 7**). Do not add additional salt or tRNA to this precipitation step.
15. Resuspend the pellet in 26 μL of RNase-free dH_2O. Calculate the cpm/μL by counting 1 μL of the sample in a scintillation counter.
16. Adjust the sample using an appropriate volume of RNase-free dH_2O to produce 100,000–200,000 cpm/μL RNA transcript as needed for the assay.
17. Store the RNA at –80°C.

3.7. In Vitro mRNA Turnover Assays: In Vitro Deadenylation

The next three protocols describe how to independently assay for deadenylase, 5'-to-3' exonucleases, and 3'-to-5' exonucleases in cytoplasmic extracts. Depending on the design of the experiment, it is important to choose the correct RNA substrate and reaction conditions to differentiate between the various enzymatic activities. This protocol specifically addresses an assay for deadenylation. The following is a reaction mixture for a single time point. Please note that for time course experiments, a larger cocktail should be prepared and aliquots taken out as necessary. The addition of cold poly(A) competitor sequesters poly(A) binding proteins and allows PARN to gain access to the poly(A) tail of the RNA substrate. An example of a deadenylation assay using extracts from three different types of mammalian cells is presented in **Fig. 1**.

1. In a microcentrifuge tube add the following reagents in this specific order:
   4 μL of 10% polyvinyl alcohol
   1 μL of 250 m*M* phosphocreatine/15 m*M* ATP mix
   1 μL of 500 ng/μL cold poly(A) (Sigma, heterogeneously sized homopolymer)
   1 μL of 200,000 cpm/μL RNA "A60" substrate (a capped transcript with a 60 base poly(A) tail prepared as described above in **Subheading 3.5.**)
   8 μL of HeLa S100 cytoplasmic extract
2. Incubate reaction at 30°C for the desired time.
3. Add 400 μL of HSCB buffer to stop the reaction. Note that for a time course, aliquot 15 μL per time point into 400 μL of HSCB.
4. Add 400 μL of phenol:chloroform:isoamyl alcohol and vortex thoroughly.
5. Separate phases in a microcentrifuge at full speed for 3 min.
6. Transfer aqueous layer to new centrifuge tube being extremely careful not to take any of the interface (*see* **Note 8**).
7. Add 1 μL of 5 mg/mL tRNA and 1000 μL of 100% ethanol. (Note that there is no need to add additional salt.)
8. Incubate on dry ice for 10 min (or until the entire time course is complete, *see* **Note 9**).
9. Pellet the RNA in a microcentrifuge at full speed for 10 min. Discard the supernatant into a radioactive waste container and wash the pellet with 80% ethanol.

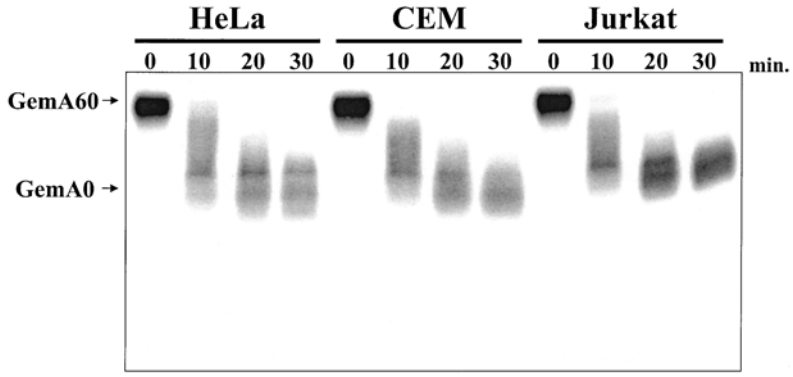

Fig. 1. Deadenylation using three independent cell extracts. Internally labeled GemA60 RNA *(1)* was incubated in cytoplasmic S100 extracts from HeLa, Jurkat, or CEM cells for the times indicated as described in **Subheading 3.7.** Reaction products were analyzed on a 5% acrylamide/8 *M* urea gel.

10. Vacuum-dry or thoroughly air-dry the pellet.
11. Resuspend the RNA pellet in 5 μL of RNA loading buffer making sure that the pellet is completely solubilized by vortexing.
12. Incubate the resuspended RNA at 90°C for 30 s and load onto a denaturing polyacrylamide gel (0.75 m*M* thick, 5% polyacrylamide, 8 *M* urea, 1X TBE). Apply sufficient power to run the gel as rapidly as possible without cracking the plates owing to excess heat.
13. Visualize the results via phosphorimaging or autoradiography.

3.8. In Vitro 3'-to-5' Exonuclease Assay

This assay relies on a nonpolyadenylated RNA substrate and is stimulated by *cis*-acting elements that encourage the exosome to load onto the RNA for further degradation. The general idea is that an RNA that still retains its 5' cap structure (^{7me}GpppG) but has been deadenylated is incubated in cytoplasmic extracts. Because decapping is very inefficient in cytoplasmic extracts under these conditions, the RNA is essentially only degraded by 3'-to-5' exonucleases. An example of a 3'-to-5' exonuclease assay in HeLa S100 cytoplasmic extract is shown in **Fig. 2**. The direction of decay can be confirmed as discussed using **Subheadings 3.10–3.13.** below.

1. In a microcentrifuge add the following reagents in this specific order:
   4 μL of 10% polyvinyl alcohol
   1 μL of 250 m*M* phosphocreatine/15 m*M* ATP
   1 μL of 200,000 cpm/μL RNA substrate that lacks a poly(A) tail
   8 μL of HeLa S100 cytoplasmic extract
2. Repeat **steps 2–13** in **Subheading 3.7.**

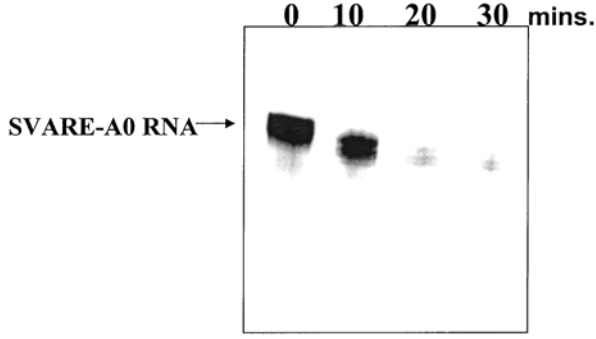

Fig. 2. Demonstration of a 3'-to-5' exonuclease assay. Internally labeled SVARE-A0 RNA *(1)* was incubated in HeLa cytoplasmic nuclear extracts for the times indicated as described in **Subheading 3.8.** Reaction products were analyzed on a 5% acrylamide/urea gel. Note that the transcript is rapidly degraded with no apparent intermediates.

3.9. In Vitro 5'-to-3' Exonuclease Assay

In this assay, a polyadenylated RNA transcript is made during transcription such that it has a 5' pG structure rather than the regular cap structure (^{7me}GpppG) or a 5' triphosphate (both of which are inhibitory to 5'-to-3' exonucleases). In addition, no unlabeled poly(A) competitor RNA is added to these reactions so that the 3' end of the transcript is protected by poly(A) binding proteins that interact with the poly (A) tail. Therefore, we can now selectively assay for 5'-to-3' exonucleases, such as human Xrn1p, in cell extracts.

1. In a microcentrifuge tube add the following:
   1 μg of DNA template (i.e., polyadenylated pGEM ARE A60)
   1 μL of 5m*M* rGMP (Sigma)
   1 μL of 10X rNTP mix (5 m*M* ATP, 5 m*M* CTP, 0.5 m*M* GTP, 0.5 m*M* UTP)
   1 μL of 10X RNA polymerase buffer (NEB)
   0.5 μL of RNase inhibitor (Roche)
   4.5 μL of α[^{32}P]UTP (800 Ci/mmol)
   1 μL of SP6 RNA polymerase (NEB)
2. Repeat **steps 2–17** in **Subheading 3.6.**
3. Now 100,000 cpms of the gel-purified RNA substrate is subjected to an exonuclease assay following the steps described in **Subheading 3.8.** with one key exception: Do not add unlabeled poly(A) to this reaction.

3.10. Assessing the Direction of Exonucleolytic Decay: 5' RNA Fragment

Riboexonucleases have been identified in many organisms with the best studied being that of unicellular organisms, yeast and bacteria, because of the ease of genetic manipulation. One method that works well in yeast and trypa-

nosomes to assess directionality of exonucleases is to use a stretch of G nucleotides to trap intermediates ***(4,20)***. The use of a G_{18} oligomer placed within the RNA body, however, has been found by several researchers to be inefficient at trapping intermediates in mammalian cells. Perhaps this is the result of a context effect or that specific factors are present in mammalian cells that target or melt these G-rich structures. We present below an approach using a synthesized RNA that contains phosphothioate modifications at specific sites in order to efficiently trap decay intermediates. These RNAs are constructed by joining two in vitro transcribed RNAs at either end of a chemically synthesized RNA middle using a DNA:RNA splinted ligation technique ***(21)***. This protocol outlines the production of the 5' RNA fragment. This fragment is a capped RNA transcribed by T3 RNA polymerase and internally labeled with [α-^{32}P]-UTP. The transcription reaction is carried out as described below.

1. In a microcentrifuge tube add the following reagents:

   1.0 µL of DNA template (i.e., linearized pBluescript II KS digested by *Eco*RV (17); prepare as described in **Subheading 3.4.**)

   1.0 µL of 5 m*M* cap analog (^{7me}GpppG)
   1.0 µL of 10X rNTP mixture
   4.5 µL of [α^{32}P-UTP (Perkin-Elmer 800 Ci/mmol)
   0.5 µL of RNase inhibitor (Roche)
   1.0 µL of 10X RNA polymerase buffer (NEB)
   1.0 µL of T3 RNA polymerase (NEB)
2. Repeat **steps 2–14** in **Subheading 3.6.**
3. Resuspend RNA in 2 µL of RNase-free dH_2O.

3.11. Assessing the Direction of Exonucleolytic Decay: 3' RNA Fragment

The downstream RNA fragment is polyadenylated and is transcribed in the presence of 500 µ*M* GMP to create a 5'-monophosphate end. This RNA is transcribed by using SP6 RNA polymerase and is also internally labeled using α-[^{32}P]UTP.

1. In a microcentrifuge tube add the following reagents:

   1.0 µL of DNA template (i.e., Gem A60 or GemARE A60; **ref. *1***)
   1.0 µL of 5 m*M* GMP
   1.0 µL of 10X rNTP mixture (5 m*M* ATP, 5 m*M* CTP, 0.5 m*M* GTP and 0.5 m*M* UTP)
   4.5 µL of α[^{32}P]UTP (800 Ci/mmol)
   0.5 µL of RNase inhibitor
   1.0 µL of 10X RNA polymerase buffer (NEB)
   1.0 µL of SP6 RNA polymerase (NEB)
2. Repeat **steps 2–14** in **Subheading 3.6.**
3. Resuspend RNA in 2 µL of RNase-free dH_2O.

3.12. Assessing the Direction of Exonucleolytic Decay: Middle RNA Fragment

The middle RNA fragment is a synthetic RNA oligonucleotide 30 nt long that contains three consecutive residues linked by phosphothioate modifications rather than standard phosphodiester bonds. A matching RNA oligo without any modification is designed to generate a wild-type control RNA. These RNA oligonucleotides can be ordered from Dharmacon.

3.13. Bridge Ligation

These three RNAs are then joined together by a three-fragment bridge ligation technique adapted from a protocol described by Moore and Sharp *(21)*. The ligation is performed in the presence of two approx 26-base DNA oligos that extend 10 bases to either side of the junction between the two fragments that are targeted for joining.

1. In a microcentrifuge add the following reagents:
   2 μL of T4 DNA ligation buffer (NEB)
   2 μL of synthetic RNA oligomer (+/– phosphothioate modifications)
   2 μL of labeled and capped RNA (T3 produced RNA)
   2 μL of labeled, polyadenylated, uncapped RNA
   2 μL of DNA 5' bridge oligomer (1 pm/μL)
   2 μL of DNA 3' bridge oligomer (1 pm/μL)
   5 μL of RNase-free dH_2O
2. Incubate the reaction at 90°C for 2 min and then allow to cool to room temperature slowly.
3. Add to the reaction 0.5 μL of RNase inhibitor, 0.5 μL of 25 m*M* ATP, 2 μL of T4 DNA ligase.
4. Incubate the ligation mix at 30°C for 2–4 h.
5. Repeat **steps 3–17** of **Subheading 3.6.**
6. This gel purified RNA is then used in any of the deadenylase (**Subheading 3.7.**) or exonuclease assays (**Subheading 3.8.** or **3.9.**) outlined above. Depending on the size of the trapped intermediates blocked by the phosphothioate modifications in the backbone of the RNA, one can determine whether the RNA is being degraded from the 5' end or the 3' end (*see* **Note 10**). An example of this assay is shown in **Fig. 3**.

3.14. Immunodepletion Assays for Identifying the Enzymes Involved: Immunodepletion by Direct Addition of Antisera to Extracts

Previously, we have used immunodepletion to identify the role of PARN and exosomal components in the HeLa S100 extracts *(9,17)*. Although this is a

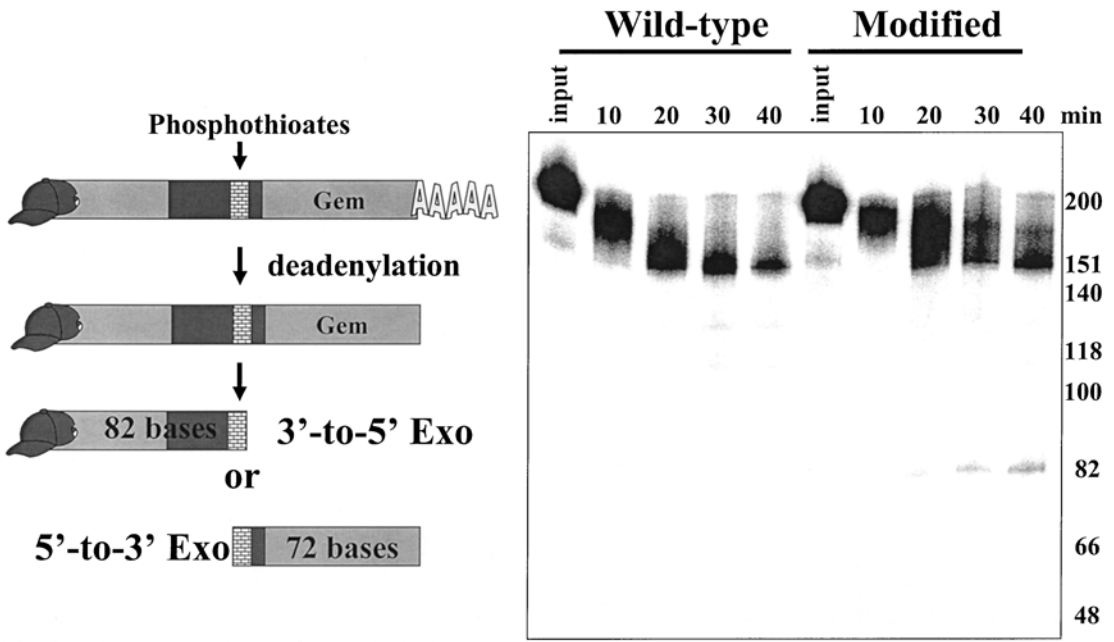

Fig. 3. An assessment of the directionality of exonucleolytic decay after deadenylation in HeLa cytoplamsic extracts. A phosphothioate-substituted GemA60 RNA substrate ***(1)*** was prepared as described in **Subheadings 3.10–3.13.** As shown in the panel on the left, trapping of an 82-base intermediate would identify a block by phosphothioate modifications to a 3'-to-5' exonuclease, whereas trapping a 72-base fragment would be consistent with decay in a 5'-to-3' direction. Results were analyzed on a 5% acrylamide/urea gel in the panel on the right. Note that only an 82-base intermediate is observed.

straightforward way of identifying enzymes involved in mRNA turnover, readdition of purified or recombinant factors should always be performed when possible to confirm the results of immunodepletion assays.

1. Add 1 μL of RNase inhibitor to each 4 μL of rabbit polyclonal antisera to neutralize endogenous nuclease activities.
2. Mix 25 μL of HeLa S100 extract with 3–4 μL of treated antisera.
3. Incubate the reaction mixture on ice for 30–40 min. Mix the reaction occasionally by tapping gently.
4. Prewash protein A-Sepharose (Sigma) with buffer D 3–4 times; 1 g swells to 4 mL.
5. Add 15–20 μL of this prewashed protein A-Sepharose to the reaction and incubate the mixture on ice for an additional 15–20 min.
6. Centrifuge the reaction at 4500*g* for 1 min to precipitate immune complexes.
7. Remove the immunodepleted extracts very carefully, so as not to disturb any protein A-Sepharose beads.
8. Now you can use these extracts in the in vitro deadenylation-degradation reactions.

3.15. Immunodepletion Assays for Identifying the Enzymes Involved: Imunodepletion Using Antibodies Prebound to Protein A-Sepharose

1. Wash protein A-Sepharose beads three to four times with buffer D.
2. Mix 4–5 μL of rabbit preimmune or anti-PM-Scl75 polyclonal antisera with 20–25 μL of washed protein A-Sepharose beads.
3. Incubate the mixture on ice for approx 30 min.
4. Then centrifuge at 4500*g* for approx 1 min and discard the supernatant. The pellet now contains antibody-bound protein A-Sepharose.
5. Wash the pellet 3–4 times with buffer D.
6. Mix 20 μL of HeLa cell S100 extracts with the antibody-bound protein A Sepharose beads and incubate the mixture on ice for approx 30–40 min.
7. Centrifuge the mixture at 4500*g* for 1 min.
8. Remove the supernatant, which is the immunodepleted extract, very carefully without disturbing or touching the protein A-Sepharose beads.

Now you can use the immunodepleted extracts in any of the deadenylase (**Subheading 3.7.**) or exonuclease assays (**Subheadings 3.8.** or **3.9.**) outlined above. It is vital to determine the efficiency of immunodepletion by Western blot because very few immunoprecipitations are 100% effective. Either of the two procedures above can be repeated on extracts to increase the effectiveness of the immunodepletion (*see* **Note 11**).

4. Notes

1. We obtain RNase-free ddH_2O by collection of house DI water in glass bottles from a distillation apparatus (Barnstead Mega-Pure MP-6A). Bottles are then autoclaved and stored at room temperature. To maintain these stock bottles

RNase-free, we never put anything into them (e.g., pipets). We pour the water out into a 50-mL conical tube before use.

2. This protocol is adapted from the one originally published by Dignam et al. ***(22)***. It is critical at this stage to make sure that all reagents, tubes, glassware, and any equipment that comes in contact with the cell lysate is RNase-free. It is also important for all bottles, tubes, solutions, and equipment to be kept cold on ice or at 4°C. It is important to have the centrifuges at 4°C so no time is taken in stabilizing the temperature during operation. The bottom line is that everything should be kept as cold as possible.
3. After taking note of the initial PCV, it is important that the cells have swelled sufficiently for lysis. For HeLa cells, we normally see a doubling in the PCV. You should see at least a 1.5-fold increase in cell volume.
4. It is important not to transfer any nuclei at this stage. Lysed nuclei are by far the greatest source of contamination we have noted in the S100 extract. Lysed nuclei contribute to a nonselective 3'-to-5' exonuclease activity that can confound the analysis of deadenylases.
5. Dialysis should be performed in the cold room and with an ice sleeve. We use a 1-L beaker in an ice bucket in the cold room on a stir plate for agitation. We have found that dialysis is optional when studying general kinetics of turnover. However, if the mechanisms of interest are dependent upon small molecules, such as ATP, and one wishes to control for their effects, then dialysis is critical.
6. Save a few microliters of the S100 to test in a Bradford Assay to determine the protein concentration. Perform a Bradford assay using 1 μL of the extract and 200 μL of Bradford Reagent (Bio-Rad) in 1 mL of total volume. We normally average 6–8 mg/mL after this procedure.
7. 5 μg tRNA may be added as a carrier at this step to ensure quantitative recovery of radiolabeled RNA. We routinely add carrier unless the expected transcription product would comigrate with tRNA on an acrylamide gel (approx 70–80 nt).
8. If the interface is too thick (white and foamy) or will not settle out from the aqueous phase, then back extraction of the phenol:chloroform will be necessary. Optionally, instead of using a mixture of phenol:chloroform, we have found that using 400 μL of Tris-saturated phenol alone to extract the RNA reaction works quite well and usually minimizes interface problems.
9. It is important to process each time point immediately. The quality of the RNA decay product may otherwise suffer. It is alright to leave the RNA decay product under ethanol on dry ice while preparing the additional time points. The tRNA is used for efficient and quantitative precipitation of all RNA products resulting from the decay reaction. It is *not* optional.
10. Placement of phosphothioate modifications at two independent places in the RNA (i.e., near either the 3' or 5' ends) can be used to rigorously demonstrate that endonucleases are not playing a role in the degradation of the RNA substrate.
11. Examples of optimization approaches include increasing the quantity of antibody or the incubation time. Excessive incubation times, however, should be avoided to maintain the activity of the extract.

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17

Application of Ligation-Mediated Reverse Transcription Polymerase Chain Reaction to the Identification of In Vivo Endonuclease-Generated Messenger RNA Decay Intermediates

Mark N. Hanson and Daniel R. Schoenberg

Summary

Most approaches to studying messenger RNA (mRNA) decay in vivo lack sufficient sensitivity to identify decay intermediates. The identification of such intermediates using in vitro decay systems can provide suggestive evidence for endonuclease-mediated degradation in vivo; to validate conclusions drawn from in vitro experiments one must demonstrate cleavage of the mRNA in vivo. Primer extension or S1 nuclease protection assays work best on relatively abundant mRNAs and even then require long exposure times. We describe a facile approach using ligation-mediated polymerase chain reaction to identify in vivo mRNA decay intermediates. In this procedure, total cellular RNA is ligated to a primer bearing a 5' phosphate and 3' amino group. Reverse transcription is primed using a complementary primer, and mRNA-specific decay intermediates are identified by polymerase chain reaction amplification using a 5'-[^{32}P]-labeled gene-specific primer. Products generated in this manner are gel purified, reamplified, and the 3' end of each decay intermediate is identified by the sequence junction of the specific mRNA and the initial ligation primer. We show an example of the time-course of appearance of several specific decay intermediates of c-*myc* mRNA in differentiating murine erythroleukemia cells.

Key Words

Endonuclease; PCR; mRNA decay; cleavage products; c-*myc*; decay intermediates; endonucleolytic cleavage; mRNA turnover.

1. Introduction

Although exonucleolytic degradation is thought to be the major process by which most messenger RNAs (mRNAs) are degraded, there are numerous,

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well-characterized examples of endonuclease-mediated mRNA degradation ***(1)***. Among these are mRNAs for apo-very low density lipoprotein II ***(2)***, c-*myc* ***(3)***, transferrin receptor ***(4)***, insulin-like growth factor II ***(5)***, α-globin ***(6)***, and albumin ***(7)***. With the exception of insulin-like growth factor II mRNA, whose unique structure results in a remarkably stable in vivo endonuclease degradation product ***(5)***, most mRNAs are degraded without the accumulation of detectable decay intermediates. In contrast, intermediates are commonly observed in experiments using crude in vitro decay systems. To prove that such intermediates reflect endonuclease-mediated mRNA decay in vivo, one must demonstrate the presence of corresponding products in cells or tissues. This presents a significant obstacle for all but a limited number of highly abundant mRNAs for which decay intermediates can be detected by primer extension or S1 nuclease protection assays. Even for abundant mRNAs, these techniques present difficulties, requiring large amounts of input RNA and long exposure times. We describe here a ligation-mediated (LM) reverse transcription polymerase chain reaction (RT-PCR) method that can be used to identify 3'-ends of degradation intermediates generated in vivo. We previously demonstrated the utility of this approach for identifying in vivo cleavage sites within both an abundant mRNA (albumin) and a rare mRNA (c-*myc*) ***(8)***.

Although endonucleolytic cleavage may initiate mRNA decay, the fact that decay intermediates do not accumulate indicates that cleaved decay intermediates must be substrates for subsequent exonucleolytic degradation, such as the components of the exosome. Such exonucleases cannot degrade mRNAs bearing a 3' phosphate. Hence, we reasoned that in vivo endonuclease decay intermediates will bear unmodified 3' termini, a result confirmed in the course of characterizing PMR1, the first identified vertebrate mRNA endonuclease ***(9)***. The LM-RT-PCR approach to identifying in vivo cleavage products builds upon this. In the first step the 3' ends of all RNAs in a population of total RNA are ligated to a primer bearing a 5'-phosphate and 3'-amino group. The 3' amino group blocks concatamerization of the primer during ligation. The conditions described below were optimized to yield approx 50% ligation efficiency. A primer complementary to the ligated primer is used for reverse transcription. PCR is then performed using a 5'-[^{32}P]-labeled primer specific to the mRNA of interest and the same primer used for reverse transcription. The PCR products are separated on a denaturing polyacrylamide/urea gel and identified by film autoradiography. We have found this can also serve as a data acquisition step and show below the accumulation of c-*myc* mRNA decay intermediates during dimethyl sulfoxide-induced differentiation of murine erythroleukemia cells. The bands identified by film autoradiography are excised, reamplified, and sequenced. The junction of the ligated primer with the mRNA identifies the 3' end of a particular degradation intermediate.

2. Materials

2.1. Primer Ligation

1. Ligation buffer: 50 m*M* Tris-HCl, pH 8.0; 10 m*M* $MgCl_2$; 20 m*M* ATP; 2 m*M* dithiothreitol; 10 μg/mL bovine serum albumin (BSA), 1 m*M* hexamine cobalt chloride, 25% (w/v) polyethylene glycol 8000, and 30 U placental ribonuclease inhibitor.
2. Primer MH11NH_3P (P-CCAGGTGGATAGTGCTCAATCTCTAGATCG-NH_3), which has a 5' phosphate (for ligation) and 3' amino termini (to prevent concatamerization).
3. T4 RNA ligase (Invitrogen, Life Technologies, Inc., Carlsbad, CA).
4. Phenol:chloroform:isoamyl alcohol (25:24:1).
5. Chloroform:isoamyl alcohol (24:1).
6. 3 *M* Sodium acetate, pH 5.5.
7. 20 mg/mL Glycogen.

2.2. Labeling of Gene-Specific Primer

1. Gene-specific primer: approx 19 nt with a T_m 56–58°C using 2X(2GC+AT).
2. 10X kinase buffer: 500 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 1 m*M* ethylenediamine tetraacetic acid, 50 m*M* dithiothreitol, 1 m*M* spermidine, pH 8.2.
3. α-[^{32}P]ATP (6000 Ci/mmol).
4. T4-polynucleotide kinase (Roche Molecular Biochemicals, Indianapolis, IN).
5. Distilled H_2O.

2.3. RT-PCR

1. Primer MH12 (5'CGAGCTAGAGATTGAGCAC), which is complementary to the first 19 nt of MH11NH_3P.
2. 5X RT buffer: 250 m*M* Tris-HCl, pH 8.3; 375 m*M* KCl; 15 m*M* $MgCl_2$; 10 m*M* dithiothreitol; and 0.5 m*M* dNTPs.
3. Diethylpyrocarbonate (DEPC)-treated sterile H_2O.
4. Superscript II reverse transcriptase (Invitrogen, Life Technologies, Inc., Carlsbad, CA).
5. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.9; 1 *M* KCl, 15 m*M* $MgCl_2$; 500 μg/mL BSA; 0.5% Tween-20 (v/v).
6. 25 m*M* $MgCl_2$.
7. 10 m*M* dNTPs.
8. Hot Start™ PCR tubes (Invitrogen, Life Technologies, Inc., Carlsbad, CA).
9. tTh polymerase from Roche Molecular Biochemicals (Indianapolis, IN).
10. Gene-specific primer: approx 19 nt with a T_m 56–58°C using 2X(2GC+AT).
11. DEPC-treated sterile H_2O.
12. Phenol:chloroform:isoamyl alcohol (25:24:1).
13. Chloroform:isoamyl alcohol (24:1).
14. 3 *M* Sodium acetate, pH 5.5.
15. 5 mg/mL Glycogen.

16. Formamide loading solution: 80% formamide; 1 m*M* ethylenediamine tetraacetic acid, pH 8.0; 0.1% bromophenol blue; 0.1% xylene cyanol.
17. 6% Sequagel from National Diagnostics (Atlanta, GA).

2.4. Product Recovery

1. X-OMAT AR film (Kodak, Rochester, NY).
2. 18-Gauge needle.
3. Scalpel or razor blade.
4. Sterile-distilled H_2O.
5. 3 *M* Sodium acetate, pH 5.5 (*see* **Note 1**).
6. 5 mg/mL Glycogen.
7. 100% Ethanol stored at –20°C.
8. 85% Ethanol.

2.5. Product Reamplification

1. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.9; 1 *M* KCl; 15 m*M* $MgCl_2$; 500 μg/mL BSA; 0.5% Tween-20 (v/v).
2. 25 m*M* $MgCl_2$.
3. 10 m*M* dNTPs.
4. Primer MH12 (5'CGAGCTAGAGATTGAGCAC), which is complementary to the first 19 nt of $MH11NH_3P$.
5. Gene-specific primer: approx 19 nt with a T_m of 56–58°C using 2X(2GC+AT).
6. Hot Start™ tube (Life Technologies, Inc.).
7. tTh polymerase from Roche Molecular Biochemicals (Indianapolis, IN).
8. DEPC-treated sterile H_2O.

2.6. Sequencing of PCR Products

1. 10 U/μL Exonuclease I, USB no. 70073 (Cleveland, OH).
2. 2 U/μL Shrimp alkaline phosphatase, USB no. 70092 (Cleveland, OH).
3. Gene-specific primer (approx 19 nt with a T_m of 56–58°C using 2X(2GC+AT).
4. T7 Sequenase™ v 2.0 Sequencing Kit from USB (Cleveland, OH).

3. Methods

3.1. Primer Ligation

1. 2 μg total RNA is added to 12 μL of ligation buffer and 1 μg of ligation primer $MH11NH_3P$ giving a final volume of 15 μL (*see* **Note 1**).
2. The primer and the RNA are ligated together for 16 h at 4°C using 15 U of T4 RNA ligase.
3. The reaction is terminated by addition of an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) and vortexing for 1 min.
4. The aqueous phase is recovered by centrifugation for 4 min at 14,000*g* (*see* **Note 2**). This is extracted again with 1 vol of chloroform:isoamyl alcohol (24:1), followed by centrifugation for 3 min at 14,000*g*.

5. The recovered aqueous layer is adjusted to 0.3 *M* sodium acetate by addition of 1/10 vol of 3 *M* sodium acetate, pH 5.5, and RNA ligated to the primer is precipitated by adding 1 µL of glycogen, 2.5 vol of ice-cold ethanol, and incubating at –80°C for 30 min.

3.2. Labeling of Gene-Specific Primer

1. 500 ng of the desired primer is added to 2.5 µL of 10X kinase buffer.
2. Sterile H_2O is added to 21 µL, followed by addition of 3 µL of γ-[^{32}P]ATP and 1 µL of T4 polynucleotide kinase then incubated for 1 h at 37°C (*see* **Note 3**).
3. The reaction is terminated by heating at 65°C for 5 min.

3.3. RT-PCR

1. The precipitated ligation reaction is resuspended in 15 µL of DEPC-treated sterile H_2O, followed by addition of 250 ng of the primer MH12 in 1 µL of water.
2. The solution is heated to 70°C for 10 min and quickly cooled on ice, followed by addition of 4 µL of 5X RT buffer, to yield a total volume of 20 µL.
3. This is heated at 42°C for 2 min followed by the addition of 200 U of Superscript II reverse transcriptase and incubated for an additional 50 min.
4. The reaction is terminated by heating at 70°C for 15 min.
5. 3 µL of the above reaction mixture is transferred to a Hot Start tube (Life Technologies, Inc.) and mixed with 2.5 µL of 10X PCR buffer, 3 µL of $MgCl_2$, and 3 µL of dNTPs.
6. The wax bead of the Hot Start tube is melted by heating at 75°C for 30 s followed by rapid cooling on ice.
7. 11 µL of DEPC water, 2 µL of 5'-γ-[^{32}P]-labeled gene specific primer, and 0.5 µL of tTh polymerase (Roche Molecular Biochemicals, Indianapolis, IN) are then added to a final volume of 25 µL.
8. The mixture is heated at 95°C for 2 min and PCR amplification is performed for 25 cycles at 95°C for 2 min, 58°C for 30 s, and 72°C for 1 min, followed by extension for 3 min at 72°C.
9. The reaction mixtures are then extracted as described in **Subheading 3.1.** with phenol:chloroform:isoamyl alcohol, and the amplified products are recovered by ethanol precipitation.
10. The recovered pellets are dissolved in 3 µL of DEPC H_2O followed by addition of an equal volume of formamide loading solution.
11. These are heated for 5 min at 95°C and electrophoresed on a denaturing 6% polyacrylamide/urea gel. After electrophoresis, the gel is transferred to Whatmann 3MM paper, dried, and exposed to X-ray film. An example of data obtained from this is shown in **Fig. 1**. These data are described in detail in **Subheading 3.7.**
12. At this point, steps should be taken to put registration marks on the dried gel and the film so they can be aligned following autoradiography. This can be done by cutting one corner of the gel and punching holes through the dried gel and the film using an 18-gauge needle.

3.4. Product Recovery

1. Using a lightbox, align the film and the dried gel at the registration marks.
2. With an 18-gauge needle, punch through the film and the gel at the four corners of each band. Use a fresh razor or scalpel blade to excise the band from the dried gel.
3. To extract the DNA the gel band is placed into a microcentrifuge tube and soaked in 200 μL of distilled H_2O for 10 min (*see* **Note 4**; **ref. *10***).
4. Seal the cap to each tube using Parafilm or a tube clamp that will prevent the lid from opening and place the tubes in boiling water or a 100°C incubator for 15 min.
5. The tubes are centrifuged at 10,000*g* for 2 min and the DNA-containing supernatant is transferred to a fresh microcentrifuge tube.
6. The DNA is precipitated by the addition of 1/10 vol of 3 *M* sodium acetate, pH 5.5, 50 μg of glycogen, and 2.5 vol of 100% ethanol, followed by freezing at –80°C for 30 min.
7. The precipitated DNA is recovered by centrifugation at 14,000*g*, 4°C for 15 min. The pellet is washed with cold 85% ethanol (*see* **Note 5**) and dissolved in 12 μL of sterile-distilled H_2O.

3.5. Product Reamplification

1. In a Hot Start tube, place 4 μL of 10X PCR buffer and 4 μL of the recovered PCR product.
2. Add $MgCl_2$ to 1.5 m*M* and dNTPs to 0.4 m*M* (*see* **Note 6**).
3. Add 1 ng each of primer MH12 and the gene specific primer, and adjust the volume to 20 μL with distilled H_2O.
4. Melt the wax bead of the Hot Start tube by heating the tube to 75°C for approx 30 s, then place the tube quickly on ice.
5. Add 2.5 U of tTh polymerase and sterile-distilled H_2O to a final volume of 40 μL.
6. PCR amplification is performed as described in **Subheading 3.3.**

3.6. Sequencing of PCR Products

1. Unincorporated PCR primers are removed by treating 8 μL of the PCR reaction mixture with 10 U of exonuclease I and 2 U of shrimp alkaline phosphatase for 20 min at 37°C. The reaction is terminated by heating for 15 min at 80°C (*see* **Note 7**).
2. 20 ng of the original gene-specific primer is annealed to the template by heating at 100°C for 3 min and cooling on ice for 5 min.
3. This is sequenced using T7 DNA polymerase (USB T7 Sequenase Kit, *see* **Notes 8** and **9**).
4. The products are denatured and electrophoresed on a 6% polyacrylamide/urea gel as described above.

3.7. Application to the Identification of In Vivo mRNA Decay Intermediates

We previously described the use of LM-PCR to identify in vivo mRNA decay intermediates for an abundant mRNA (serum albumin) and a rare

mRNA (c-*myc*; **ref. *8***). In addition to its utility in identifying precise sites of endonucleolytic cleavage, the LM-PCR assay can be used to characterize the appearance of cleavage products resulting from a stimulus that alters the half-life of a particular mRNA within the cell or tissue under study. This application takes advantage of the semi-quantitative nature of the initial PCR amplification step using the [^{32}P]-labeled mRNA-specific primer, and is performed after one has conducted a complete analysis of the decay products as described above. An example of this is shown in **Fig. 1**. c-*myc* mRNA undergoes both exonucleolytic and endonucleolytic decay ***(11)***. Ross and coworkers identified a site for ribosome pausing within the coding region ***(12)*** that can be cleaved in vitro by a polysome-associated endonuclease ***(3)***. Previous work showed that the site identified in vitro by those authors was indeed cleaved in vivo in murine erythroleukemia cells ***(8)***. This site (number 4) is identified by the underscore in **Fig. 1B**. The sequence identified here also corresponds to a site for ribosome pausing ***(12)***. The other sites identified in **Fig. 1B** represent additional in vivo cleavage sites that were not previously detected in vitro ***(8)***. A characteristic of MEL cells is their ability to differentiate to an erythroid state upon addition of dimethyl sulfoxide. The experiment in **Fig. 1A** shows a time course for the appearance of cleavages within c-*myc* mRNA during a 12-h period after the addition of dimethyl sulfoxide. Notable in these data are the presence of three constitutive cleavage sites (sites 1–3) and the regulated appearance of a cleavage product corresponding to the main in vitro cleavage at 8 h. It is unlikely this would have been observed using either primer extension or S1 nuclease protection assays.

4. Notes

1. It is important to only use sodium acetate precipitated nucleic acids throughout this procedure because residual ammonium acetate can inhibit the RNA ligase.
2. The ligation reaction should be increased to 100–150 μL to minimize the loss of the sample.
3. Although labeling reactions can be carried out in 15 min, we find that 1 h gives a much better signal. The T4 Polynucleotide Kinase from Roche Molecular Biochemicals was found to work better than other brands tried.
4. This volume should be enough to completely soak the gel slice and the paper backing, and should be adjusted for significantly larger or smaller band.
5. It is important to use 85% ethanol to prevent the loss of DNA that can occur at lower concentrations.
6. The lower concentration of $MgCl_2$ and dNTPs is important to prevent the amplification of contaminants in this reamplification.
7. It is imperative to completely denature these enzymes or else the sequencing will not work.

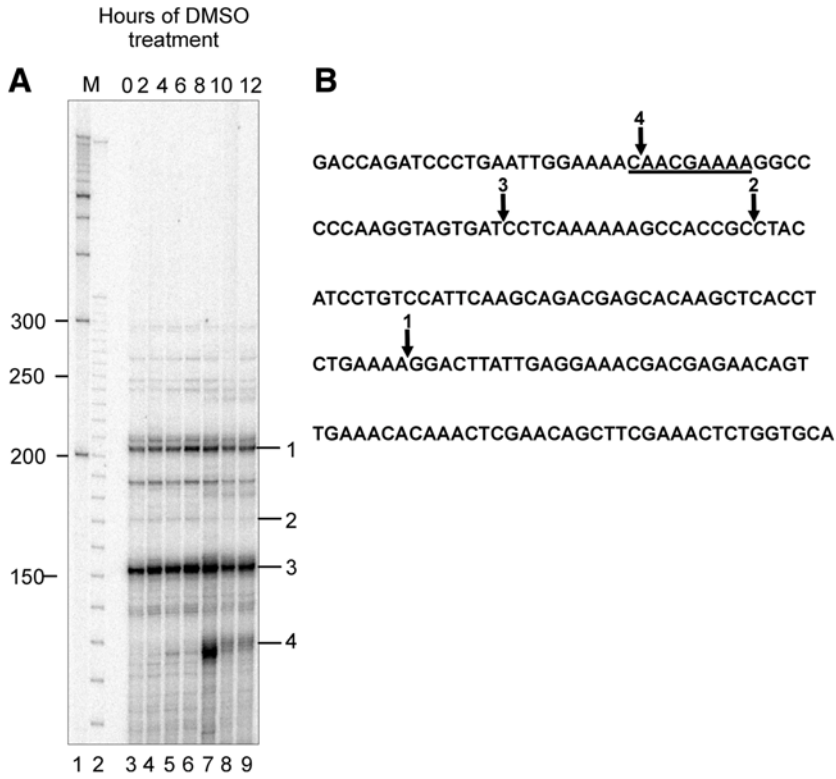

Fig. 1. Time course appearance of endonucleolytic cleavage products of c-*myc* mRNA in DMSO-treated MEL cells. Murine erythroleukemia cells were cultured for the indicated times in medium containing 2% DMSO. **(A)**, Total RNA isolated at each time point was processed through the first LM-RT-PCR amplification step using a 5'[^{32}P]-labeled primer for c-*myc* mRNA. The products were separated on a denaturing 6% polyacrylamide/urea gel and identified by autoradiography. The positions of the previously mapped ***(8)*** endonuclease cleavage sites are numbered on the right side. **(B)**, The sequence of the c-*myc* coding region instability determinant is shown with the sites corresponding to in vivo and in vitro cleavage indicated with numbered arrows. The underlined sequence at site 4 corresponds to the major site of cleavage in vitro.

8. We have found that either using the ITP labeling mix and dNTPs and/or increasing the reaction temperatures so that elongation occurs at 37°C and termination occurs at 42°C can improve the quality of the sequence.
9. Sequencing with α-[^{32}P]ATP works best if the nucleotide is several weeks old, which cuts down on the intensity of the signal, creating more distinct bands, and less background.

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18

Biochemical Dissection of RNA Silencing in Plants

Guiliang Tang and Phillip D. Zamore

Summary

Although RNA silencing was first discovered in plants, thus far it has been studied biochemically only in animals, where it is known as RNA interference (RNAi). In animals, two components of the RNAi pathway have been identified: Dicer, a multidomain RNase III that converts long double-stranded RNA (dsRNA) into small interfering RNA (siRNA) and the RNA-induced silencing complex (RISC), as siRNA-containing protein–RNA complex that targets complementary mRNA for destruction. We have developed methods for the biochemical dissection of plant RNA silencing. In this chapter, we describe in detail how to use wheat germ extract to study two distinct Dicer-like activities, RNA-dependent RNA polymerase (RdRP), and endogenous microRNA-programmed RISC activities. These comprehensive protocols should prove useful in the further dissection of the plant RNA silencing pathway, as well as for the validation of the predicted targets of endogenous plant microRNAs.

Key Words

RNA silencing; RNAi; PTGS; siRNA; miRNA; RISC; Dicer; RdRP.

1. Introduction

RNA silencing is a universal phenomenon in eukaryotes ***(1–7)***. It describes a group of related phenomena in which aberrant or double-stranded RNA (dsRNA) triggers a dramatic reduction in either the transcription of the corresponding gene or the direct degradation of the corresponding messenger RNA (mRNA). In animals, posttranscriptional RNA degradation induced by exogenous double-stranded RNA (dsRNA) is called RNA interference (RNAi); in plants, it is called posttranscriptional gene silencing (PTGS). Biochemical approaches have been successfully used to study RNAi in *Drosophila* and mammals ***(8,9)***. In RNAi, long dsRNA is fragmented into 21–23 nt small interfering RNAs (siRNAs) by members of the Dicer family of RNase III enzymes

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(10–13). siRNAs assemble with specific proteins to form an RNA-induced silencing complex (RISC) that directs target RNA cleavage, thereby silencing gene expression ***(8,11–21)***.

In plants, endogenous gene silencing triggered by extra copies of a transgene with the same sequence is known as cosuppression ***(22)***. Cosuppression can reflect either transcriptional repression (transcriptional gene silencing [TGS]) or PTGS. PTGS is the plant RNA silencing phenomenon most closely related to RNAi ***(23)***. In IR-PTGS, dsRNA generated from the transcription of inverted repeats efficiently silences the corresponding mRNA in transgenic plants ***(24,25)***. Unlike RNAi, PTGS can also be initiated by transgenes that produce sense RNA (S-PTGS) or antisense RNA (AS-PTGS). S-PTGS and AS-PTGS initiated by highly expressed transgenes is genetically distinct from IR-PTGS, in which silencing is initiated by transgenes specifically designed to generate dsRNA ***(26)***. The unifying feature of RNAi and PTGS is the production of small RNAs of 21–25 nt in length ***(8,15,27–31)***. In contrast to the single population of small RNAs produced in *Drosophila*, two distinct populations of siRNAs, approx 21 nt and 24–25 nt, are generated in plants ***(32–34)***. The two classes of small RNAs have been proposed to play distinct functional roles in plant RNA silencing, with the long one correlating with systematic silencing and DNA methylation and the short one with local RNA silencing ***(33)***.

In addition to Dicer-like enzymes, an RNA-dependent RNA polymerase (RdRP) is involved in RNA silencing in plants, worm, *Neurospora* and *Dictyostelium*, but not in *Drosophila* or human cells ***(35–45)***. Notably, the requirement for the putative RdRP protein SGS2 (SDE1) is bypassed when silencing in plants is initiated by transgenes specifically engineered to produce dsRNA ***(26)***. RdRPs have been proposed to use aberrant RNAs as templates and copy them into complementary RNAs (cRNAs) to form dsRNA ***(23,46)***. This newly synthesized dsRNA is thought to be a substrate for Dicer-like enzymes ***(47)***. What features make an RNA aberrant remains unknown. Furthermore, a novel population of small RNAs is synthesized directly by a purified recombinant *Neurospora* RdRP (QDE1) ***(48)***. The function of these RdRP-generated small RNAs is likewise unknown.

Dicer also produces microRNAs (miRNAs), small, 21- to 24-nt single-stranded RNAs proposed to control gene expressions in eukaryotes. The first miRNAs discovered were *lin-4* and *let-7*, which regulate the timing of development in *Caenorhabditis elegans* ***(49,50)***; to date, more than a hundred miRNAs have been identified ***(51–56)***. miRNAs are encoded in genes that produce RNA transcripts that can fold into imperfect stem-loop structures. These stem-loops are the direct precursors of miRNAs and are called pre-miRNAs. Dicer cleaves each miRNA from one side or the other of the stem of the pre-miRNA ***(12,13,52–57)***. Animal and plant miRNAs have been proposed to func-

tion by different mechanisms. In animals, *lin-4* and *let-7* are thought to base pair imperfectly with sites within the 3' untranslated region of their target mRNAs, triggering translational repression of the mRNA targets ***(58,59)***. In contrast, miRNAs from plants act as natural siRNAs to mediate the cleavage of target mRNAs because of their near perfect complementarity between the miRNAs and their target RNAs ***(32,60–62)***. Regardless of how they function, miRNAs generally are associated with a common RISC-like protein complex. Current evidence suggests that the extent of complementarity between miRNAs and their target RNAs determines whether miRNAs regulate translation or direct target cleavage ***(20,63,64)***.

Although RNA silencing was first discovered in plants, the plant-silencing pathway has not been subject to extensive biochemical analysis in part because a suitable in vitro system was unavailable. Here, we describe an in vitro system, wheat germ extract, useful for analyzing several key features of RNA silencing in plants. Using this in vitro system, we have shown previously that the RNA silencing pathways in plants and animals are remarkably similar ***(32)***. Wheat germ extract contains Dicer-like enzymes that produce siRNAs and RNA-dependent RNA polymerases (RdRPs) that copy exogenous single-stranded RNA (ssRNA) into dsRNA. In the in vitro system, plant Dicer-like enzymes convert dsRNAs into two distinct classes of siRNAs, long and short siRNAs. Furthermore, endogenous plant miRNAs can direct mRNA degradation in wheat germ extract. In this chapter, we describe the preparation and use of wheat germ extract to dissect the RNA-silencing pathway in plants.

2. Materials

2.1. Preparation of Cell-Free Extracts

1. Raw wheat germ (e.g., Fearn Nature Fresh Raw Wheat Germ, Bread and Circus).
2. Fresh cauliflower.
3. *Drosophila melanogaster* embryo (0–2 h) lysate.
4. 1X lysis buffer: 100 m*M* potassium acetate; 30 m*M* HEPES-KOH, pH 7.4; 2 m*M* magnesium acetate.
5. 1 *M* Dithiothreitol (DTT), stored in aliquots at –20°C.
6. Complete, mini-EDTA-free protease inhibitor cocktail tablets (Roche).

2.2. Production of Transcription Templates by Polymerase Chain Reaction (PCR)

2.2.1. DNA Primers (see **Note 1**)

1. *Renilla reniformis* luciferase 501-bp (*Rr*-luc501) T7 template primers:
   a. Sense template primers: 5' PCR primer GCGTAATACGACTCACTATA GGAAAAACATGCAGAAAATGC and 3' PCR primer GAAGAATGGTTC AAGATATGCTG.

   b. Antisense template primers: 5' PCR primer GCGTAATACGACTCACT ATAGGAATGGTTCAAGATATGCTG and 3' PCR primer GAAGAAAA ACATGCAGAAAATGC.
2. Wild-type *PHAVOLUTA* (*PHV*) T7 template primers: 5' primer containing T7 promoter GCGTAATACGACTCACTATAGGCGCCGGAACAAGTTGAAG and 3' primer GACAGTCACGGAACCAAGATG.
3. Mutant *phv* T7 template primers: First pair is 5'-*PHV* primer (the same as the wild-type *PHV* T7 template 5' primer) and CCACTGCAGTTGCGTGAA ACAGCTACGATACCAAT AGAATCCGG-ATCAGGCTTCATCCC. Second pair is 5'-*PHV* primer (the same as the wild-type *PHV* T7 template 5' primer) and GACAGTCACGGAACCAAGATGGACGATCTTTGAGGATTTCAGCGAC CTTCATGGGTTCTAAACTCACGAGGCCACAGGCACGTGCTGCTATTCCAC TGCAGTTGCGTG AAACAGC.

2.2.2. PCR Templates

1. *Renilla reniformis* luciferase (*Rr*-luc) cDNA clone as PCR template for *Rr*-luc501.
2. *Arabidopsis* flower cDNA library (CD4-6) (*Arabidopsis* Stock Center, Ohio) as PCR template for cloning wild-type *PHV*.

2.2.3. 10X PCR Buffer

100 m*M* Tris-Cl, pH 8.3; 500 m*M* KCl; 15 m*M* $MgCl_2$; 0.1% w/v gelatin.

2.2.4. Taq Polymerase

2–5 U/μL (Roche or Invitrogen).

2.3. RNA Synthesis

1. 10X T7 transcription buffer: 400 m*M* Tris-Cl, pH 7.9; 25 m*M* spermidine; 260 m*M* $MgCl_2$; 0.1% v/v Triton X-100.
2. 2X proteinase K (PK) buffer: 200 m*M* Tris-Cl, pH 7.5; 25 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0; 300 m*M* NaCl; 2% w/v sodium dodecyl sulfate (SDS).
3. 2X Urea stop mix/loading dye: 8 *M* urea; 25 m*M* EDTA, pH 8.0; 0.025% w/v xylene cyanol FF; 0.025% w/v bromophenol blue.
4. SP6-*Pp*-luc (*Pyralis photinus* luciferase) plasmid template for RNA transcription.
5. T7 RNA polymerase (New England Biolabs).
6. SP6 RNA polymerase (New England Biolabs).
7. SequaGel System (National Diagnostics).
8. 40 U/μL RNasin (Promega).
9. Super RNasin (Ambion).

2.4. RNA Labeling

1. Radiochemicals: α-[^{32}P] adenosine triphosphate (ATP; 40 mCi/ml, 3000 Ci/mmol, ICN), α-[^{32}P] guanosine triphosphate (GTP; 40 mCi/mL, 3000 Ci/mmol, ICN), α-[^{32}P] uridine triphosphate (UTP; approx 10 mCi/mL, 25 Ci/mmol, ICN), and α-[^{32}P] ATP (6000 Ci/mmol, ≥10 mCi/mL, NEN or ICN).

2. T4 polynucleotide kinase (PNK, 10,000 U/mL, New England Biolabs).
3. 10X T4 polynucleotide kinase buffer: 700 m*M* Tris-HCl, pH 7.6; 100 m*M* $MgCl_2$; 50 m*M* DTT.
4. Formamide loading dye: 98% v/v deionized formamide; 10 m*M* EDTA, pH 8.0; 0.025% w/v xylene cyanol; 0.025% w/v bromophenol blue.
5. 10X Guanylyl transferase buffer: 500 m*M* Tris-Cl, pH 8.0; 60 m*M* KCl; 12 m*M* $MgCl_2$; 25 m*M* DTT.
6. *S*-adenosyl methionine (SAM) dilution buffer (5 n*M* H_2SO_4 in 10% v/v ethanol): 6.96 μL of 36 M H_2SO_4, 5 mL of absolute ethanol, and 45 mL of deionized H_2O.
7. Guanylyl transferase ***(65)***.
8. SAM (Sigma).
9. Typical RNA oligonucleotides (Dharmacon): siRNAs (sense and antisense strands) corresponding to miR165, miR166, *PHV*, and mutant *phv* target positions were miR165, UCGGACCAGGCUUCAUCCCCC and GGGAUGAAG CCUGGUCCGAGG; miR166, UCGGACCAGG CUUCAUUCCCC and GGAA UGAAGCCUGGUCCGAGA; *PHV*, CCGGACCAGGCUUCAUCCCAA and GGGAUGAAGC CUGGUCCGGAU; and mutant *phv*, CCGGAUCAGGCUU CAUCCCAA and GGGAUGAAGCCUGAUCCGGAU. The 21-nt siRNA inhibitor comprised CGUACGCGGAAUACUUCGAUU annealed with UCGAAG UAUUCCGC GUACGUG; the 25-mer siRNA inhibitor comprised AUCACG UACGCGGAAUACUUCGAUU annealed with UCGAAGUAUUCCGCGUA CGUGAUUG.

2.5. RNA Processing

1. dsRNA/RNAi processing mix: 50 μL of H_2O, 20 μL of 500 m*M* creatine monophosphate (Fluka, prepared fresh from powder), 20 μL of amino acid stock (Sigma, 1 m*M* each amino acid), 2 μL of 40 U/μL RNasin (Promega), 4 μL of 100 m*M* ATP, 1 μL of 100 m*M* GTP, 6 μL of 2 U/μL creatine phosphokinase (Cal-Biochem), prepared by diluting 2 μL of a 20 U/μL stock in 18 μL of 1X lysis buffer containing 2 m*M* DTT, 16 μL of 1 *M* potassium acetate.
2. 10X RNase digestion buffer: 3 *M* NaCl; 100 m*M* Tris-Cl, pH 7.4; 50 m*M* EDTA, pH 7.5.
3. 5X Borax/boric acid buffer (300 m*M*, pH 8.6): 150 m*M* borax (MW 381.4) and 150 m*M* boric acid (FW 61.83).

2.6. siRNA Structure Analysis

1. 1X Borax/boric acid buffer, pH 9.5: dilute 5X borax/boric acid buffer (300 m*M*, pH 8.6) in water and adjust pH with 10 *M* NaOH.
2. 200 m*M* Periodate ($NaIO_4$) solution (prepared freshly before use).
3. Phosphatase, calf intestinal (CIP, Promega or New England Biolabs).

2.7. Northern Blot Analysis

1. 20X SSPE: 3 *M* NaCl, 0.25 *M* NaH_2PO_4, and 0.02 *M* EDTA, adjust to pH 7.4.
2. 20X SSC: 3 *M* NaCl and 0.3 *M* sodium citrate, adjust to pH 7.0.

3. 50X Denhardt's reagent: 1% w/v Ficoll (type 400, Pharmacia), 1% w/v polyvinylpyrrolidone, 1% w/v bovine serum albumin (Fraction V, Sigma).
4. Formamide.
5. 10% SDS.
6. 10 mg/mL Herring sperm DNA (Invitrogen).
7. Hybond N+ membrane (Amersham Pharmacia Biotech).
8. Semidry electrotransfer apparatus.

3. Methods

3.1. Lysate Preparation

Developing cell-free systems is a key step to begin biochemical studies on RNA silencing. In RNAi, both the trigger dsRNA and the target mRNA are specifically cleaved by components of the silencing machinery. To detect these specific cleavages, the cell-free system should be free of nonspecific RNases. Special care should be taken during lysate preparation. All containers and extracting equipment should be made free of RNases by baking overnight. Wear gloves during all steps in lysate preparation.

1. Wheat germ extracts is prepared from frozen or vacuum-packed raw wheat germ (e.g., Fearn Nature Fresh Raw Wheat Germ, Bread and Circus) as described ***(66)***. The extract was centrifuged at 14,500*g* at 4°C for 25 min; the supernatant was then frozen in aliquots in liquid nitrogen and stored at –80°C.
2. For cauliflower extract, the outer layer of fresh cauliflower (Shaws Supermarket) is harvested with a razor blade and ground to a powder under liquid nitrogen in a pre-chilled mortar and pestle. Immediately transfer the powder to another prechilled mortar and pestle and homogenize it by grinding for additional 5 min on ice in 3 mL of 1X lysis buffer containing 5 m*M* DTT and EDTA-free protease inhibitor tablet (1 minitablet per 10 mL of buffer) per gram of plant tissue. The extract is centrifuged, and the supernatant is flash frozen in liquid nitrogen and stored at –70°C.
3. *Drosophila* embryo lysates are prepared as described previously ***(9)***.

3.2. Generation of T7-PCR Templates (see Note 1)

To study RNA cleavage in vitro, all the RNAs are generated by transcription in vitro. The easiest way to generate RNA transcription templates is to make templates by PCR. Using PCR, you can rapidly create different dsRNAs and target RNAs and introduce mutations in the RNAs. By way of example we describe here, in detail, the preparation of the RNA templates used in our recent study ***(32)***.

3.2.1. Rr-luc501 T7-PCR Templates

1. Set up PCR to amplify T7-sense-*Rr*-luc501 templates using *Rr*-luc cDNA clone as initial template, 5' PCR primer GCGTAATACGACTCACTATAGGAA

AAACATGCAGAAAATGC and 3' PCR primer GAAGAATGGTTCAAG ATATGCTG.

2. Set up PCR to amplify T7-antisense-*Rr*-luc501 templates using *Rr*-luc cDNA clone as initial template, 5' PCR primer GCGTAATACGACTCACTATAG GAATGGTTCAAGATATGCTG and 3' PCR primer GAAGAAAAACATGC AGAAAATGC.

3.2.2. PHV T7-PCR Templates

1. Wild-type *PHV* T7-PCR template: *Arabidopsis PHV* cDNA sequences containing the miR165/166 complementary sequences are amplified from an *Arabidopsis* flower cDNA library (CD4-6) by PCR using the following primer pairs: 5'-*PHV* primer, GCGTAATACGACTCACTATAGGCGCCGGAACAAGTTG AAG, and 3'-*PHV* primer, GACAGTCACGGAACCAAGATG.
2. Mutant *phv* T7-PCR template: The *Arabidopsis* G to A mutant *phv* template is initially amplified using the 5'-*PHV* primer (the same 5' primer as for wild-type *PHV*) and CCACTGCAGTTGCGTGAAACAGCTACGATACCAATAGAA TCCGGATCAGGCTTCATCCC. This PCR product is then diluted 100-fold and reamplified with the 5'-*PHV* primer (the same 5' primer as for wild-type *PHV*) and GACAGTCACGGAACCAAGATGGACGATCTTTGAGGATTTCAGCG ACCTTCATGGGTTCTAAACTCACGAGGCCACAGGCACGTGCTGCTATTCCAC TGCAGTTGCGTG AAACAGC.

3.2.3. Precipitation of T7-PCR Templates

The PCR products are precipitated directly from the reaction by the addition of 2.5 vol of absolute ethanol and 1/10 vol 3 *M* sodium acetate. The precipitate is recovered by centrifugation, washed in 70% ethanol, dried under vacuum, and redissolved in 1X T7 transcription buffer to give a stock of DNA template 10-fold more concentrated than the original PCR.

3.3. In Vitro RNA Transcription

1. Generation of uniformly ^{32}P-radiolabeled RNA: Assemble a 50-µL reaction containing 5 µL of 10X T7 buffer, 0.5 µL of 1 *M* DTT, 2.5 µL of 10 m*M* ATP, 2.5 µL of 100 m*M* UTP, 2.5 µL of 100 m*M* cytidine triphosphate (CTP), 4 µL of 100 m*M* GTP, 25 µL of α-^{32}P ATP (40 mCi/mL, 3000 Ci/mmol), 2.5 µL of (3–6 µg) PCR template (as described above), 1 µL of RNasin (40 U/µL, Promega), 2.5 µL of H_2O, and 2 µL of purified recombinant, histidine-tagged T7 RNA polymerase (6.8 mg/mL). Incubate at 37°C for 1–2 h. Stop the reaction by adding an equal volume of 8 *M* urea stop mix/loading buffer.
2. Production of nonradioactive RNA: Prepare a 100-µL reaction containing 10 µL of 10X T7 transcription buffer, 3 µL of (3–6 µg) PCR template (described above), 0.5 µL of 1 *M* DTT, 5 µL of 100 m*M* ATP, 5 µL of 100 m*M* CTP, 5 µL of 100 m*M* UTP, 8 µL of 100 m*M* GTP, 61.5 µL of H_2O and 2 µL of T7 RNA polymerase (6.8 mg/mL). Incubate at 37°C for 2 h. Stop the reaction by adding an equal volume of 8 *M* urea stop mix.

3. Production of nonradioactive capped mRNA: We use 3 μL of a 2 mg/mL stock of linearized plasmid or PCR transcription template as described above for a 100-μL reaction. From 100 m*M* stock solutions, add 5 μL each of ATP, CTP, and UTP, plus 1 μL of GTP. To this, add 10 μL of10X T7 reaction buffer (or SP6 reaction buffer if the promoter is SP6), 0.5 μL of 1 *M* DTT, 26.5 μL of water, 1 μL of RNasin (40 U/μL, Promega), 40 μL of 10 m*M* cap analog [7mG(5')ppp(5')G, New England Biolabs] and 2 μL of the appropriate recombinant bacteriophage RNA polymerase (e.g., SP6 20 U/μL or T7 6.8 mg/mL). Incubate for 2 h at 37°C. Stop the reaction by adding 7 μL of 500 m*M* EDTA. Precipitate the RNA transcripts with 0.8 *M* LiCl for 1 h on ice, centrifuge at 13,000*g* for 10 min, wash with 80% ethanol, and resuspend the pellet in 75 μL of H_2O. Examine 5 μL on a denaturing 2% agarose gel and visualize with ethidium bromide. To the remaining 70 μL, add 10 μL of 10X RQ1 DNase buffer (Promega), 5 μL of RQ1 DNase (1 U/μL, Promega), and incubate for 30 min at 37°C to eliminate any residual DNA template. Phenol/chloroform/isoamylalcohol extract and precipitate with 2.5 vol absolute ethanol. Resuspend purified transcripts in H_2O and determine concentrations by absorbance at 260 nm.

3.4. Gel Purification of In Vitro Transcribed RNA (see Note 2)

Gel purification is essential to eliminate abortive transcripts during in vitro transcription. Often, in vitro transcripts are a population of lengths. Gel purification allows selection of full-length transcripts.

1. Heat the stopped transcription reaction at 95°C for 2 min and then purify the transcribed RNA by electrophoresis on a denaturing acrylamide gel. RNA is located on gel by UV shadowing or phosphorimagery.
2. The gel portion containing RNAs is excised and the transcripts are recovered by soaking the gel slice in 1–2 mL of 2X PK buffer tumbling overnight at room temperature.
3. The elute is then phenol/chloroform/isoamylalcohol extracted and the RNA precipitated with 2.5 vol of cold absolute ethanol. The RNA precipitate is resuspended in pure, sterile H_2O.
4. To remove traces of copurifying DNA templates, use RNase-free DNase to treat the RNA at 37°C for 30 min (*see* **Subheading 3.3., step 3**). This step is only used when the template and transcript lengths are similar.
5. Phenol/chloroform/isoamylalcohol extract the RNA again to remove DNase.
6. Precipitate the DNA-free RNA with 2.5 volumes of –20°C absolute ethanol.
7. After purification, the concentration of each transcript is determined.
8. Measure the absorbance at 260 nm of 2 μL of RNA stock diluted into 198 μL of H_2O. Concentration is calculated by the following equation: $C = (A260 \times \text{dilution factor})/(10313 \times \text{number of nucleotides})$ where C is in molar and the dilution factor in this instance is 100. This calculation assumes a 1-cm path length in the cuvette.

3.5. Annealing the In Vitro Transcribed ssRNAs into dsRNA

dsRNA is the substrate for Dicer-like enzymes. After annealing, it is important to check on a gel that annealing is complete and dsRNA has been generated. This is essential when the dsRNAs are radiolabeled because incomplete annealing will leave uncapped ssRNAs, which are not stable in most cell-free systems and will produce a high background in subsequent assays.

1. Equimolar amounts of the sense and antisense RNA strands are annealed by mixing them with an equal volume of 2X lysis buffer to make 0.5–1 μ*M* dsRNA.
2. The mixture is heated for 5 min at 95°C and then annealed overnight at 37°C.
3. Analyze a small portion the dsRNA on a native agarose gel. ssRNA for both sense and antisense strands of the dsRNA should be run adjacent to the dsRNA product. dsRNA, unlike ssRNA, typically runs with the same mobility as dsDNA, so we use dsDNA markers as size standard on the gel. The RNA is visualized by staining the gel with ethidium bromide or, for ^{32}P-RNAs, by drying the gel under vacuum onto a positively charged nylon membrane and detecting the RNA by phosphorimagery.

3.6. Analysis of Plant Dicer-Like Activities in Wheat Germ Extracts

Dicer-like enzymes are multidomain members of RNase III family. These enzymes cleave dsRNA into small fragments 21–25 nt in length. Therefore, Dicer-like activities can be monitored by detecting the production of small RNAs on a 15% denaturing polyacrylamide sequencing gel.

1. Assemble a typical Dicer-like activity assay as follows: for a 10-μL reaction, add 5 μL of wheat germ extract (use *Drosophila* embryo lysate as a positive control), 3 μL of dsRNA/RNAi processing mix, 1 μL of H_2O, and 1 μL of 50 n*M* uniformly [^{32}P]-labeled dsRNA (5 n*M* final concentration).
2. Incubate 1–3 h at 25°C.
3. Quench by adding 100 μL of 2X PK buffer.
4. Deproteinize by adding 10 μL of 20 mg/mL proteinase K (dissolved in water) and 1 μL of 20 mg/mL glycogen carrier (Roche) to the reaction. Incubate 15 min at 65°C.
5. Extract with phenol/chloroform/isoamylalcohol and precipitate the RNA with 3 vol of –20°C absolute ethanol. Wash the precipitate with 80% ethanol and dry the precipitate under vacuum.
6. Dissolve the pellet in 20 μL of formamide loading dye. After heating 2 min at 95°C, resolve the small RNA products produced by Dicer-like enzymes by electrophoresis on a 15%, 0.2- to 0.5-mm thick denaturing polyacrylamide sequencing gel at constant 20–25 W. Stop the electrophoresis before bromophenol blue dye runs out of the gel. 5' ^{32}P-radiolabeled synthetic RNA oligonucleotides (14, 19, 21, 25, and 34 nt) are used as size markers (*see* **Subheading 3.9.2.**, **step 1**).

3.7. Characterization of Small RNA Structures

Dicer-like activities in wheat germ extract produce two populations of small RNAs with distinct lengths, approx 21 nt and 24–25 nt. To ask whether small RNAs are *bona fide* small interfering RNAs (siRNAs) produced by Dicer-like enzymes, analyze the structure of the small RNAs. There are several criteria for siRNAs: (1) small, 21–25 nt; (2) initially double-stranded; (3) 3' 2-nt overhangs; and (4) 5' monophosphate and 3' hydroxyl termini.

3.7.1. Gel Filtration Assay

1. Scale up the Dicer assay in **Subheading 3.6.** 5–10-fold. Perform **steps 1–3** both for wheat germ extracts and Drosophila lysate (control).
2. Deproteinize the reaction at 25°C for 1 h with proteinase K without subsequent phenol/chloroform/isoamylalcohol extraction. (Phenol facilitates RNA annealing.)
3. Precipitate the RNA with 3 vol of –20°C absolute ethanol.
4. Wash the precipitate with 80% ethanol and dissolve the pellet in 100–200 µL 1X lysis buffer.
5. Immediately analyze the small RNAs by gel filtration at room temperature on a Superdex-200 HR 10/30 column (Pharmacia) at 0.75 mL/min in lysis buffer using a BioCad Sprint (PerSeptive Biosystems) or similar chromatography system, as described previously ***(19)***.
6. Collect all the fractions, add 1 µL of glycogen (20 mg/mL) to each fraction, and precipitate with 3 vol of –20°C absolute ethanol.
7. Analyze each fraction by electrophoresis on a 15% denaturing polyacrylamide sequencing gel together with size markers.
8. Compare the small RNAs with *Drosophila* Dicer products. Single and dsRNA (siRNA duplex) markers should also be used to calibrate the column.

3.7.2. RNase Protection Assay for Analyzing dsRNA With 3' 2-nt Overhangs

RNase protection assay uses RNase A and T1 in the presence of high salt to digest ssRNA portion but not dsRNA portion of siRNA duplexes. siRNA is dsRNA with 3' 2-nt overhangs (single-stranded 3' "tails"). Therefore, after RNase A and T1 treatment, the siRNA is shortened by 1–2 nt, which is readily detected on a 15% denaturing polyacrylamide sequencing gel.

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts as in **Subheading 3.7.1.**, **steps 1** and **2**.
2. Wash the precipitate with 80% ethanol and dissolve the pellet in 1X RNase digestion buffer by dilution of 10X RNase digestion buffer (*see* **Subheading 2.5.**). Set up a 10-µL reaction containing 5 µL of RNAs in 1X RNase digestion buffer, 0.5 µL 10X RNase digestion buffer (add this before RNase A and T1 to avoid diluting the salt concentration so as to prevent the dsRNA being digested by the RNase), and varying amounts of RNase A and T1. The amount of RNase A and T1 should be

titrated before hand using a reaction containing radiolabeled synthetic siRNA duplex (*see* **Subheading 3.9.2.**, **step 1**) incubated in the presence of nonradioactive total wheat RNA isolated from 5 μL of wheat germ extract.
3. Stop the RNase protection reaction by adding 1 μL of 10% SDS and treat with 1 μL of 40 mg/mL proteinase K at 25°C for 1 h.
4. Precipitate the RNase protection products and analyze them by electrophoresis on a 15% denaturing polyacrylamide sequencing gel as described previously. In wheat germ extract, the result should be that each of the two populations of wheat small RNAs are shortened by RNase. Positive controls, synthetic siRNA duplexes should also be shortened by 1–2 nt.

3.7.3. Analysis of 5' and 3' End Structures

Dicer products, like all RNase III products, have 5' monophosphate and 3' hydroxyl termini. To test for this type of end structure, we analyzed the siRNAs using CIP, PNK, and periodate treatment followed by β-elimination ***(67)***. CIP can remove all terminal phosphates (tri-, di-, or monophosphate at 5' or 3' end) from any RNA. PNK adds the γ-phosphate of ATP to a free hydroxyl group at the 5'-end of nucleic acids. If an RNA has a single monophosphate terminus, it should have its original mobility restored when treated sequentially with CIP then PNK. If the input RNA has a 5' triphosphate terminus, for example, the untreated RNA will have a faster gel mobility than the same RNA treated with CIP then PNK. Unambiguous assignment of a monophosphate to the 5' end requires analysis of 3' end structures using periodate-treatment/β-elimination. Periodate only reacts with RNAs containing vicinal diols, which are oxidized to aldehydes. That is, adjacent 2' and 3' hydroxyl groups must be present ***(67)***. The aldehydes are then reacted with base (borax/boric acid) to catalyze β-elimination, resulting in an RNA one nucleotide shorter at its 3' end but with a 3'-monophosphate bearing two negative charges in place of the singly charged phosphodiester bond ***(67)***. Therefore, an RNA must have 5' monophosphate and 3' hydroxyl termini if it both is able to be β-eliminated after periodate treatment and has a slower mobility when treated with CIP, but its original mobility when the CIP-treated RNA is treated with PNK and ATP.

3.7.3.1. Analysis of 5' End Structure

1. Scale up and process uniformly ^{32}P-labeled dsRNA in wheat germ extract as for the assay for Dicer-like activities (**Subheading 3.6.**, **steps 1–6**).
2. Purify the small RNAs from a denaturing polyacrylamide gel. Next, the RNA is dissolved in water. Aliquot the RNA for CIP (Promega) and PNK (Promega) treatments.
3. For CIP treatment, the small RNAs are reacted with 1–2 U of CIP in a 10-μL volume at 37°C for 0.5–1 h. Stop the reaction by adding 10 μL of formamide loading buffer.

4. For CIP plus PNK treatment, the small RNAs are first treated with CIP as above but stop the reaction by treating with 100 µL of 2X PK buffer, 1 µL of glycogen (20 mg/mL), and 10 µL of proteinase K at 65°C for 15 min. Phenol/chloroform/isoamylalcohol extract the reaction and precipitate the RNAs as described previously. The RNAs are then treated with PNK 1 h at 37°C in 10 µL (1 µL 10X T4 polynucleotide kinase buffer, 1 µL 10,000 U/mL PNK, 1 µL 10–20 m*M* ATP, and 7 µL H_2O). Stop the reactions by adding 10 µL of formamide loading buffer.
5. As a control, the small RNAs are treated directly with PNK, without CIP treatment.
6. Heat the various small RNAs in formamide loading buffer at 95°C for 2 min: no treatment, CIP-treated, CIP plus PNK-treated, and PNK only. Analyze the relative gel mobilities of the RNAs by electrophoresis on a 15% denaturing polyacrylamide sequencing gel.

3.7.3.2. 3' End Analysis

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts and purify the RNA (*see* **Subheading 3.6., steps 1–5**).
2. Dissolve RNA pellets in 13.5 µL of H_2O, 4 µL of 5X borax/boric acid buffer (pH 8.6), and 2.5 µL of 200 m*M* $NaIO_4$.
3. Incubate 10 min at 25°C.
4. Add 2 µL of glycerol to the reactions to quench unreacted $NaIO_4$.
5. Dry under vacuum.
6. Dissolve the pellet in 50 µL of 1X borax/boric acid buffer (pH 9.5).
7. Incubate 90 min at 45°C (β-elimination reaction).
8. Add 3 µL of 5 *M* NaCl and 150 µL of ethanol and incubate on ice for 2 h.
9. Collect the pellet by centrifugation. Wash the pellet with 80% ethanol.
10. Dissolve the pellet in 20 µL of H_2O.
11. Analyze 5 µL of RNA plus 5 µL of 8 *M* urea stop mix by electrophoresis through a 15% denaturing polyacrylamide sequencing gel as described above. Include a control without periodate treatment and β-elimination.

3.8. Testing Dicer-Like Activities for Susceptibility to Product Inhibition

Wheat germ extracts contain two distinct Dicer-like activities that produce two different lengths of small RNAs, 21 nt and 24–25 nt. To distinguish whether these two different siRNAs are generated by distinct Dicer-like enzyme, we use synthetic siRNA duplexes of the corresponding size as Dicer inhibitors. If the enzyme has high affinity for its small RNA products, the synthetic siRNA duplex will act as an inhibitor. Conversely, if the enzyme has low affinity for its product, the synthetic siRNAs will not inhibit the enzyme reaction. Results from our studies showed that the enzyme responsible for the production of 24–25 nt siRNA has high affinity for its siRNA products, whereas the enzyme that produces the approx 21 nt siRNAs does not. We concluded that wheat germ extracts contain at least two distinct Dicer-like enzymes ***(32)***. To test whether

Dicer-like enzymes are inhibited by their products, assay the conversion of a fixed amount of uniformly radiolabeled long dsRNA into siRNA in the presence of increasing concentrations of unlabeled synthetic siRNA duplexes.

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts (with fly embryo lysate as a control) as **Subheading 3.6.** but in the presence of increasing amount (0–800 n*M*) of the cold synthetic siRNA duplexes.
2. Work up the processed RNAs as described above and dissolve the purified RNA in 10 μL of formamide loading buffer as described above.
3. Analyze the two populations of small radioactive RNAs produced by electrophoresis on a 15% denaturing polyacrylamide sequencing gel. Plot the amount of siRNA produced vs inhibitor siRNA duplex concentration. In our experiments, the data fit well to a single-exponential, and we used τ to compare the sensitivity of different enzymes to inhibitor or the effectiveness of different lengths of siRNA duplexes.

3.9. Analysis of RdRP Activity

RdRP is an enzyme that uses RNA as template to synthesize complementary RNA (cRNA). We have shown that wheat RdRPs can synthesize both primed and unprimed cRNA ***(32)***. In addition, RdRPs can also produce a novel population of small RNAs of approx 24–25 nt in length. These RdRP-related small RNAs can be the products of a specific wheat Dicer-like enzyme that uses the dsRNA synthesized by wheat RdRP or produced directly by the RdRP itself ***(48)***.

When assaying for RdRP activity in crude extracts, special care should be taken in interpretation of the results. In crude extracts, terminal transferase activities can label RNA templates directly using the input radionucleotides. Such terminal transferase activity is present in *Drosophila* embryo lysate. To detect authentic RdRP products, control reactions with 3' deoxynucleotides should be performed. When 3' deoxynucleotides are incorporated into RNA, RNA synthesis is terminated because of the lack of a 3' hydroxyl. Terminal transferases require only a single type of NTP rather than all four, so they are not necessarily blocked by a single 3' dNTP in the presence of the other three NTPs. This labeled template RNA is easily misinterpreted as an RdRP product. Using a radiolabeled primer to assay RdRP is a more reliable way to exclude terminal transferase activity. However, in our hands, such primer extension reactions are inefficient. Primer extension also may reflect reverse transcriptase activity, so control experiment using 2', 3' ddNTPs should be performed to verify that an RdRP, not an RNA-dependent DNA polymerase is being detected. Resistance to RNase-free, DNase treatment can also establish that the RNA primer was used to initiate RNA synthesis. It is important to note that undialyzed *Drosophila* embryo lysates contain high concentration of endogenous nucleotides, making RdRP activities impossible to detect using exogenous

radioactive NTPs. Using a radiolabeled primer can overcome this problem. Wheat germ extracts are typically gel-filtered, and therefore contain low concentration of endogenous NTPs.

3.9.1. Analyzing Unprimed RdRP Products

1. Set up a 10-μL reaction containing 5 μL of wheat germ extract (or fly embryo lysate as a control), 100 μ*M* GTP, 100 μ*M* CTP, 500 μ*M* ATP, 20 μ*M* UTP, 5 μCi of α-[^{32}P]UTP (25 Ci/mmol, approx 10 mCi/mL), 10 m*M* creatine phosphate, 10 μg/mL creatine phosphokinase, 5 m*M* DTT, 0.2 U/μL Super-RNasin (Ambion), and various concentrations of 7-methyl-G- or A-capped RNA template.
2. After incubation at 25°C for 3 h, deproteinize the reaction with proteinase K in 200 μL of 2X proteinase K buffer at 65°C for 15 min.
3. After phenol/chloroform/isoamylalcohol extraction, precipitate the aqueous phase with three volumes of cold ethanol, then resuspend in 10 μL of 2X formamide loading buffer.
4. Resolve the labeled RdRP products on 7 or 10% polyacrylamide sequencing gel.

3.9.2. Analyzing Primed RdRP Products

1. Label antisense and control sense RNA primers with PNK and γ-[^{32}P]ATP: In a 10-μL reaction, add 10 pmol RNA oligo, 1 μL of γ-[^{32}P]ATP, 1 μL 10X PNK buffer, and 1 μL of PNK. Incubate the reaction at 37°C for 1 h and stop by passing through a G-25 spin column (Roche). Finally gel-purify the labeled RNA oligo as described above. It is important that all traces of γ-[^{32}P]ATP be removed, as contaminating γ-[^{32}P]ATP can be used by enzymes in crude extracts to 5' radiolabel abundant, endogenous RNAs.
2. For primed RdRP product assays, capped RNAs are preincubated with single-stranded radiolabled 21-nt antisense RNA primer, sense RNA primer or siRNA duplexes at room temperature for 10 min before the remaining reaction components are added.
3. Process the reactions as for analyzing unprimed RdRP products (*see* **Subheading 3.9.1., steps 1–4**).

3.10. Detection of Endogenous miRNAs in Wheat Germ Extracts (see Notes 3 and 4)

Although wheat germ extracts can produce siRNAs, the siRNAs seem unable to direct target RNA cleavage (unpublished data). There are several explanations: (1) wheat germ extracts might lack cofactors that are essential for RISC assembly; (2) RISC components are limited; or (3) RISC components are fully occupied by endogenous miRNAs. In wheat germ extract, endogenous miRNAs assembled in RISC complexes can direct target RNA cleavage. Unlike animal miRNAs, plant miRNAs target specific mRNA cleavage because of their extensive complementarity to specific mRNAs. We choose the wheat ortholog of *Arabidopsis* miR165, miR165/166, and an Arabidopsis target mRNA,

PHABULUTA (*PHV*), to test this idea and give here a protocol for these experiments. We also describe procedures for demonstrating that a miRNA can cleave its wild-type target mRNA but not a mutant RNA ***(32)***.

3.10.1. Quantitative Northern Detection of Wheat miRNAs in Wheat Germ Extracts

1. Synthesize sense and antisense miR165 or corresponding DNA oligonucleotides as probes and controls.
2. Proteinase K, treat 30 μL of wheat germ extract in 200 μL of 2X PK buffer at 65°C for 0.5–1 h.
3. Precipitate the total RNAs as above, then dissolve the RNA pellet in 10–20 μL of formamide loading buffer.
4. Heat the sample 2 min at 95°C and load on a 15% 2 mm-thick polyacrylamide sequencing gel together with synthetic sense miRNA or corresponding DNA oligonucleotides as a positive control. Make a dilution series of the corresponding synthetic miR165 to serve as concentration standards.
5. Transfer the small RNAs from the gel to Hybond N+ (Amersham Pharmacia Biotech) membrane using a semidry electrotransfer apparatus.
6. Rinse the membrane with 2X saline/sodium phosphate/EDTA (SSPE), pH 7.4.
7. UV-crosslink the membrane in UV-Stratalinker 2400.
8. Prehybridize the membrane for at least 2 h at 42°C in 50% formamide, 5' SSPE (pH 7.4), 5' Denhardt's reagent, 0.5% SDS, denatured herring sperm DNA (160 μg/mL).
9. Radiolabel antisense miR165 RNA or corresponding DNA oligo with PNK and γ-[^{32}P]ATP (ICN).
10. Purify the radiolabeled probe by passing through G-25 spin column (Roche).
11. Carefully add the purified probe to the hybridization solution without directly touching the membrane and continue the hybridization overnight.
12. Discard the hybridization solution into radioactive waste and rinse the membrane immediately with 2X saline sodium citrate (SSC) and 0.1% SDS at 42°C.
13. Wash the membrane twice more, 20 min per wash, at 42°C in 2X SSC and 0.1% SDS. Check the background signal after each wash.
14. Wrap the membrane with saran and exposure overnight under film.

3.10.2. Detection of miRNA-Programmed RISC Activity

1. Preparation of cap-radiolabeled partial PHV mRNA containing the miRNA 165/166 target region.
   a. PCR generate T7-*PHV* transcription template as described above.
   b. Transcribe uncapped *PHV* RNA using T7 RNA polymerase as described above.
   c. After gel purification, 10 pmol of the uncapped mRNA target (in 1.75 μL of H_2O) is added to 5 μL of α-[^{32}P]GTP (3000 Ci/mmol, 40 mCi/mL, custom order, ICN), 1 μL of 10X guanylyl transferase buffer, 0.25 μL of 40 U/μL RNasin (Promega), 1 μL of 10 m*M S*-adenosyl methionine (SAM, Sigma) in SAM dilution buffer, and 0.5 μL of recombinant, heterodimeric vaccinia virus guanylyl transferase.

d. Incubate for 2 h at 37°C, adjust the reaction to 100 μL with H_2O and pass through a G-50 spin column (Roche).
e. Gel-purify the radiolabeled target as mentioned previously.

2. Assay for miRNA-directed target RNA cleavage.
   a. Set up reactions as for wheat Dicer-like activity assay (**Subheading 3.6.**) except use 5 n*M* purified, cap-radiolabeled wild-type *PHV* or mutant *phv* RNA targets instead of the uniformly [^{32}P]-labeled dsRNA.
   b. Incubate the reaction 0–80 min at 25°C.
   c. In parallel, RNAi control reactions, as previously described ***(30)***, should be conducted in fly embryo lysate, with a synthetic siRNA duplex in which one strand is the miRNA. It is useful to use as a control an siRNA duplex with perfect complementarity to the target region corresponding to the miRNA.
   d. Deproteinize and precipitate the reactions, then analyze the cleaved products on a 5% polyacrylamide sequencing gel. Include appropriate size markers. We typically use the 100-nt RNA ladder (Ambion) labeled with Guanylyl transferase, α-[^{32}P]GTP, and SAM.

4. Notes

1. When designing primers for generating PCR DNA templates for the transcription of RNA, the 5' PCR primer should include at its 5' end the T7 promoter sequence. In principle, T3 or SP6 could be used. The T7 promoter is followed by 15–20 bases of sequence complementary to the desired target RNA sequence. In addition to the final, transcribed G of the T7 promoter, one or two additional guanosines should be included immediately after the promoter sequence to ensure efficient transcription. A 20 nt, 3' PCR primer is designed to be complementary to the target sequence 200–1000 bases downstream from the 5' sequences. Two separate primer pairs are used to generate templates for the transcription of sense and antisense RNA strands.
2. Polyacrylamide/urea gel purification of transcribed RNA is appropriate for RNA transcripts ≤600 nt. However, longer RNA is difficult to recover in good yield from polyacrylamide gels. Instead, we use LiCl to precipitate the transcribed RNAs as described in **Subheading 3.3.**, **step 3**.
3. Plant miRNAs were initially discovered in *Arabidopsis*. Many miRNAs are conserved between *Arabidopsis* and rice. To assess the presence of miRNAs in wheat germ, we perform Northern analysis using synthetic 21 nt RNA or DNA probes complementary to individual *Arabidopsis* miRNAs. Our results show that at least nine orthologs of *Arabidopsis* miRNAs are present in wheat germ extracts.
4. *PHV* (*PHAVOLUTA*), like *PHB* (*PHABULOSA*), is a member of homeodomain, leucine-zipper transcription factors, which were initially discovered in *Arabidopsis*. *PHB* is abundant on the adaxial face of leaf primordial but not on the abaxial face ***(68)*** and has been proposed to specify adaxial fates during leaf development. Dominant gain-of-function *Arabidopsis* mutants with in-frame or point mutations in the putative sterol/lipid-binding domains show loss of leaf

asymmetry and express mutant *phb* transcript on both adaxial and abaxial region in the leaf primordium ***(68)***. miRNA 165 was predicted to pair with the region in *PHB* mRNAs where the mutations are found ***(61)***. Therefore, the wild-type *PHB* mRNA was proposed to be cleaved by a protein–RNA complex containing miR165 ***(61)***. The mutant *phb* mRNA was proposed to be resistant to such cleavage because of its reduced complementarity to the miRNA.

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RNA Interference of mRNA Processing Factors in *Drosophila* S2 Cells

Alicia M. Celotto and Brenton R. Graveley

Summary

RNA interference (RNAi) is a useful tool for degrading targeted messenger RNAs (mRNAs) and thus "knocking down" the abundance of the encoded protein. We have been using RNAi in cultured *Drosophila* cells to evaluate the effect of "knocking down" numerous mRNA processing factors on the alternative splicing of specific pre-mRNAs. This relatively simple technique has allowed us to identify a number of splicing factors that impact the alternative splicing of particular alternatively spliced exons. This approach can be extended to examine the splicing of nearly any gene.

Key Words

RNA interference (RNAi); *Drosophila melanogaster*; Schneider (S2) cells; knock-down.

1. Introduction

The fruit fly *Drosophila melanogaster* is an excellent organism in which to study alternative splicing. First, *Drosophila* is probably the best-characterized multicellular organism. In addition to having a complex developmental program, fruit flies also display multiple behaviors. Second, the sequence of the euchromatic portion of the *D. melanogaster* genome has been available for some time now ***(1)*** and is fairly well annotated (http://www.fruitfly.org, http://www.flybase.org). Analyses of the fly genome have revealed that approximately 50% of genes are alternatively spliced ***(2)***. Moreover, the extent of alternative splicing of a single gene varies widely: whereas some genes give rise to only two isoforms, others, such as the *Dscam* gene ***(3)***, can give rise to tens of thousands. Because most of the known human splicing factors have clear orthologs in the fly ***(4)***, it is likely that many of the principles of splicing regu-

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lation revealed by studies in *Drosophila* will be directly applicable to alternative splicing in humans. *Drosophila* is also an excellent organism to use for genetic analyses and many mutant stocks are freely available. Numerous cell culture lines (i.e., S2, Kc, BG2-C6, etc.) have been established ***(5)*** and biochemical studies can be performed using extracts from cultured cells ***(6,7)*** or embryos ***(8,9)***. The most recent additions to the arsenal of tools available to *Drosophila* researchers is RNA interference (RNAi; **ref. *10***). RNAi is a phenomenon whereby dsRNA induces the degradation of homologous messenger RNAs (mRNAs), thereby depleting the protein encoded by the targeted mRNA. In *Drosophila*, RNAi can be performed in either cultured cells ***(11)*** or the intact organism ***(12)***. A major advantage of performing RNAi in cultured *Drosophila* cells rather than mammalian cells is that long, inexpensive, enzymatically synthesized dsRNAs can be used in *Drosophila* cells whereas short, expensive, chemically synthesized dsRNAs must be used in mammalian cells ***(13)***.

Performing RNAi in cultured *Drosophila* cells is quite easy: simply add dsRNA to the culture medium ***(11)***. As a result, it is straight forward to use RNAi to test the role of a large number of proteins in nearly any process or pathway. We have used this approach to identify known mRNA processing factors (*see* **Table 1**) that affect the alternative splicing of different pre-mRNAs in cultured *Drosophila* cells. In this chapter, we describe this approach in detail.

2. Materials

2.1. Equipment

1. Cell culture incubator: An incubator capable of maintaining a constant temperature of 25–27°C is required. It is not necessary to use an incubator with a CO_2 supply line.
2. Cell culture hood.

2.2. Supplies

1. Cell culture plasticware: 100-mL plastic Erlenmeyer flasks for maintaining cell stocks, six-well dishes for performing RNAi.
2. Schneider (S2) cells: Dmel-2 cells (Invitrogen, cat. no. 10831-014) or S2 cells (Invitrogen, cat. no. R690-07). Cells are also available from the ATCC (CRL-1963).
3. Cell culture medium: *Drosophila*-SFM (Invitrogen, cat. no. 10797-017), supplemented with 1X penicillin-streptomycin (Invitrogen, cat. no. 15140-122, 100X stock), and 2 m*M* glutamine (Invitrogen, cat. no. 25030-081, 100X stock).
4. Oligonucleotides: T7 primer, TAATACGACTCACTATAGGG; SP6 primer, ATTTAGGTGACACTATAG, gene-specific primers to clone complementary DNA (cDNA) fragments.
5. Cloning vector: pCRII-TOPO TA cloning kit (Invitrogen, cat. no. K4600-01).
6. Transcription reagents: Ribomax Large Scale RNA Production Kits (Promega, SP6 kit, cat. no. P1280, T7 kit, cat. no. P1300).

Table 1
mRNA Processing Factors Targeted by RNAi

Accession number	Gene name
CG8749	dU1-70K
CG5454	dU1C
CG4528	snf/dU1A/U2B”
CG7564	dLuc-7
CG3582	dU2AF38
CG9998	dU2AF50
CG5442	dSC35
CG6987	dASF/SF2
CG10851	B52
CG10203	d9G8
CG17136	RBP1
CG1987	RBP1-like
CG4602	dSRp54
CG9085	dSRPKD2
CG1658	CLK kinase/DOA
CG3193	Crooked neck/CLF1
CG12085	Halfpint/dPUF-60
CG10328	dPSF
CG2094	dPTB/hnRNPI
CG11107	dPrp43
CG16725	dSMN
CG17454	dSPF30
CG10419	dSIP1
CG7942	Debranching enzyme
CG5655	RSF1
CG11266	Splicing factor (CC1.3)
CG8912	PSI
CG10128	Tra2
CG18350	Sxl
CG16724	tra (female-specific)
CG15481	dRrp41p
CG3931	dRrp4p
CG8395	dRrp42p
CG4043	dRrp46p
CG4792	Dicer-1
CG6493	Dicer-2
CG5422	Rox8
CG6319	Bruno
CG8144	Pasilla
CG17540	dSPF45

7. RNA isolation reagents: Trizol™ reagent (Invitrogen).
8. Reverse transcription polymerase chain reaction (PCR) reagents: Standard reagents, that is, dNTPs, reverse transcriptase, *Taq* DNA polymerase, reaction buffers, and so on.

2.3. Solutions

1. 10X annealing buffer: 1 *M* NaCl; 200 m*M* Tris-HCl, pH 8.0; 10 m*M* ethylenediamine tetraacetic acid.
2. 1X Laemlli loading buffer: 62.5 m*M* Tris-HCl, pH 6.8; 2% sodium dodecyl sulfate; 10% glycerol; 0.025% bromophenol blue; 2.5% (v/v) β-mercaptoethanol.
3. Phenol:chloroform:isoamyl alcohol (25:24:1).
4. Chloroform:isoamyl alcohol (24:1).
5. Chloroform.

3. Methods

The methods described below discuss the manner in which RNAi is performed in cultured *Drosophila* cells in our laboratory. These involve (1) the cloning of cDNA fragments for the desired RNAi targets, (2) generating transcription templates, (3) RNA synthesis, (4) RNA annealing, (5) treating of the cells with dsRNA, and analyzing the effect of "knocking down" the target on (6) protein levels, or (7) alternative splicing. An outline of this procedure is shown in **Fig. 1**.

3.1. Clone cDNA Fragments for the mRNAs to be Targeted by RNAi

The first step is to generate a transcription template that will be used to synthesize dsRNA homologous the mRNA you are interested in targeting. There are various ways to go about producing dsRNA. For example, this can be done by performing RT-PCR on RNA, or PCR on a cDNA clone, with gene-specific primers containing a T7 or SP6 RNA polymerase promoter at the 5' end ***(14)***. The resulting PCR product can be transcribed directly. However, because of the large number of splicing factors we were interested in testing, rather than appending a T7 or SP6 RNA polymerase promoter sequence to the end of each oligonucleotide, we found it to be more cost-effective to first clone cDNA fragments into a vector in which the cloning site is flanked by T7 and SP6 promoters. For this purpose, we typically use the pCRII-TOPO-TA vector (Invitrogen). When size permits, it is a good idea to use primers that amplify the entire open reading frame of your cDNA of interest in the event that you want to use the clone for future experiments. Although it is not necessary to use the whole open reading frame, the larger the dsRNA trigger, the more efficiently the RNAi response will be. We have successfully performed RNAi using dsRNAs ranging in size from 75 bp to 1500 bp. On average, approx 800-bp

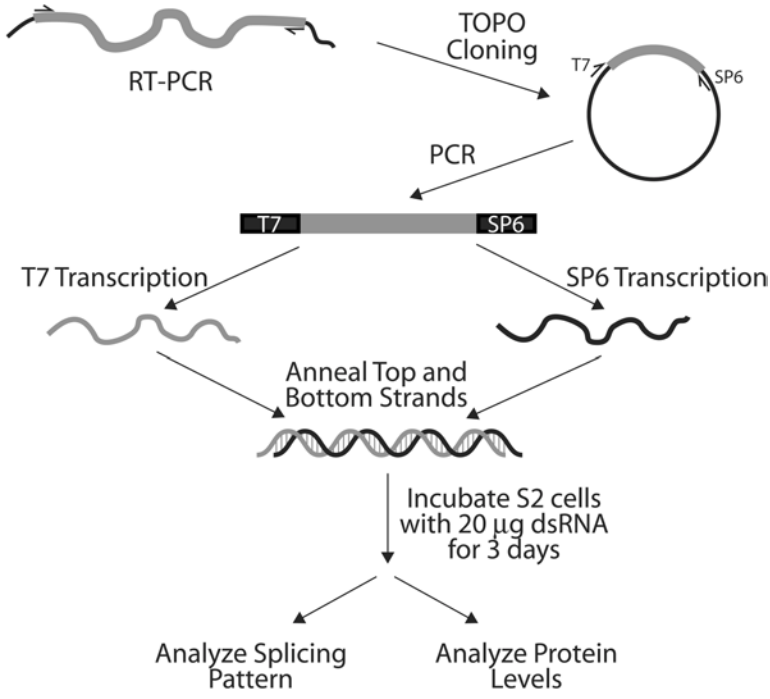

Fig. 1. Overview of the RNAi procedure. Complementary DNAs that will be used to generate transcription templates are cloned by performing RT-PCR and cloning the resulting PCR product into the pCRII-TOPO-TA vector. These vectors are used as templates in PCR reactions with the T7 and SP6 RNA polymerase promoter primers to yield a PCR product containing the cDNA sequence flanked by T7 and SP6 RNA polymerase promoters. The transcription templates are separately transcribed with T7 and SP6 RNA polymerase to yield top- and bottom-strand RNAs. The two RNA strands are annealed and 20 µg of the RNA is incubated with S2 cells for 3 d. After incubation with the dsRNA, protein levels can be analyzed by Western blotting and changes in alternative splicing can be monitored by RT-PCR and/or RNase protection.

fragments should be used when possible. Thus, the first step is to design oligonucleotides to the mRNAs of interest that will be used for RT-PCR (*see* **Note 1**). Once the oligonucleotides are in hand, RT and PCRs should be performed using standard procedures ***(15)*** to amplify the cDNA fragment of interest. As a template, RNA isolated from either cultured *Drosophila* cells or from flies can be used.

The presence of the correctly sized PCR product should be verified by agarose gel electrophoresis. Subsequently, the PCR product should be cloned into

the pCRII-TOPO-TA vector (Invitrogen). To further reduce the cost of this procedure, we have scaled down the volume of the TOPO cloning protocol provided by the manufacturer as follows:

1. Assemble a 1.9-μL reaction containing 1.3 μL of RT-PCR product, 0.3 μL of salt solution (included in the TOPO cloning kit), and 0.3 μL of pCRII-TOPO-TA Vector.
2. Incubate the reaction at room temperature for 5–30 min.
3. Use the entire reaction to transform *Escherichia coli* using standard procedures ***(15)*** and plate onto LB-Amp plates containing X-gal.
4. The next day, pick several colonies to grow up and screen for the presence of an insert by restriction mapping.
5. Sequence one representative clone to ensure that it contains the cDNA of interest. These cDNA clones will be used in the next section to generate transcription templates.

3.2. Generating Transcription Templates

Once clones containing the target cDNAs are in hand, the next step is to generate the transcription template. To do this, PCR is performed using T7 and SP6 primers, which anneal to the plasmid on either side of the cloning site. The resulting PCR product will contain the cDNA flanked on either side by a T7 and SP6 RNA polymerase promoters.

1. In 0.2-mL tubes, assemble a 100-μL reaction containing 5 μL of DNA template (1 ng/μL), 2 μL of 10 m*M* dNTPs, 8 μl of 2 μ*M* SP6 primer, 8 μL of 2 μ*M* T7 primer, 10 μL of 10X PCR buffer without $MgCl_2$, 3 μL of 50 m*M* $MgCl_2$, 0.5 μL of *Taq* DNA polymerase (1 U/μL), and 63.5 μL of H_2O.
2. Cycle the reactions 35 times at 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min, followed by a 3-min incubation at 72°C.
3. Check for the presence of the desired PCR product by running 5 μL of the reaction on a 1% agarose gel and staining the gel with ethidium bromide.
4. Clean up the PCR products by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitation with 2.5 vol ethanol.
5. Resuspend the pellet in 10 μL of H_2O and quantify by spectrophotometry.

3.3. RNA Synthesis

The next step is to synthesize RNA from the transcription template. Because the transcription template is a PCR product containing a T7 promoter on one end and an SP6 promoter on the other, separate transcription reactions are required to synthesize the top and bottom RNA strands. We typically use the RiboMax Large Scale RNA Production Kit (Promega).

1. Set up a 50-μL SP6 transcription reaction containing 10 μL of SP6 transcription 5X buffer, 10 μL of rNTPs at 25m*M* each (adenosine triphosphate [ATP], cyti-

dine triphosphate [CTP], guanosine triphosphate [GTP], uridine triphosphate [UTP]), 5–10 μg of PCR product, and 5 μL of SP6 enzyme mix.
2. Set up a separate 50-μL T7 transcription reaction containing 10 μL of T7 transcription 5X buffer, 15 μL of rNTPs at 25 m*M* each (ATP, CTP, GTP, UTP), 5–10 μg of PCR product, and 5 μL of T7 enzyme mix.
3. Incubate both reactions at 37°C for 4 h.
4. Add 5 μL of DNase I to each reaction and continue the incubation for 15 additional minutes at 37°C to degrade the transcription template.
5. Extract the reactions with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by a second extraction with an equal volume of chloroform:isoamyl alcohol (24:1).
6. Precipitate the RNA by adding 1/10 vol 3 *M* sodium acetate, pH 5.2, and 2.5 vol ethanol.
7. Resuspend the pellet in 50 μL of H_2O.

3.4. Annealing the RNA to Make dsRNA

Once the two strands of the RNA are made, they must be annealed to generate dsRNA prior to adding it to the cells.

1. Assemble a 50-μL reaction containing 20 μg of top-strand RNA, 20 μg of bottom-strand RNA, and 5 μL of 10X annealing buffer.
2. Heat the samples to 68°C for 10 min followed by an incubation at 37°C for 30 min. If desired, the efficiency of annealing can be determined by running the single strands and the annealed dsRNA on an agarose gel.

3.5. Adding dsRNA to S2 Cells

Once you have made the dsRNA, you can use it to perform RNAi on the cultured cells. A variety of Schneider cell lines are in use in different laboratories. The majority of our studies have used the Dmel-2 cells (Invitrogen), which have been optimized for growth in serum free medium. Because serum can interfere with the uptake of dsRNA into the cells, using this cell line makes the process of performing RNAi significantly easier. For more information on performing RNAi on cell lines that require serum for growth, please refer to **ref. *14*** or the Dixon lab website (http://dixonlab.biochem.med.umich.edu).

1. Dilute S2 cells to a concentration of 1×10^6 cells/mL in *Drosophila*-SFM media supplemented with penicillin/streptomycin and glutamine.
2. Add 2 mL of the diluted cells to each well of a six-well dish.
3. Add the dsRNA directly to the culture medium in each well.
4. Incubate the cells at 27°C for the desired amount of time (a good starting point is 3 d). It is not necessary, and not recommended that you change the culture medium during the first day of treatment. However, for protocols requiring more than 3 d of incubation with the dsRNA, it is advisable to change the medium every 2 or 3 d.

Three major variables should be optimized for each dsRNA. The first is the concentration of dsRNA to be used. For most targets we have tested, 20 μg of dsRNA/well is sufficient. However, you may find that more dsRNA is necessary or that you can effectively deplete your protein using much less dsRNA. A second variable is the amount of time required to observe efficient depletion of the protein of interest. We have found 3 d to be sufficient to effectively deplete most proteins. In fact, often proteins are significantly depleted after only one day of treatment. However, for stable and/or abundant proteins longer incubations, up to 6 d, may be necessary. Finally, for proteins that are particularly difficult to deplete, it may be necessary to treat the cells with the dsRNA multiple times. For example, some users have needed to treat the cells with 20 μg of dsRNA every 2 d for 6 d to effectively deplete the targeted protein. All of these variables must be empirically tested. Finally, it is possible to simultaneously deplete more than one protein by simply incubating the cells with multiple dsRNAs at the same time.

3.6. Preparation of Cell Lysate for Protein Analysis

The next step is to determine the degree to which the targeted protein was depleted. This can really only be performed if antibodies specific to the target protein are available. Although we have been unable to test the degree of protein depletion for many of the proteins we have targeted, for those that we have tested, we find the protein levels are reduced from at least twofold to more than 10-fold.

1. Completely remove the culture medium.
2. Add 500 μL of 1X Laemmli loading buffer to each well.
3. Pipet the solution up and down several times and transfer to a 1.5-mL microcentrifuge tube. The extent of depletion can be measures by electrophoresing the cell lysate on an SDS-protein gel and performing a Western blot using standard procedures ***(15)***. Ten microliters of cell lysate is generally sufficient to detect the protein of interest by Western blot.

3.7. RNA Isolation

The final step is to isolate RNA from the cell to examine the splicing pattern of the pre-mRNA of interest. We find this is most easily done using Trizol™ reagent (Invitrogen).

1. Completely remove the culture medium from the six-well dishes.
2. Add 1 mL of Trizol to each well.
3. Gently pipet the solution up and down a few times and then transfer to a 1.5-mL microcentrifuge tube.
4. Add 100 μL of chloroform to each tube and briefly vortex.
5. Incubate the tubes on ice for 5 min.

6. Centrifuge the samples at 12,000*g* for 15 min at 4°C.
7. Remove the aqueous layer to a new tube and add 500 μL of isopropanol to precipitate the RNA.
8. Centrifuge the samples at 12,000*g* for 15 min at 4°C.
9. Remove the supernatant, briefly dry the pellet on the bench, and then dissolve the pellet in 50 μL of H_2O.
10. Quantify the RNA by spectrophotometry and analyze by desired method (i.e., RT-PCR, RNase protection, etc.). If the RNA is not to be used immediately it should be stored at –80°C.

4. Notes

1. It is important to keep in mind that 22 nt siRNAs mediate target recognition and cleavage during RNAi. Therefore, you should search the *Drosophila* genome with the sequence of the dsRNA you will be using to determine whether the dsRNA has the potential to codeplete other proteins.

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RNA Interference by Short Hairpin RNAs Expressed in Vertebrate Cells

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Summary

RNA interference (RNAi) is now established as a general method to silence gene expression in a variety of organisms. Double-stranded RNA (dsRNA), when introduced to cells, interferes with the expression of homologous genes, disrupting their normal function. In mammals, transient delivery of synthetic short interfering RNAs (siRNAs), which resemble the processed form of standard double stranded RNAi triggers, is effective in silencing mammalian genes. Issues related to transfer efficiency and duration of the silencing effect, however, restrict the spectrum of the applications of siRNAs in mammals. These shortcomings of siRNAs have been solved by the cellular expression of short hairpin RNAs (shRNAs) from DNA vectors. shRNAs are indistinguishable from siRNAs in terms of efficacy and mechanism but can be produced within cells from standard mammalian expression vectors. In this way, shRNA expression makes possible the creation of continuous cell lines and transgenic animals in which suppression of a target gene is stably maintained by RNAi. As a result, the types of RNAi-based gene function analysis that can be carried out in mammals have been greatly expanded. We describe methods for the construction and transfer of stable shRNA expressing vectors suitable for generating loss of function alleles in mammalian cells in vitro or in vivo.

Key Words

RNAi; gene silencing; retrovirus; knock-outs; mammalian genetics.

1. Introduction

The recent completion of the human and mouse genomes has brought mammalian gene function analysis to the forefront of biology ***(1–3)***. Until recently, the techniques available for mammalian gene function analysis were far less effective than those commonly used in model genetic organisms. This was especially true in the generation of loss-of-function mutations. Rapid methods for directed gene ablation in a number of model organisms have existed for 20 yr.

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In contrast, directed gene disruption by homologous recombination or antisense or ribozyme techniques in mammalian cells, although useful, are often costly, time-consuming, ineffective, or some combination thereof.

The recent development of RNA interference (RNAi) has significantly closed the technology gap between model organisms and mammals ***(4,5)***. RNAi was first described in *Caenorhabditis elegans* as a method in which exogenously supplied double-stranded RNA (dsRNA) resulted in the suppression of expression of homologous genes ***(6)***. Although nonspecific effects of long dsRNA were an initial hurdle in the use of RNAi in mammals ***(7,8)***, research into its mechanism yielded clues important for its eventual generalized implementation (for review, *see* **ref. *9***). In our current understanding of RNAi, dsRNA is processed by an RNAse III-like enzyme named Dicer into small approx 21 nt RNAs known as short-interfering RNAs (siRNAs) siRNAs are incorporated into RNA-induced silencing complexes and act as specificity determinants in the degradation of homologous mRNAs. The discovery that the application of Dicer product-like siRNAs were sufficient to trigger RNA interference in mammalian somatic cells revolutionized the genetics of tissue culture cells ***(10)***.

Despite the triumph of siRNAs in mammalian cells, significant differences still existed between RNAi based on exogenously-supplied dsRNA in *C. elegans* and mammalian cells. Mammalian cells do not take up exogenously applied dsRNA efficiently and the persistence of the RNAi response, which is amplified in *C. elegans*, is limited to approximately six to eight cell doublings in mammals ***(11)***. For this reason, several types of mammalian experiments require that dsRNA silencing triggers must be repeatedly supplied to mammalian cells with cationic lipids or via electroporation. Although this only complicates in vitro experiments, it precludes the use of siRNAs in most animal experiments.

These limitations sparked the development of stable RNAi-based silencing in mammals. Similar approaches in which long dsRNA hairpins, typically 500–1000 nt in length, are expressed from within cells have modernized RNAi analysis of gene function in lower organisms because they provide uniform delivery and duration of the silencing ***(12–15)***. Largely because of the nonspecific responses to long dsRNA, these approaches are of limited use in mammals. However, basing their designs loosely on the small, hairpin-structured, noncoding microRNAs that are substrates of the RNAi pathway, several groups solved the problems related to persistence and transfer efficiency in mammalian cells by expressing short hairpin RNAs (shRNAs; **refs. *16–21***). Although somewhat variable in structure, shRNAs are expressed from mammalian promoters on DNA vectors that are introduced to cells by transfection or infection and possess double stranded stems less than 30 nt in length that serve as sub-

strates for Dicer. This permits the creation of stably silenced cell lines, which complements the use of siRNAs in tissue culture. For silencing genes in mammalian cells in vivo, shRNAs are the only viable technique ***(22–25)***.

The utility of shRNAs as a genetic tool lies in the generation of cells in which the shRNA expression cassette is stable. For this reason, gene-transfer methodologies, which are inherently stable, are better platforms for the expression of shRNAs. Perhaps the best-characterized stable expression technologies for mammalian cells are systems based on retroviral integration (for a review of these methods, *see* **ref. *26***). We typically house shRNA expression cassettes on an MSCV-based retrovirus that contains a mammalian selectable drug-resistance marker. RNA polymerase III, which normally initiates and terminates small, highly structured RNA transcripts precisely ***(27)***, is responsible for transcription of shRNA sequences contained immediately downstream of the human U6-snRNA promoter ***(18–20)***. This promoter is active in most if not all embryonal and somatic cell types and has, to date, offered similar levels of constitutive expression in a variety of settings.

Here we present detailed protocols for the design, construction and delivery of shRNA expression vectors for use in gene silencing experiments in mammals. The protocol makes use of the pMSCV-SHAG BbsBlue vector to produce clones identical to those used in various silencing experiments (**ref. *24*** and our unpublished results). In this vector, DNA oligonucleotides encoding shRNAs are cloned into a *Bbs*I site for optimal expression by the human U6 promoter. Displacement of a *lacZ*α fragment aids in the identification of shRNA-carrying clones. Once constructed, these vectors can be used to direct the production of retroviruses that silence a specific gene in a variety of host cell types. These methods can be used for gene function analysis of mammalian gene function in cultured cells or in whole animals.

2. Materials

2.1. Hairpin Oligos

1. Annealing buffer: 10 m*M* Tris-HCl, pH 7.5; 50 m*M* NaCl; 1 m*M* ethylenediamine tetraacetic acid (EDTA).
2. Oligo A (described in detail in **Subheading 3.1.**).
3. Oligo B (described in detail in **Subheading 3.1.**).

2.2. Polynucleotide Kinase Reaction

1. 10X Kinase buffer: 500 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 1 m*M* EDTA, 50 m*M* dithiothreitol, 1 m*M* spermidine, pH 8.2.
2. T4-polynucleotide kinase (Roche Molecular Biochemicals, Indianapolis, IN).
3. 10 m*M* ATP.
4. Distilled H_2O.

2.3. Vector Preparation

1. pMSCV-SHAG BbsBlue vector (*see* **Fig. 1**).
2. *Bbs*I (New England Biolabs, Beverly, MA, cat. no. R0539S).
3. 10X *Bbs*I buffer: 500 m*M* NaCl, 100 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 10 m*M* dithiothreitol, pH 7.9.
4. Distilled H_2O.
5. Phenol:chloroform:isoamyl alcohol (25:24:1).
6. Chloroform:isoamyl alcohol (24:1).

2.4. Ligation Reaction

1. 10X ligase buffer: 500 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 100 m*M* dithiothreitol, 10 m*M* ATP, 250 mg/mL bovine serum albumin, pH 7.8.
2. Distilled H_2O.
3. DNA ligase (New England Biolabs, Beverly, MA, cat. no. M0202S).
4. Glycogen (5 mg/mL).
5. 3 *M* Sodium acetate, pH 4.8.
6. 100% Ethanol.
7. 70% Ethanol.

2.5. Transformation

1. Competent *Escherichia coli* (*see* **Note 1**).
2. LB plates containing ampicillin and X-gal (Sigma cat. no. B4252; *see* **Note 2**).

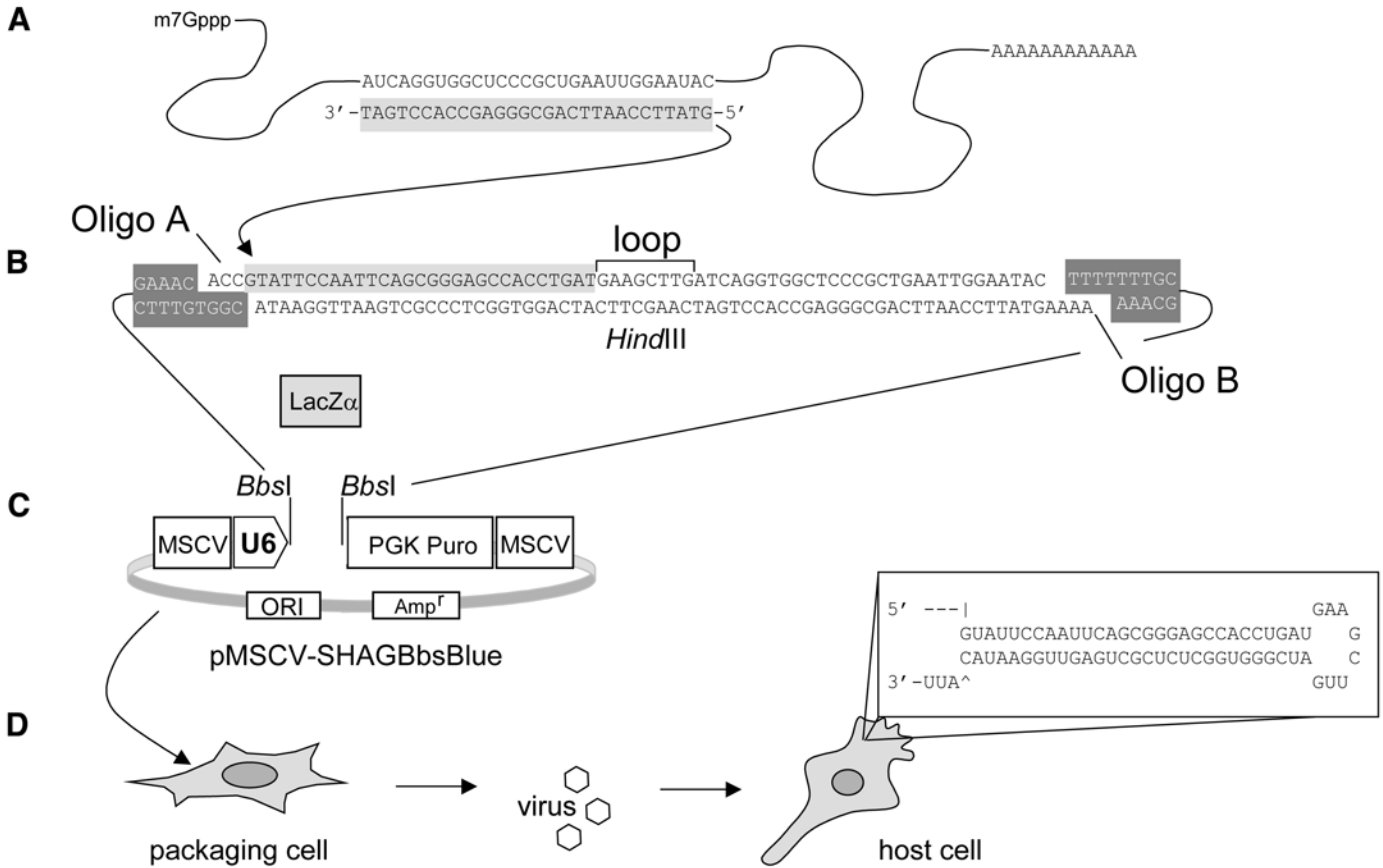
2.6. Sequencing Analysis

1. U6 -130 primer (5'-TACAAAATACGTGACGTAGA-3').

2.7. Cell Culture

1. Retroviral packaging cell line. Various suitable retroviral packaging lines are available from the American Type Culture Collection. Methods for the use of these cells have already been published in this series ***(26)***.
2. Host cells in which gene is to be silenced.
3. 10X $CaCl_2$ 2.5 *M*.

Fig. 1. (*see opposite page*) Strategy for the construction of stable silencing constructs in mammalian cells. **(A)**, A target sequence within an mRNA of interest is selected and used to design oligos that when expressed will form a short hairpin structure. **(B)**, The annealed oligos, with 5' ends shown, are ligated directly into *Bbs*I-digested pMSCV-SHAG BbsBlue **(C)**, *Bbs*I digestion of this vector releases a *lacZ*α fragment leaving ends compatible with the insertion of the designed oligo at the first transcribed base. **(D)**, The resulting clones are packaged into retroviral particles which in turn are used to infect a cell type of interest. Expression from the U6 promoter results in expression of an shRNA, which silences the cognate target via the RNAi pathway.


A
m7Gppp
AUCAGGUGGCUCCCGCUGAAUUGGAAUAC
3′-TAGTCCACCGAGGGCGACTTAACCTTATG-5′
AAAAAAAAAAAA
B
Oligo A
loop
GAAAC ACCGTATTCCAATTCAGCGGGAGCCACCTGATGAAGCTTGATCAGGTGGCTCCCGCTGAATTGGAATAC TTTTTTTGC
CTTTGTGGC ATAAGGTTAAGTCGCCCTCGGTGGACTACTTCGAACTAGTCCACCGAGGGCGACTTAACCTTATGAAAA AAACG
HindIII
Oligo B
LacZα
C
BbsI
BbsI
MSCV
U6
PGK Puro
MSCV
ORI
Ampr
pMSCV-SHAGBbsBlue
D
5′ ---|
GAA
GUAUUCCAAUUCAGCGGGAGCCACCUGAU G
CAUAAGGUUGAGUCGCUCUCGGUGGGCUA C
3′-UUA^
GUU
packaging cell
virus
host cell

4. 2X BES-buffered solution (2X BBS): 50 m*M* *N*,*N*-*bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, Adjust to optimal pH with 1 N NaOH, room temperature (*see* **Note 3**). Sterilize by filtration through a 0.45-μm filter and store at –70°C.
5. 8 mg/mL Polybrene: hexadimethrine bromide (Sigma, H9268).
6. 1 mg/mL Puromycin dihydrochloride: cell culture-tested (Calbiochem, cat. no. 540411).

3. Methods

3.1. Oligonucleotide Design for Insertion Into BbsI-Cut pMSCV-SHAG Vector (see Note 4)

1. Obtain the coding sequence of any gene.
2. Select any 29 base sequence within the gene that does not contain stretches of three or more consecutive adenines or three or more consecutive thymidines and ends with cytosine (*see* **Note 5**):
   5'-N1 N2 N3 .... N26 N27 N28 C-3'
   (where N1, N2, etc., is any nucleotide).
3. Determine the reverse complement of the sequence in **step 2**:
   5'- G N28' N27' N26' .....N3' N2' N1'-3'
   (where N1', N2', are the complementary bases to nucleotides N1, N2, and so on).
4. Add 5'-GAAGCTTG-3' to the 3' end of **step 3** to get:
   5'- G N28' N27' N26' .....N3' N2' N1'GAAGCTTG -3'.
5. Add the sequence in **step 2** to the 5' end of the sequence in **step 4** to get:
   5'- G N28' N27' N26' ..N3' N2' N1'GAAGCTTG N1 N2 N3 .... N26 N27 N28 C -3'.
6. Add ACC to the 5' end of the sequence in **step 6** to generate the sequence of oligonucleotide A: 5'-ACC G N28' N27' N26' ..N3' N2' N1'GAAGCTTG N1 N2 N3 .N27 N28 C -3'.
7. For oligonucleotide B, first prepare the reverse complement of the sequence in **step 6** to generate: 5'-G N28' N27' N26' ..N3' N2' N1'CAAGCTTC N1 N2 N3 .... N26 N27 N28 -3.'
8. Add AAAA to the 5' end of the sequence in **step 6** to generate oligonucleotide B: 5'-AAAA G N28' N27' N26' ..N3' N2' N1'CAAGCTTC N1 N2 N3 ... N26 N27 N28 -3'.
9. Oligonucleotides used here are obtained by standard oligonucleotide synthesis (*see* **Note 6**).

3.2. Oligonucleotide Hybridization

1. Mix 2 μL of each complementary oligonucleotide with 16 μL of annealing buffer in a 1.5-mL microfuge tube.
2. Place tube in a standard heating block at 95°C.
3. Remove the heating block from the apparatus and allow to cool to room temperature (or at least below 30°C) on the workbench. Briefly centrifuge to recover liquid. Store on ice or at 4°C until ready to use (*see* **Note 7**).

3.3. Phosphorylation of Oligonucleotides

1. To a 1.5-mL microfuge tube add: 5 μL H_2O, 2 μL of annealed oligonucleotides, 1 μL of 10X T4 polynucleotide kinase buffer, 1 μL of 1 m*M* ATP (stock is 10 m*M*), 1 μL of T4 polynucleotide kinase.
2. Incubate for 30 min at 37°C.
3 Heat-inactivate by incubation for 10 min at 70°C.

3.4. Vector Preparation

1. Digest 1 μg of pMSCV-SHAG Bbs vector in a 1.5-mL microfuge tube in a reaction with 2 μL of 10X buffer, 5 units of *Bbs*I, and distilled H_2O to 20 mL total for 60 min (*see* **Note 8**).
2. Heat inactivate the enzyme by incubation for 10 min at 70°C .
3. Remove protein from the reaction by addition of an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1). Briefly vortex and centrifuge at 14,000*g* for 2 min.
4. Recover the aqueous phase to a new tube and extract with one volume of chloroform:isoamyl alcohol (24:1). Briefly vortex and centrifuge at 14,000*g* for 2 min.
5. Recover the aqueous phase to a new tube and adjust to 0.3 *M* sodium acetate by addition of 1/10 vol of 3 *M* sodium acetate, pH 4.8. Precipitate the vector by adding 1 μL of glycogen, 2.5 vol of ice-cold 100% ethanol, and incubating at 0°C for 10 min. Centrifuge at 14,000*g* for 10 min to recover the vector DNA.
6. Wash the vector pellet with 100 μL of 70% ethanol, dry briefly and resuspend in 10 μL of H_2O.

3.5. Insertion of Oligonucleotides Into the Expression Vector

1. In a 1.5-mL microfuge tube, combine 14 μL of H_2O, 2 μL of 10X ligase buffer, 1 μL of prepared vector, and 2 μL of the phosphorylated oligonucleotides.
2. Add 1 μL of DNA ligase.
3. Incubate at 14°C overnight.
4. Use 2 μL to transform standard competent cells (>10^7 colonies/μg).
5. Plate on LB plates containing ampicillin and X-gal.
6. Incubate at 37°C overnight.
7. Pick white colonies to prepare template for DNA sequence analysis (*see* **Note 9**).

3.6. Sequencing of Hairpin Clones

1. Prepare template DNA.
2. Analyze the DNA sequence using the U6-130 primer.
3. Confirm that the sequence is correct by comparing it to oligonucleotide A designed in **Subheading 3.1.**, **step 7**.
4. Make large-scale preparations of clones that yield correct sequence. Various commercially available kits are ideal for production of transfection-ready DNA.

3.7. Transient Production of Silencing Retroviruses in Packaging Cell Lines After Transfection (see Note 10)

1. Production, day 1: Plate exponentially growing packaging cells at a density of 1×10^6 cells per 10-cm culture dish.
2. Production, day 2: Inspect each plate from day 1 to ensure that each has cells at 50–80% confluency.
3. Production, day 2: 1–2 h before transfection, remove the media from the previous day and replace with fresh medium.
4. Production, day 2: Prepare the transfection mixture: Also you may want to incubate your solutions at room temperature or 37°C before mixing to standardize the precipitate characteristics. For each 10-cm plate to be transfected, add 15 μg of plasmid DNA and distilled H_2O to a total volume of 900 μL in a polypropylene tube (Falcon 2063).
5. Production, day 2: Add 100 μL of $CaCl_2$ and mix.
6. Production, day 2: Add 1 mL of 2X BBS while bubbling the solution vigorously for approx 10 s by forcing air with an automatic pipettor through a pipet positioned at the bottom of the tube.
7. Production, day 2: Immediately (within approx 1–2 min) add dropwise (one drop every other second) the transfection mixture to the cell monolayer while rocking the plate. Briefly rock the plates back and forth and side to side to evenly distribute DNA/$CaPO_4$ particles and place plates in 37°C incubator.
8. Production, day 3: Replace the medium with 10–15 mL of fresh, prewarmed medium.
9. Production, day 3: For virus production move the plates to a 32°C incubator 2 d before infection (*see* **Note 11**).
10. Production, day 5: Carefully remove the retrovirus-containing media and filter this through a 0.45-μm syringe filter. If infection is planned for that day the viral filtrate should be stored at +4°C. Longer term storage should be at –80°C.

3.8. Introduction of Silencing Retroviruses Into Host Cell Lines

1. Infection, day 1: Plate exponentially growing host cells at a density that will allow them to proliferate for at least 3 d. Be sure to have at least one plate as an uninfected control.
2. Infection, day 2: For each 10 cm plate to be infected prepare an infection mixture containing 5 mL of host cell media, 5 mL of viral supernatant from **Subheading 3.7.**, **step 10**, and 10 μL 8 mg/mL polybrene (*see* **Note 12**).
3. Infection, day 2: Remove the media from host cell and replace with infection mix.
4. Infection, day 2: Incubate for 8–24 h at 32°C.
5. Infection, day 3: Carefully replace the medium with the normal host cell medium and return the cells to the 37°C incubator.
6. Infection, day 5: Add an appropriate dilution of puromycin to the plates (*see* **Note 13**).

7. Infection, day 7: Inspect uninfected control plates for extensive cell death. Although infection rates vary depending on cell type, infected plates should have surviving cells on the second day of selection.
8. Expand the surviving cell cultures and test for silencing of the gene of interest and resulting phenotype.

4. Notes

1. Commercially available competent cells that yield $>10^7$ colonies/μg are ideal for this purpose. Any of a variety of strains that carry the *lacZ*(M15) mutation should be appropriate for blue–white screening. Such strains include JM109, XL-1 Blue, and DH5α. Despite the presence of tandemly repeated sequences, we find that hairpin constructs are stable in most commonly used bacterial strains.
2. To premade LB plate containing appropriate antibiotics, spread 40 μL of a stock solution of X-gal (20 mg/mL in dimethylformamide) and 4 μL of a stock solution of IPTG (200 mg/mL) on the surface of the plate. These volumes can be scaled up and added to media immediately before pouring.
3. The pH (pH 6.95–7.10) of the 2X BBS solution is critical. When preparing 2X BBS buffer, pH should be checked against a *bona fide* reference stock when available. Alternatively, several test batches can be produced at once that differ from each other by 0.05 pH units, for example, pH 6.90, 6.95, 7.00, 7.05, and 7.10. Each test batch can be examined for transfection efficiency using vectors that express easily scored markers, such as β-galactosidase or green fluorescent protein. Store the optimal solutions at –70°C in 50-mL aliquots.
4. The design of shRNA constructs is relatively flexible. Double stranded stems between 18 and 29 nt in length are approximately equivalent in efficacy ***(28)***. Either strand of the stem structure should be complementary to the sense strand of the targeted mRNA. It does not seem to matter whether it is the 5' stem strand or the 3' stem strand ***(16–21)***. The loop sequences are unimportant. Various sequences between 3 and 9 nt in length work well, but longer loops appear to be deleterious ***(28)***. At present, RNAi target sequences within mRNAs are poorly defined. The somewhat imprecise published guidelines with which to select hairpin target sites suggest a target sequence near the 5' end of the gene with a GC content of approx 50%. Many target sites that do not share these criteria are highly effective including several cases in which the 3' end of the gene was the best choice ***(29,30)***. The protocol shown here in **Subheading 3.1.** has been incorporated into an online design tool for short hairpin construct design: http://katahdin.cshl.org:9331/RNAi/ . Several other similar sites exist: http://jura.wi.mit.edu/bioc/siRNA/, http://www.dharmacon.com/, http://www.ambion.com/techlib/misc/siRNA_finder.html.
5. The oligonucleotide must end in a C so that RNA polymerase III, which initiates at a G in the U6 promoter, will initiate precisely at the first base of the antisense strand. Runs of A's or T's will cause premature termination by RNA polymerase III.

6. Oligonucleotides can be obtained from any commercial supplier. A 0.05-μmol synthesis scale is sufficient. Resuspend the oligonucleotides at 100 μ*M* before annealing.
7. A thermal cycler can also be used to anneal oligonucleotides. Add 10-μL aliquots of mixed oligonucleotides into polymerase chain reaction tubes (500-μL size). Place the tubes in a thermal cycler and set up a program to perform the following profile: step 1: heat to 95°C and remain at 95°C for 2 min, step 2: ramp cool to 25°C over a period of 10 min, step 3: proceed to a storage temperature of 4°C.
8. The completeness of digestion can be monitored by agarose gel electrophoresis. Although not generally necessary, digested vector can also be purified following agarose gel electrophoresis.
9. White colonies result from the displacement of the *lacZ*α gene, which drastically reduces the vector background in the cloning reaction. The inclusion of the *Hin*dIII site in the loop of the hairpin allows for rapid identification of clones containing a hairpin. Digestion of successful hairpin constructs with *Hin*dIII produces a 570-bp band. A few colonies for each hairpin construct should be sequenced.
10. Retroviruses produced by these methods are potentially hazardous! Caution should be exercised in their production and use. Although a powerful technique for gene transfer, appropriate NIH and other regional guidelines should be followed to ensure the safety of those working in the laboratory.
11. Retroviruses are more stable at 32°C than 37°C. Although incubation at 32°C during production and infection is not necessary, it will improve titers.
12. The volume of virus will depend on several factors including the titer of virus, the susceptibility of the strain to infection, and so on. A good starting point is to infect the host cells using a 0.5X dilution of the retroviral media supernatant collected in **Subheading 3.7.**, **step 10**.
13. Reverse transcription and integration of viral genomes generally take place within the first 24–36 h. Although cells can be assayed for the phenotype of interest at this time, selection of infected cells with puromycin greatly improves the penetrance of most phenotypes. For each host cell line, the minimum puromycin concentration required to kill uninfected cells should be determined in titration experiments before infection. One mg/mL final concentration is a good starting point for titration. It should be noted that puromycin typically kills most cells after only 2-d exposure.

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