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Exon-Specific RNA Interference A Tool to Determine the Functional Relevance of Proteins Encoded by Alternatively Spliced mRNAs

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1. Introduction

The majority of metazoan genes encode pre-mRNAs that are subject to alternative splicing. For example, it has recently been estimated that as many as 74% of human genes encode alternatively spliced mRNAs (1). An alternatively spliced gene can generate anywhere from 2 different isoforms to as many as 38,016 isoforms in the case of the *Drosophila Dscam* gene (2). Thus, alternative splicing serves to greatly expand the diversity of the proteins encoded by a genome (3).

In many cases, it has been shown that different protein isoforms synthesized from a single gene have distinct functions (3). In our efforts to determine the functional significance of alternative splicing, we developed a variant of RNA interference (RNAi) we call exon-specific RNAi (4). This technique involves the selective design of a double-stranded RNA (dsRNA) trigger that is complementary to a specific alternative exon (*see* Fig. 1). We have shown that in cultured *Drosophila* cells, these exon-specific dsRNA triggers specifically induce the degradation of mRNA isoforms containing the targeted exon, but they do not affect the stability of mRNA isoforms synthesized from the same gene that lack the targeted exon. Thus, it should be possible to use this technique to determine the function of proteins encoded by alternatively spliced mRNAs.

It is important to note that exon-specific RNAi is unlikely to work in organisms that contain RNA-dependent RNA polymerases (RdRPs), such as worms and plants (5). This is because these enzymes are responsible for a phenomenon referred to as transitive RNAi (6). RdRPs can use the siRNA triggers to catalyze the synthesis of dsRNA complementary to the mRNA upstream of the targeted sequence. This, in turn, induces the degradation of mRNAs complementary to the secondary siRNAs. Thus, if the dsRNA trigger is homologous to an alternative exon, the RdRPs will synthesize secondary siRNAs complementary to the upstream constitutive exon and, consequently, all mRNA isoforms from the targeted gene will be degraded (7). However, exon-specific RNAi can be used in organisms such as *Drosophila*, mouse, and humans, which lack RdRPs (5). In this chapter, we discuss the methods and approaches to perform exon-specific RNAi with a specific emphasis on using this technique in *Drosophila* cells.

2. Materials

2.1. Equipment

The equipment required for these experiments are common to most labs routinely performing molecular biology experiments.

1. Electrophoresis supplies: Power supplies and standard agarose gel electrophoresis units will be needed.
2. Thermal cycler: This is required for polymerase chain reaction (PCR), but can also be used for other reaction incubations if needed.
3. 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.
4. 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 cloning kit (Invitrogen, cat. no. K4600-01).
6. Transcription reagents: Ampliscribe High Yield Transcription Kits (Epicentre, SP6 kit, cat. no. AS3106, T7 kit, cat. no. AS3107).
7. Electrophoresis chemicals: agarose, ethidium bromide, and so forth.
8. Reverse transcription–polymerase chain reaction (RT-PCR) reagents: standard reagents (i.e., dNTPs, reverse transcriptase, *Taq* DNA polymerase, reaction buffers, etc.).
9. RNA isolation reagents: Trizol reagent (Invitrogen).

2.3. Solutions

1. 10X Annealing buffer: 1 M NaCl, 200 mM Tris-HCl, pH 8.0, 10 mM ethylenediamine tetraacetic acid.
2. Phenol: chloroform : isoamyl alcohol (25 : 24 : 1, v : v : v).
3. Ethanol.

3. Methods

The methods described in this section discuss (1) designing the exon-specific dsRNA trigger, (2) cloning the transcription template for the exon-specific dsRNA trigger, (3) synthesis of the exon-specific dsRNA trigger, (4) application of the exon-specific dsRNA trigger, and (5) testing the efficiency and specificity of the exon-specific dsRNA trigger.

3.1. Design of the Exon-Specific dsRNA Trigger

When performing RNAi in *Drosophila* cells, it is preferable to use long dsRNAs, as you can add these directly to the culture medium. The dsRNAs enter the cells and are processed into siRNAs by Dicer. We, therefore, typically use exon-specific dsRNA triggers that encompass

the entire alternative exon (*see* Note 1). As we will describe later, it is important to ensure that the exon-specific dsRNA does not share enough homology with other regions of the same gene, let alone other genes, such that a subset of the siRNAs liberated from the dsRNA by Dicer induce the degradation of untargeted mRNA iso-forms. This length is typically about 18 nt (nucleotides).

3.2. dsRNA Trigger Transcription Template

Once a target sequence has been selected, a transcription template must be synthesized. We typically do this by designing oligonucleotide primers to be used for RT-PCR that encompass the targeted sequence. We then perform RT-PCR on total cellular RNA and clone the PCR product into a vector containing bacteriophage RNA polymerase promoters flanking the cloning site. The transcription template is then generated by PCR using oligonucleotide primers complementary to the RNA polymerase promoters.

3.2.1. Reverse Transcription of Total Cellular RNA—We typically isolate total cellular RNA to be used as a template for RT-PCR from the source that exon-specific RNAi will be performed on (i.e., cells, animals, etc.). The reverse transcription reaction is carried out as follows:

1. Assemble a 20- μ L reaction containing 1 μ L of RNase inhibitor (Invitrogen), 1 μ L of a 265 ng/ μ L stock of Random Hexamers, 5 μ g of total *Drosophila* cellular RNA, 4 μ L of First Strand Buffer (provided with Superscript II), 2 μ L of 0.1 M dithiothreitol (DTT), 1 μ L of 10 mM dNTPs, and 1 μ L of Superscript II (Invitrogen).
2. Incubate the reaction at 42°C for 1 h.
3. At this point, the reaction can be stored at –20°C indefinitely.

3.2.2. Polymerase Chain Reaction to Amplify the DNA Encoding the dsRNA Trigger—Next, the cDNA synthesized by reverse transcription is used as a template for PCR. The PCR is carried out as follows:

1. Assemble a 50- μ L reaction containing 3 μ L cDNA from **Subheading 3.2.1.**, 1 μ L of 10 mM dNTPs, 4 μ L of a 2- μ M stock of the primer complementary to the 5' end of the dsRNA target sequence, 4 μ L of a 2- μ M stock of the primer complementary to the 3' end of the dsRNA target sequence, 5 μ L of 10X PCR buffer without MgCl₂, 1.5 μ L of 50 mM MgCl₂, 0.25 μ L of *Taq* DNA polymerase (1 unit/ μ L), and 31.25 μ L H₂O.
2. The reactions are 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. The annealing temperature and extension time should be optimized for each DNA fragment being amplified.
3. Run an aliquot of each reaction on an agarose gel to verify that a PCR product of the correct size was produced.

3.2.3. Cloning of the PCR Product—Next, the PCR product obtained in **Subheading 3.2.2.** should be cloned into the pCRII-TOPO (Invitrogen) vector.

1. Assemble a 1.9- μ L reaction containing 1.3 μ L of the PCR product, 0.3 μ L of salt solution (included in the TOPO cloning kit), and 0.3 μ L of pCRII-TOPO vector.
2. Incubate the reaction at room temperature for 5–30 min.

¹We have successfully used dsRNAs as large as 1500 bp and as small as 79 bp.

3. Use the entire reaction to transform *Escherichia coli* using standard procedures and plate onto Luria–Bertani (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. The cDNA will next be used to generate transcription templates.

3.2.4. Generating the Transcription Template—Once clones containing the target sequence have been obtained, you will need to generate the actual transcription template. To do this, PCR is performed using T7 and SP6 primers, which anneal the plasmid on either side of the cloning site. The resulting PCR product will contain the dsRNA trigger sequence flanked by RNA polymerase promoters (*see* Note 2).

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 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. Run a portion of the reaction on an agarose gel to verify that a product was generated.
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 of ethanol.
5. Resuspend the pellet in 10 μ L of H₂O and quantify by spectrophotometry.

3.3. dsRNA Synthesis

The next step is to synthesize RNA from the transcription template. This is done by transcribing the two RNA strands and then annealing them together.

3.3.1. RNA Synthesis—Separate transcription reactions are required to synthesize the top and bottom strands of RNA because the transcription template is a PCR product containing a T7 promoter on one end and an SP6 promoter on the other. A variety of methods can be used for large-scale synthesis, but we typically use the Ampliscribe High Yield transcription kits from Epicentre.

1. Set up a 20- μ L reaction T7 transcription reaction containing 1 μ g of the transcription template, 2 μ L of 10X AmpliScribe T7 reaction buffer, 1.5 μ L of 100 mM ATP, 1.5 μ L of 100 mM CTP, 1.5 μ L of 100 mM GTP, 1.5 μ L of 100 mM UTP, 2 μ L of 100 mM DTT, and 2 μ L of the Ampliscribe T7 Enzyme Solution.
2. Set up a separate 200- μ L SP6 transcription reaction containing 1 μ g of the transcription template, 2 μ L of 10X AmpliScribe SP6 reaction buffer, 1 μ L of 100 mM ATP, 1 μ L of 100 mM CTP, 1 μ L of 100 mM GTP, 1 μ L of 100 mM UTP, 2 μ L of 100 mM DTT, and 2 μ L of the AmpliScribe SP6 Enzyme Solution.
3. Incubate both reactions at 37°C for 2 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.

²Alternatively, primers specific to the target sequence can be designed that each contain a T7 promoter such that the PCR product can be bidirectionally transcribed with T7 RNA polymerase.

5. Extract the reactions with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and precipitate the RNA by adding 1/10 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of ethanol.
6. Resuspend the pellet in 50 µL of H₂O.

3.3.2. RNA Annealing—After the two RNA strands are synthesized, they are annealed together to produce dsRNA. The annealing reaction is carried out as follows.

1. Assemble a 50-µL reaction containing 20 µg of each RNA strand and 5 µL of 10X annealing buffer.
2. Heat the samples to 78°C for 10 min, followed by incubation at 37°C for 30 min (*see* Note 3).

3.4. dsRNA Application

Once you have made the dsRNA, you can use it to perform RNAi on the cultured cells. We typically use the *Drosophila* Dmel-2 cell line (Invitrogen), which is a variety of S2 cells that have been optimized for growth in serum-free medium. Serum interferes with dsRNA uptake. Thus, using Dmel-2 cells significantly streamlines the RNAi process (*see* Note 4).

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 3 d (*see* Note 5).

3.5. Determining the Efficiency and Specificity of the dsRNA Trigger

The exact method that is used to determine the efficiency and specificity of exon-specific RNAi will depend on the reagents available to you. For example, if antibodies exist that are specific to each isoform produced from the target gene, Western blots of the cell lysate could be performed. However, it is unlikely that such reagents will be available. Thus, the most common method will be to analyze the abundance of the different mRNA isoforms from the target gene. We typically check this by extracting total cellular RNA from the cells, perform RT-PCR to amplify the different mRNA isoforms, and examine the abundance of each by electrophoretic methods.

3.5.1. RNA Isolation—We typically isolate RNA 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.

³You can check for the efficiency of RNA annealing by running an aliquot of both single strands and the dsRNA on an agarose gel. The dsRNA should migrate more slowly than each single strand.

⁴Detailed protocols performing RNAi on cell lines that require serum for growth are available in **ref. 14** or the Dixon lab website (http://cmm.ucsd.edu/Lab_Pages/Dixon/).

⁵There are at least three variables that determine the efficiency of RNAi: the length of time the cells are exposed to the dsRNA, the amount of dsRNA added to the cells, and the frequency of dsRNA addition. Each of these values must be determined empirically, but we have found that a single treatment of 20 µg of dsRNA for 3 d is sufficient for most targets. However, in some cases, we have needed to treat cells every day for 6 d to efficiently deplete the target protein.

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 µL 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 µL of H₂O.
10. Quantify the RNA by spectrophotometry and analyze by RT-PCR using protocols optimized for your target gene. If the RNA is not to be used immediately, it should be stored at -80°C.

Figure 2 shows an example of the efficiency and specificity of exon-specific RNAi. In this case, we have attempted to degrade different isoforms of the *Drosophila Dscam* mRNA. The portion of the *Dscam* gene we targeted, the exon 4 cluster, contains 12 alternative exons that are included in the mRNA in a mutually exclusive manner (see Fig. 2A). We treated Dmel2 cells with each of 12 dsRNAs containing the entire sequence of each exon 4 variant. In nearly every case, the abundance of the mRNA containing the targeted exon was reduced while the abundance of the other 11 isoforms remained unchanged. One exception is seen with the exon 4.1 and 4.8 dsRNA triggers, which each induced the degradation of transcripts containing both exons (see Fig. 2B, lanes 1 and 8). For example, the exon 4.1 dsRNA degrades both exon 4.1 and 4.8 containing transcripts as does the exon 4.8 dsRNA trigger (the exon 4.8 band is difficult to observe because of its normal low abundance). A similar phenomenon is observed for the exon 4.6 and 4.7 dsRNAs (lanes 6 and 7). Finally, the exon 4.11 dsRNA trigger does not efficiently induce degradation of the exon 4.11 containing mRNAs (lane 11). However, all of the other dsRNA triggers efficiently and specifically induce degradation of the targeted mRNA isoforms.

The basis of and solution to the crossreactivity of some of the dsRNA triggers is best seen with the exon 4.1 and 4.8 dsRNAs. Figure 3A depicts a nucleotide sequence alignment of these two exons. These two exons share a region of 23 consecutive identical nucleotides. Thus, a subset of the siRNAs liberated from each of these dsRNAs would be complementary to both exon 4.1 and 4.8 containing mRNAs. To circumvent this problem, we synthesized smaller dsRNA triggers containing only the first 79 nucleotides of each exon. As shown in Fig. 3B, each of these shorter dsRNAs specifically induced degradation of the targeted mRNA without affecting the stability of the nontargeted mRNA. These results stress the importance of ensuring that the sequence of the dsRNA trigger is specific to the targeted mRNA isoforms. In addition, these results show that even for highly similar exons, it is possible to design specific dsRNA triggers.

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Fig. 1. Overview of exon-specific RNAi

The hypothetical gene shown contains four exons, two of which—B and C—are alternatively spliced in a mutually exclusive manner. dsRNAs corresponding to a common exon, such as exon A, would induce the degradation of all mRNA isoforms synthesized from this gene. In contrast, dsRNAs corresponding to the alternative exons would specifically induce the degradation of mRNAs containing the targeted exon without affecting the stability of mRNAs lacking the targeted exon.

Fig. 2. Example of exon-specific RNAi in action

(**A**) Diagram of the exon 4 region of the *Drosophila Dscam* gene. This gene contains 12 variants of exon 4, which are alternatively spliced in a mutually exclusive manner. (**B**) An SSCP gel demonstrating the efficiency and specificity of exon-specific RNAi. *Drosophila* cells were treated with dsRNA to each of the 12 exon 4 variants for 3 d. Total cellular RNA was isolated and the abundance of each *Dscam* isoform analyzed by RT-PCR and SSCP gel electrophoresis.

Fig. 3. Circumventing crossreacting dsRNA triggers

(A) An alignment of the nucleotide sequences of exons 4.1 and 4.8. The region likely responsible for the cross-reaction is shaded in gray. (B) Smaller dsRNA triggers result in isoform-specific mRNA degradation. Small dsRNAs corresponding to the first 79 nt of exon 4.1 and 4.8 were synthesized and used to treat *Drosophila* cells. The abundance of each *Dscam* isoform was analyzed as described in Fig. 2 and the results for exon 4.1 and 4.8 are shown graphically.