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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 *Drosophila melanogaster Dscam* gene, which contains 12 mutually exclusive exons. The ease and the quantitative nature of this technique should be very useful.

Keywords

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. However, 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 sharktooth 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 phosphorimager. 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 -[³²P]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 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol.
3. Elution buffer: 300 mM sodium acetate, pH 6.1, 0.2% sodium dodecyl sulfate, 1 mM 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 [³²P] 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 H₂O to volume), 4 μL First Strand Buffer (provided with Superscript II), 2 μL dithiothreitol (0.1 M), 1 μL dNTPs (10 mM), 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 μM oligonucleotide, 4 μL 5X forward reaction buffer, 2 μL γ-[³²P]ATP (3000 Ci/mmol; 10 mCi/mL), 12 μL H₂O, 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 mM Tris-HCl, pH 8.0; 50 mM NaCl; 0.5 mM 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.

¹For 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.

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 50g 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 675g for 2 min to remove any residual buffer.
7. Once the kinase reaction is complete, add 30 μ L 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 675g 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- μ L reaction containing 3 μ L cDNA from **Subheading 3.2.** or a 25 ng/ μ L solution of a cDNA clone from **Subheading 3.6.**, 1 μ L dNTPs (10 mM), 1 μ L of 5'-end-labeled primer from **Subheading 3.3.**, 4 μ L 5' primer (2 μ M), 4 μ L 3' primer (2 μ M), 5 μ L 10X PCR buffer without MgCl_2 , 1.5 μ L MgCl_2 (50 mM), 0.25 μ L *Taq* DNA polymerase (1 U/ μ L), and 30.25 μ L 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 μ L of 10% ammonium persulfate, and 20 μ L 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 sharktooth 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 μ L of SSCP stop solution with 2 μ L 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 μ L of each sample into the wells created by the sharktooth 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 μ L 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 μ L of 1 mg/mL glycogen as a carrier. Wash the pellet with 75% ethanol and resuspend the pellet in 10 μ L of H₂O.

²If 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 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.

³It 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.

6. Amplify the DNA fragments isolated from the gel by PCR using 1 μ L 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.

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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.