Chapter 15

Complementation of U4 snRNA in *S. cerevisiae* Splicing Extracts for Biochemical Studies of snRNP Assembly and Function

Martha R. Stark and Stephen D. Rader

Abstract

Pre-messenger RNA splicing is a surprisingly complex and dynamic process, the details of which remain largely unknown. One important method for studying splicing involves the replacement of endogenous splicing components with their synthetic counterparts. This enables changes in protein or nucleic acid sequence to be tested for functional effects, as well as the introduction of chemical moieties such as cross-linking groups and fluorescent dyes. To introduce the modified component, the endogenous one must be removed and a method found to reconstitute the active splicing machinery. In extracts prepared from *S. cerevisiae*, reconstitution has been accomplished with the small, nuclear RNAs U6, U2, and U5.

We describe a comparable method to reconstitute active U4 small, nuclear RNA (snRNA) into a splicing extract. In order to remove the endogenous U4 it is necessary to target it for oligo-directed RNase H degradation while active splicing is under way, i.e., in the presence of a splicing transcript and ATP. This allows complete degradation of endogenous U4 and subsequent replacement with an exogenous version. In contrast to the procedures described for depletion of U6, U2, or U5 snRNAs, depletion of U4 requires concurrent active splicing. The ability to reconstitute U4 in yeast extract allows a variety of structural and functional studies to be carried out.

Key words U4 snRNP, Splicing extract, *S. cerevisiae*, Functional complementation, Functional reconstitution, Pre-mRNA splicing, snRNA, snRNP

1 Introduction

Alternative splicing of pre-messenger RNA (pre-mRNA) gives rise to much of the amazing diversity of human proteins, despite the relative paucity of actual genes. Pre-mRNA splicing is also important for human gene expression because even small errors in splicing can have catastrophic consequences, as illustrated by the vast number of diseases whose cause can be found in splicing errors (reviewed in refs. 1, 2). It has recently been proposed that up to 60 % of all hereditary human diseases may be caused by the disruption of normal splicing patterns [3]. The large number of proteins

Klemens J. Hertel (ed.), *Spliceosomal Pre-mRNA Splicing: Methods and Protocols*, Methods in Molecular Biology, vol. 1126, DOI 10.1007/978-1-62703-980-2_15,

involved in pre-mRNA splicing as well as the many macromolecular rearrangements that must occur during the process have hampered our understanding of the detailed molecular mechanism by which the splicing machinery carries out its functions.

The chemical steps of pre-mRNA splicing are relatively straightforward: in the first step, the 2′ hydroxyl of the branch point adenosine reacts with the 5′ splice site, breaking the phosphodiester bond between the last exonic nucleotide and the first intronic one. This results in the formation of the so-called lariat intron intermediate with the branched adenosine connected to the intronic loop on one side and the downstream exon on the other. The 5′ exon is not covalently attached to the remainder of the transcript after the first chemical step. In the second step, the 3′ hydroxyl of the upstream (5′) exon reacts with the 3′ splice site, thereby ligating the two exons together and releasing the intervening intron as a lariat.

In contrast to the simplicity of the chemical steps, the assembly, catalytic mechanism, and regulation of splicing are so complicated that after 30 years of study we still understand only the broadest outlines of these processes. Assembly of the splicing complex, known as the spliceosome, appears to happen as an ordered series of reversible events (reviewed in ref. 4). The spliceosome is composed of five small, nuclear ribonucleoproteins (snRNPs), each consisting of a small, nuclear RNA (snRNA) and a set of proteins, as well as a number of other proteins and protein complexes. Two of these snRNPs, U1 and U2, recognize the 5′ splice site and branch point of the pre-mRNA, respectively, via direct base pairing between snRNAs and transcript. The other three, U4, U5, and U6, join the assembling spliceosome together as a preassembled tri-snRNP. After a number of rearrangements, the active spliceosome, consisting of the U2, U5, and U6 snRNPs, remains to catalyze the chemical steps of splicing.

General functions for U5 (in holding the exons together) and U6 (in catalyzing the chemical steps) have been suggested, but U4's role in the splicing process remains enigmatic, as it dissociates prior to the chemical steps. U4 is known, however, to be closely associated with U6 via extensive base pairing. This association is necessary for the introduction of U6 into the assembling spliceosome, but whether it serves some additional regulatory function and what prevents U6 from assembling by itself are unknown.

A number of genetic and biochemical studies have suggested that the main role of U4, aside from base pairing to U6, is to facilitate assembly with U5 to form the tri-snRNP [5]. In addition, recent clinical work has linked mutations in U4 to a type of congenital dwarfism [6, 7]. Nevertheless, many aspects of U4's functions remain to be worked out, including the mechanism by which it associates with U6, its role in promoting assembly of the tri-snRNP, and the mechanism by which it dissociates from the spliceosome.

The method presented here provides a powerful tool for studying this enigmatic molecule.

The normal method for reconstituting snRNAs involves either DNA oligonucleotide-targeted degradation of the endogenous RNA by RNase H [8–14] or the removal of the RNA using streptavidin-agarose affinity selection with 2′-*O*-methyl RNA oligos complementary to the snRNA [15–18]. This is followed by addition of an exogenous version of the snRNA. The first challenge is to identify a region of the snRNA that is accessible to the targeting oligonucleotide, and frequently a number of oligos must be tested before an effective one is found. The second complication is that snRNAs may exist in more than one form in a static extract (i.e., an extract in which nothing is happening biochemically), some of which may be accessible to oligo binding and others not. Third, and finally, the added exogenous snRNA may not assemble properly with other splicing components.

In the method presented here, the region of U4 targeted for degradation is the 5′ end of the molecule, the part that forms the majority of the base pairing interactions with U6 (Fig. 1). Degradation of this region most effectively eliminates U4 function [19] but is not accessible in the majority of U4 molecules, as they are base paired to U6. Consequently, to make this region accessible it is necessary to ensure that U4 is actively cycling in and out of the spliceosome. In yeast extract this requires the addition of ATP as

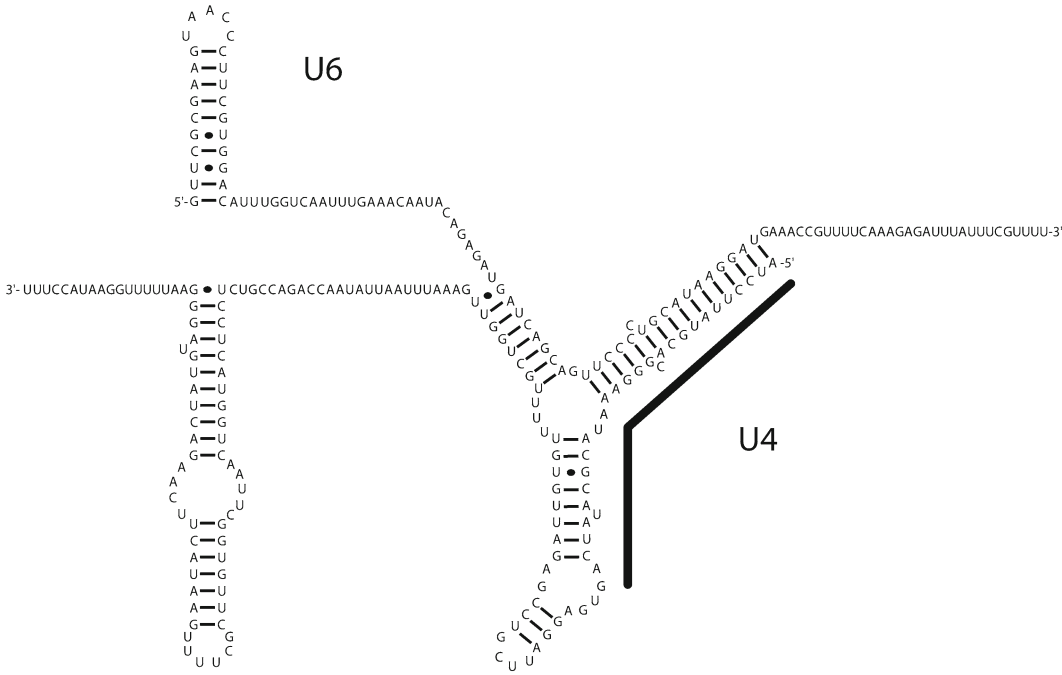

Fig. 1 Base pairing between U6 snRNA (*top*) and U4 snRNA (*bottom*). The location of the DNA oligo used in this study to degrade U4 is indicated by the *thick*, *black line*

well as an intron-containing RNA transcript. Once endogenous U4 has been sufficiently depleted, exogenous U4 is added to reconstitute splicing activity. A large excess of U4 relative to other splicing components was found to be necessary for maximal splicing, in part due to degradation of the exogenous U4 and perhaps due to its misfolding as well [5].

2 Materials

Take all possible precautions to avoid nuclease contamination: purchase nuclease-free plasticware, use ultrapure water in all buffers and solutions, and filter all solutions that will be added to reactions containing RNA through high-protein-binding nitrocellulose filters.

2.1 Prepare Splicing Extract

Make yeast splicing extract from protease-deficient strain BJ2168 [20] following the protocol in Chapter 9.

2.2 Prepare Unlabeled U4 snRNA and Actin Pre-mRNA

1. Plasmids containing the T7 promoter followed by a partial actin gene (pJPS149; [21] (*see* **Note 1**)) and the U4 gene [22].
2. Restriction enzymes HindIII and StyI, and appropriate buffers.
3. MEGAshortscript Kit for in vitro transcription (Invitrogen).
4. Vertical gel system; glass gel plates approximately 16 cm × 14 cm, 0.75 mm spacers.
5. 20× Tris–borate–EDTA (TBE) gel running buffer (1.8 M Tris base, 1.8 M boric acid, 25 mM EDTA). Autoclave.
6. 6 % (19:1), 7 M urea polyacrylamide gel.
7. Formamide.
8. Fluor-coated thin-layer chromatography plate.
9. Handheld UV light.
10. Disposable 1.5 mL microcentrifuge tube pestle (Kontes Scientific).
11. DTR gel filtration cartridge (Edge BioSystems).
12. 20 mg/mL glycogen in water.
13. 3 M NaOAc, pH 5.2.
14. 70 and 100 % EtOH.

2.3 Prepare Radioactive Actin Pre-mRNA and U4 Northern Probe

1. U4 Northern probe (14B), 200 μM stock in water: 5′AGGTATTCCAAAAATTCCCTAC3′.
2. T4 polynucleotide kinase (PNK) and 10× buffer.
3. T7 RNA polymerase and 10× buffer.
4. Superasin RNase inhibitor (Invitrogen).
5. 100 mM ATP, CTP, GTP, UTP.

6. α^{32}P-GTP and γ^{32}P-ATP, 10 mCi/mL, 3,000 Ci/mmol.
7. TE, pH 7.5 (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Filter sterilize.
8. G-25 spin columns.
9. Scintillation counter.

2.4 Deplete Endogenous U4

1. U4-targeting oligonucleotide, 200 μM stock in water: 5′CTGATATGCGTATTTCCCGTGCATAAGGAT3′.
2. 5× splicing buffer (12.5 mM $MgCl_2$, 300 mM KPO_4, pH 7.0, 15 % PEG 8000). Make fresh each time from stocks that have been autoclaved, filter sterilized, and stored at −80 °C.
3. 100 mM ATP.

2.5 Test Effectiveness of Depletion

1. Splicing stop buffer (300 mM NaOAc, pH 5.2, 1 mM EDTA, 0.1 % SDS, 34 μg/mL *E. coli* tRNA). Filter sterilize. Store at room temperature. Add the tRNA just before use.
2. Phenol/chloroform, pH 6.7 and chloroform.
3. Urea loading buffer (4.2 g urea, 500 μL 20× TBE, 20 μL 0.5 M EDTA, 2.5 mg xylene cyanol, 2.5 mg bromophenol blue, water to 10 mL). Filter sterilize. Aliquot and store at −20 °C.
4. Hybond + nylon membrane (GE Healthcare).
5. Whatman paper.
6. Semidry electroblotter (e.g., Owl).
7. UV cross-linking apparatus (e.g., Stratalinker).
8. Rapid Hyb hybridization buffer (GE Healthcare).
9. Hybridization oven.
10. 20× SSC (3 M NaCl, 0.3 M sodium citrate; pH to 7.0 with a few drops of concentrated HCl).
11. Northern wash buffer (6× SSC, 0.2 % SDS).
12. Phosphorimager, screen, and cassette.

2.6 Reconstitution with Exogenous U4

Same materials as in Subheadings 2.2–2.5.

2.7 Measure Pre-mRNA Splicing

Same materials as in Subheadings 2.2–2.5.

3 Methods

3.1 Prepare Splicing Extract

See Chapter 9 in this volume.

3.2 Prepare Unlabeled U4 snRNA and Actin Pre-mRNA

1. Linearize the U4 IVT template. Digest 10 μg of the pT7U4 plasmid by mixing the DNA with 10 μL 10× restriction buffer, 10 μL StyI, 1 μL 10 mg/mL BSA, and water to 100 μL. Incubate for 4 h at 37 °C (*see* **Notes 2** and **3**). Add 100 μL water to the digestion reaction, phenol/chloroform extract, and precipitate (*see* **Note 4**). Resuspend pellet in 12 μL water and determine the concentration by measuring the A_{260} on a spectrophotometer.
2. Linearize the actin IVT template. Digest 10 μg of the pJPS149 (actin pre-mRNA template) plasmid by mixing the DNA with 3 μL 10× restriction buffer, 1.5 μL HindIII, 3 μL 1 mg/mL BSA, and water up to 30 μL. Incubate for 1 h at 37 °C. Prepare DNA for transcription by extraction and precipitation, as above.
3. Transcribe U4 RNA and actin pre-mRNA using an in vitro transcription kit (e.g., MEGAshortscript) with the linearized DNA templates from **steps 1** and **2** (*see* **Note 5**). Use approximately 2–4 μg linearized pT7U4 and 1 μg pJPS149 per 20 μL reaction. Incubate for 3–4 h at 37 °C.
4. Make the denaturing polyacrylamide gel once the transcription reactions have been set up (*see* **Note 6**). Near the end of the transcription incubation step, pre-run the gel for 15–30 min at 400 V (*see* **Note 7**).
5. Separate full-length IVT products from any truncated RNAs. Add 20 μL formamide to each transcription reaction and heat for 3 min at 65 °C. Load samples onto the prepared gel (*see* **Note 8**). Run for 1 h at 400 V.
6. Visualize the RNA transcripts by UV shadowing (*see* **Note 9**). Move the wrapped gel onto a clean, scratchproof surface. Using a sterile scalpel, cut the marked fragment of gel away from the remainder and place in a microcentrifuge tube.
7. Elute the RNA from the gel fragment. Crush with a disposable pestle, add 400 μL water, crush some more, and then heat at 70 °C for 10 min.
8. Remove the acrylamide from the eluted RNA. While heating the gel solution, pre-spin the DTR cartridge at 850 × *g* for 3 min in a microcentrifuge. Transfer the cartridge to a new tube. Load the entire gel solution onto the column and spin again for 3 min. Discard the cartridge containing the acrylamide.
9. Precipitate the eluted RNA with 0.01 volume 3 M NaOAc, pH 5.2 plus 2.5 volumes cold 100 % EtOH, adding 15 μg glycogen as a carrier (*see* **Note 4**). Resuspend the RNA in 25–50 μL water and determine the concentration by UV spectrophotometry (*see* **Note 10**).

3.3 Prepare Radioactive Actin Pre-mRNA and U4 Northern Probe

1. In vitro transcribe radiolabeled actin. To 500 ng linearized pJPS149 (in a maximum volume of 4.5 μL) add 1 μL 10× T7 RNA polymerase buffer, 0.5 μL 10 mM each NTP (ATP, CTP, UTP), 0.5 μL 0.5 mM GTP, 0.5 μL Superasin, 2.5 μL α^{32}P-GTP, 0.5 μL T7 RNA polymerase, and water to 10 μL (*see* **Note 11**). Incubate for 1.5 h at 37 °C.
2. 5′-end radiolabel the U4 DNA oligo. To 25 pmol U4 14B oligo add 2.5 μL 10× PNK buffer, 2.5 μL γ^{32}P-ATP, 1.5 μL PNK, and water to 25 μL. Incubate for 1 h at 37 °C.
3. Remove unincorporated nucleotides from the IVT and kinasing reactions, and calculate the efficiency of ^{32}P incorporation. Pre-spin 2 G-25 spin columns according to the manufacturer's instructions. Dilute the labeling reactions to 50 μL with TE, pH 7.5. Count 1 μL in a scintillation counter. Load the remainder onto the G-25 column and spin according to instructions. Count 1 μL of the flow through in a scintillation counter. Determine the percent ^{32}P-GTP incorporation into the actin transcript and the cpm/fmol actin (*see* **Note 12**). Dilute the actin to 4 fmol/μL in TE.

3.4 Deplete Endogenous U4

1. To 4 μL of yeast splicing extract add 1.6 μL 5× splicing buffer, 0.64 μL 10 μM U4-targeting oligo, 15–80 fmol unlabeled IVT actin pre-mRNA, 0.8 μL 100 mM ATP, and water to 8 μL (*see* **Note 13**). At the same time set up a mock-depleted reaction, leaving out the U4 oligo. Incubate for 30 min at 30 °C (*see* **Note 14**).

3.5 Test Effectiveness of Depletion

1. Terminate reactions by adding 200 μL splicing stop buffer. Phenol/chloroform extract to remove the proteins from the splicing extract, and precipitate the RNA with 3 volumes of cold 100 % EtOH (*see* **Note 4**).
2. Resuspend pellets in 8 μL urea loading buffer. Heat for 3 min at 65 °C and load on a pre-run 6 %, 7 M urea denaturing polyacrylamide gel. Run in 1× TBE 400 V for 30 min.
3. Transfer RNA to a nylon membrane using a semidry electroblotter (*see* **Note 15**). Transfer in 1× TBE for 20 min at 2.5 mA/cm^2 of membrane.
4. Cross-link RNA to membrane using a UV cross-linker (approximately 120,000 μJ using a 254 nm light source for 25–50 s).
5. Pre-hybridize the cross-linked membrane in 5–10 mL Rapid hyb buffer in a roller tube in a hybridization oven at room temperature for 30 min.
6. Add 20 μL ^{32}P-labeled U4 14B probe, and hybridize the probe to the RNA at room temperature for 1–2 h.

7. Pour off the probe and wash 3 × 5 min in Northern wash buffer to remove any probe that has bound nonspecifically to the membrane (*see* **Note 16**).
8. Remove membrane from tube with forceps, wrap in plastic wrap, and expose to phosphor screen overnight.
9. Scan the phosphor screen in a phosphorimager, and determine the efficiency of U4 degradation by quantitatively comparing the intensity of full-length U4 in the depleted reaction to that in the mock-depleted reaction (Fig. 2a).

3.6 Reconstitution with Exogenous U4

1. To 8 μL of depleted splicing extract (**step 1**, Subheading 3.4) add either 0.48 μL of water (control) or 5 μM U4 IVT (283 nM final concentration). Incubate for 12 min at 23 °C to allow time for snRNP assembly.

3.7 Measure Pre-mRNA Splicing

1. Add 4 fmol internally ^{32}P-GTP-labeled actin pre-mRNA transcript and incubate for an additional 30 min at 23 °C.

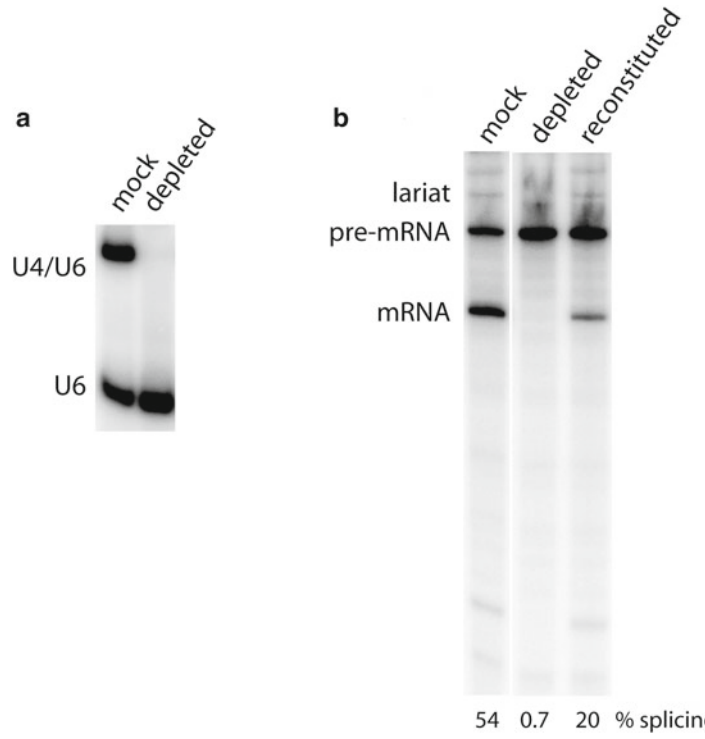

Fig. 2 Example of U4 depletion and reconstitution of splicing. (**a**) Non-denaturing northern blot of mock-depleted (*lane 1*) and U4-depleted (*lane 2*) splicing extract probed for U6. In the absence of U4, all of the U6 snRNA runs as free U6. (**b**) Denaturing autoradiogram of pre-mRNA splicing in mock-depleted (*lane 1*), U4-depleted (*lane 2*), and reconstituted (*lane 3*) extract. Positions of pre-mRNA, mRNA, and lariat intron are marked on the *left*

2. Stop the reaction with 200 μL splicing stop buffer, extract with phenol/chloroform, and precipitate with 3 volumes of cold 100 % EtOH (*see* **Note 4**).
3. Resuspend the pellets in 8 μL urea loading buffer, heat for 3 min at 65 °C, and electrophorese through a 6 % (19:1) 7 M urea denaturing polyacrylamide gel for 1 h at 400 V.
4. Remove the top plate from the gel. Press a piece of Whatman paper onto the gel, and carefully peel the gel off of the gel plate. Wrap the gel in plastic wrap and expose to a phosphor screen at −80 C for a few hours.
5. Scan the phosphor screen (Fig. 2b), and calculate the splicing efficiency (*see* **Note 17**).

4 Notes

1. We have experienced problems with the pJPS149 plasmid when purified from a glycerol stock of DH5α. Consequently, always freshly transform DH5α cells from the original plasmid stock when more plasmid is needed.
2. Use a large excess of StyI and digest for 4 h–overnight as the enzyme cuts very inefficiently.
3. Analyze 200 ng of the cut plasmid alongside 200 ng of uncut plasmid on a 0.8 % agarose gel containing 0.5 μg/mL EtBr in 1× TBE to make sure that the plasmid is completely linearized.
4. Extract one time with an equal volume of phenol/chloroform. Back extract with 1 volume of chloroform and then precipitate with 0.1 volume of 3 M NaOAc, pH 5.2 and 2 volumes of cold 100 % EtOH. Spin for 30 min at 4 °C, aspirate the supernatant, wash pellet with 70 % EtOH, spin for 5 min, and aspirate supernatant. Allow pellet to air-dry for 5 min.
5. The IVT U4 and actin RNAs are 163- and 590-nt long, respectively. The MEGAshortscript kit has been optimized for high yields of RNA in the 20–500 nt range, but we have found it to also give excellent yields of the slightly larger actin RNA. The transcription reaction can also be carried out without a kit, although the RNA product yields may be lower, especially for the small U4 RNA.
6. Mix 2.25 mL 40 % (19:1) acrylamide, 750 μL 20× TBE, 6.3 g urea, and 7.3 mL water in a 50 mL glass beaker with a stir bar. Stir until the urea has completely dissolved. Add 150 μL 10 % ammonium persulfate and 15 μL TEMED. Mix and pour into gel cassette. Push comb most of the way into the gel in order to make deep wells. Allow to solidify for at least 1 h.
7. Remove the comb from the polyacrylamide gel, and place the gel in the gel box. Fill with 1× TBE buffer. Use a syringe to rinse

out each well with running buffer, making sure that there are no air bubbles trapped in the wells or in the space under the gel.

8. Rinse the wells again before loading the samples. Load the entire sample into one well. Leave an empty well or two between the samples to prevent any spillover contamination of the transcripts.
9. Remove the glass plates from the gel, and wrap the gel in plastic wrap. Place gel on a fluorescent thin-layer chromatography plate. Using a handheld shortwave UV lamp, identify the location in the gel of the desired RNA product by looking for the shadow cast by the RNA. Mark the location of the band by drawing a box on the plastic wrap with a permanent marker.
10. Store RNA transcripts at −80 °C. Dilute some of the stock to a working concentration and aliquot into several tubes so that the stock tube is not repeatedly frozen/thawed.
11. Assemble the IVT reaction at room temperature. The spermidine in the T7 polymerase buffer can cause the DNA template to precipitate at 4 °C.
12. Calculate the molar activity of the transcript.

    Proportion of GTP incorporated into actin:

    (cpm/μL after G25 column)/(cpm/μL before column) = % ^{32}P GTP incorporated.

    Total moles of ^{32}P GTP in the IVT reaction = 8.3×10^{-9} mmol (8.3 pmol).

    Total moles of unlabeled GTP in the IVT reaction = 2.5×10^{-7} mmol (250 pmol).

    Total moles of GTP in the IVT reaction:

    8.3 pmol + 250 pmol = 2.58×10^{-7} mmol (258 pmol).

    Total moles of GTP incorporated into actin:

    Total GTP in reaction *x*% incorporated = 2.58×10^{-7} mmol GTP *x*% ^{32}P GTP incorporated.

    Moles of actin synthesized:

    Total moles of GTP incorporated/(moles of G/mole of actin) = total moles of GTP incorporated into actin/(118 moles G nucleotides per mole actin).

    Molar activity of actin transcript:

    Total cpm for IVT reaction/moles of actin (expressed in cpm/fmol).
13. Varying amounts of pre-mRNA transcript are required for complete degradation of U4, dependent on the specific splicing extract being depleted. Titrate the transcript in the depletion reaction to determine the lowest amount necessary for efficient degradation of U4 and inhibition of splicing.

14. The splicing extract contains sufficient endogenous RNase H activity to degrade the DNA oligo:RNA duplex. Therefore, it is not necessary to add any exogenous RNase H.
15. Cut a piece of nylon membrane to cover the portion of the gel containing the RNA. The U4 will be in the top half of the gel, so it is not necessary to transfer the bottom half. Label the back of the membrane with a pencil. Cut six pieces of Whatman paper slightly larger than the membrane. Remove the top plate from the gel, and press one piece of Whatman paper onto the portion of the gel for transfer. Carefully peel the Whatman paper with the gel stuck to it off of the bottom gel plate. Make a sandwich on the electroblotter platform consisting of two pieces of Whatman paper wetted in 1× TBE buffer, followed by the Whatman/gel (gel facing up; if the Whatman paper on the gel does not get completely wet, add more buffer underneath it; gently roll a pipet over the surface of the gel to remove any trapped air bubbles), and then three more wet pieces of Whatman paper on top. Carefully place electroblotter lid on top of stack, and begin transfer.
16. The probe in Rapid hyb buffer can be saved at 4 °C and used up to three times.
17. To calculate percent splicing, divide the intensity of bands corresponding to product (mRNA and lariat) by the total intensity of the starting material plus the products (pre-mRNA, lariat, and mRNA):

$$\text{Splicing efficiency} = \text{products}/\text{total} = (\text{mRNA} + \text{lariat})/(\text{pre-mRNA} + \text{lariat} + \text{mRNA}).$$

Acknowledgments

This work was supported by NSERC Discovery Grant 298521 and UNBC Office of Research awards to SDR.

References

1. Faustino NA, Cooper TA (2003) Pre-mRNA splicing and human disease. Genes Dev 17:419–437
2. Wang G-S, Cooper TA (2007) Splicing in disease: disruption of the splicing code and the decoding machinery. Nat Rev Genet 8:749–761
3. López-Bigas N, Audit B, Ouzounis C et al (2005) Are splicing mutations the most frequent cause of hereditary disease? FEBS Lett 579:1900–1903
4. Will CL, Lührmann R (2011) Spliceosome structure and function. Cold Spring Harbor Perspect Biol 3:1–23
5. Hayduk AJ, Stark MR, Rader SD (2012) In vitro reconstitution of yeast splicing with U4 snRNA reveals multiple roles for the 3′ stem-loop. RNA 18:1075–1090
6. Edery P, Marcaillou C, Sahbatou M et al (2011) Association of TALS developmental disorder with defect in minor splicing component U4atac snRNA. Science 332: 240–243
7. He H, Liyanarachchi S, Akagi K et al (2011) Mutations in U4atac snRNA, a component of the minor spliceosome, in the developmental disorder MOPD I. Science 332:238–240

8. McPheeters DS, Fabrizio P, Abelson J (1989) In vitro reconstitution of functional yeast U2 snRNPs. Genes Dev 3:2124–2136
9. Fabrizio P, McPheeters DS, Abelson J (1989) In vitro assembly of yeast U6 snRNP: a functional assay. Genes Dev 3:2137–2150
10. O'Keefe RT, Norman C, Newman AJ (1996) The invariant U5 snRNA loop 1 sequence is dispensable for the first catalytic step of pre-mRNA splicing in yeast. Cell 86:679–689
11. Pan ZQ, Ge H, Fu XY et al (1989) Oligonucleotide-targeted degradation of U1 and U2 snRNAs reveals differential interactions of simian virus 40 pre-mRNAs with snRNPs. Nucleic Acids Res 17:6553–6568
12. Pan ZQ, Prives C (1988) Assembly of functional U1 and U2 human-amphibian hybrid snRNPs in Xenopus laevis oocytes. Science 241:1328–1331
13. Hamm J, Dathan NA, Mattaj IW (1989) Functional analysis of mutant Xenopus U2 snRNAs. Cell 59:159–169
14. Hamm J, Dathan NA, Scherly D et al (1990) Multiple domains of U1 snRNA, including U1 specific protein binding sites, are required for splicing. EMBO J 9:1237–1244
15. Wersig C, Bindereif A (1992) Reconstitution of functional mammalian U4 small nuclear ribonucleoprotein: Sm protein binding is not essential for splicing in vitro. Mol Cell Biol 12:1460–1468
16. Wolff T, Bindereif A (1992) Reconstituted mammalian U4/U6 snRNP complements splicing: a mutational analysis. EMBO J 11:345–359
17. Ségault V, Will CL, Sproat BS, Lührmann R (1995) In vitro reconstitution of mammalian U2 and U5 snRNPs active in splicing: Sm proteins are functionally interchangeable and are essential for the formation of functional U2 and U5 snRNPs. EMBO J 14:4010–4021
18. Will CL, Rümpler S, Klein Gunnewiek J et al (1996) In vitro reconstitution of mammalian U1 snRNPs active in splicing: the U1-C protein enhances the formation of early (E) spliceosomal complexes. Nucleic Acids Res 24: 4614–4623
19. Black DL, Steitz JA (1986) Pre-mRNA splicing in vitro requires intact U4/U6 small nuclear ribonucleoprotein. Cell 46:697–704
20. Ansari A, Schwer B (1995) SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing. EMBO J 14:4001–4009
21. Vijayraghavan U, Parker R, Tamm J et al (1986) Mutations in conserved intron sequences affect multiple steps in the yeast splicing pathway, particularly assembly of the spliceosome. EMBO J 5:1683–1695
22. Ghetti A, Company M, Abelson J (1995) Specificity of Prp24 binding to RNA: a role for Prp24 in the dynamic interaction of U4 and U6 snRNAs. RNA 1:132–145