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

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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.

Keywords

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 regulation 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 CO₂ 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 mM 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).
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 mM Tris-HCl, pH 8.0; 10 mM ethylenediamine tetraacetic acid.
2. 1X Laemlli loading buffer: 62.5 mM 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 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- μ L reaction containing 1.3 μ L of RT-PCR product, 0.3 μ L of salt solution (included in the TOPO cloning kit), and 0.3 μ L 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.

¹It 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.

1. In 0.2-mL tubes, assemble a 100- μ L reaction containing 5 μ L of DNA template (1 ng/ μ L), 2 μ L of 10 mM dNTPs, 8 μ L of 2 μ M SP6 primer, 8 μ L of 2 μ M T7 primer, 10 μ L of 10X PCR buffer without MgCl₂, 3 μ L of 50 mM MgCl₂, 0.5 μ L of *Taq* DNA polymerase (1 U/ μ L), and 63.5 μ L of H₂O.
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 μ L 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 μ L of H₂O 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- μ L SP6 transcription reaction containing 10 μ L of SP6 transcription 5X buffer, 10 μ L of rNTPs at 25mM each (adenosine triphosphate [ATP], cytidine triphosphate [CTP], guanosine triphosphate [GTP], uridine triphosphate [UTP]), 5–10 μ g of PCR product, and 5 μ L of SP6 enzyme mix.
2. Set up a separate 50- μ L T7 transcription reaction containing 10 μ L of T7 transcription 5X buffer, 15 μ L of rNTPs at 25 mM each (ATP, CTP, GTP, UTP), 5–10 μ g of PCR product, and 5 μ L of T7 enzyme mix.
3. Incubate both reactions at 37°C for 4 h.
4. Add 5 μ L 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 μ L of H₂O.

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- μ L reaction containing 20 μ g of top-strand RNA, 20 μ g of bottom-strand RNA, and 5 μ L 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 measured 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 μ L of chloroform to each tube and briefly vortex.
5. Incubate the tubes on ice for 5 min.
6. Centrifuge the samples at 12,000g for 15 min at 4°C.
7. Remove the aqueous layer to a new tube and add 500 μ L of isopropanol to precipitate the RNA.
8. Centrifuge the samples at 12,000g for 15 min at 4°C.
9. Remove the supernatant, briefly dry the pellet on the bench, and then dissolve the pellet in 50 μ L of H₂O.
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.

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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.

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