Use of terminal transferase-dependent antisense RNA amplification to determine the transcription start site of the *Snrpn* gene in individual neurons

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ABSTRACT

We describe here a very sensitive technique for RNA structure analysis and the determination of transcription start sites and demonstrate its use for mapping the start site of the imprinted *Snrpn* gene in individual hippocampal neurons. The method is adapted from reverse transcription-terminal transferase-dependent PCR (RT-TDPCR) to include amplification of the antisense sequence by *in vitro* transcription just prior to the final PCR step. The method should be useful for analysis of all genes for which variation in promoter usage and/or differences in RNA secondary structure may be specific to a given cell type or developmental stage.

INTRODUCTION

Variation in promoter usage plays a wide role in gene expression of both prokaryotes and eukaryotes (reviewed in 1) and recent data suggests that it may be involved in the mechanism of X chromosome inactivation (2) and imprinting (3,4). For genes that are expressed or imprinted at different stages in development or in different cell types, determination of transcription start sites may require methods sensitive enough for detection in one or a few cells. Standard methods based on primer extension of a radioactively labeled primer may require up to 100 µg of RNA (5–7). Amplification-based methods described thus far (8,9), including the use of template switching (10), require less starting material (~1 µg), but are not sensitive enough for analysis of specific cells during development. We describe here a method that provides a strong single cell signal for the transcription start site of the *Snrpn* gene, which is highly expressed in neurons. The method should be generally applicable to analysis of any gene from one or a small number of cells. Furthermore, combined with quantitative assays for allele specificity (see for example 11), the method can be used to correlate variation in transcription start site and/or RNA structure with imprinting or allelic exclusion.

The method combines reverse transcription-terminal deoxynucleotidyl transferase-dependent PCR (RT-TDPCR) (12) with *in vitro* transcription prior to the final amplification by

PCR. A previously described technique utilizing *in vitro* transcription, termed antisense RNA (aRNA) amplification (13), was based on the original procedure of Gubler and Hoffman (14). We have found the procedure to be considerably less sensitive than the RT-TDPCR-based method described here in which terminal deoxynucleotidyl transferase (TdT) is used to create cohesive termini for ligation of a universal linker (15,16). Combining the *in vitro* transcription step of the aRNA amplification method with RT-TDPCR, we developed the technique outlined in Figure 2, which we term TdT-dependent antisense RNA (TD.aRNA) amplification.

We demonstrate here use of the method to determine the transcription start site of the imprinted *Snrpn* gene in individual neurons of the mouse hippocampus. The *Snrpn* gene codes for small nuclear ribonucleoprotein polypeptide N (SmN), a 29 kDa spliceosomal protein that is expressed predominantly in brain and heart. In human, the 5'-region of the gene maps to the smallest deletion associated with both Prader-Willi syndrome and loss of imprinting of neighboring genes, suggesting that it may serve as an imprinting center (17,18). The transcription start site we report here is within a few nucleotides of the site(s) we and others have observed using primer extension and 5'-RACE experiments (19) and helps provide a basis for future analysis of DNA-protein interactions in the promoter region.

MATERIALS AND METHODS

Isolation of RNA

Individual neurons were isolated by micromanipulation from dissociated hippocampi of mouse F₁ hybrids (*Mus musculus* × *Mus musculus castaneus*) as previously described (20). Samples were stored in 50–100 µl RNazol (Tel-Test Inc., Friendswood, TX) at –70°C and RNA was extracted immediately prior to use (21).

Primers and linkers

Snrpn-specific primers were as follows (Fig. 1): primer 1, ttaatacgactactataggGCGTTGCAAATCACTCCTCAGA (lower case letters correspond to the T7 promoter sequence, upper case letters to the complement of the sequence shown in Fig. 1); primer 2, ATCACTCCTCAGAACCAAGCGT. The

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-40 gcacgctag ggagccgc gacaaacctg agccattg
 +1 gcaagactag cgcagagagg agagggagcc ggagatgcca
 +41 gacgcttggt tctgaggagt gatttgcaac gcaatggagc
 +81 gaggaaggtc agctgggctt gtggattcta gtagtgaag
 +121 tgcacccat ttgacaaaa cattctagat ttgggcttat

primers 1 and 2 are indicated by arrows. The vertical arrow shows the start site of transcription determined here; the 3'-terminus of the first exon corresponds to position +87 in the figure (19,28). The sequence shown is taken from Blaydes *et al.* (29) and our unpublished data.

Figure 1. The *Snrpn* promoter region. Primers 1 and 2 are complementary to the underlined sequences. The vertical arrow shows the start site of transcription determined here; the 3'-terminus of the first exon corresponds to position +87 in the figure (19,28). The sequence shown is taken from Blaydes *et al.* (29) and our unpublished data.

universal linker containing a 3'-overhang of three cytosines in the upper strand and a 5'-phosphorylated lower strand with an amino-blocked 3'-terminus has been described previously (16). Primer 3 is the upper strand of the universal linker lacking two of the cytosines at the 3'-terminus.

TD.aRNA amplification

All reactions were performed on ice unless otherwise stated. Total RNA isolated from single cells was reverse transcribed by use of Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco BRL). RNA samples incubated with M-MLV RT were initially denatured at 85°C for 5 min in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂ and 0.6 U/μl RNasin (Promega) (buffer 1) also containing 2.5 mM each dNTP and 1 μM primer 1 in a final volume of 10 μl. Following gradual cooling to 42°C, an additional 10 μl of buffer 1 containing 50 U M-MLV RT was added to each sample, which was then incubated at 42°C for 45 min and heat inactivated at 99°C for 5 min.

Ethanol precipitation, the tailing reaction using TdT and ligation of the linker were all performed as previously described (16). Following an additional ethanol precipitation step, samples were resuspended in 10 μl of 1 mM Tris, pH 8.0, 0.1 mM EDTA (0.1× TE buffer).

PCR was carried out with primers 1 and 3 for three cycles using the buffer and temperatures previously described for LM-PCR (final volume 50 μl) (22). Reactions were carried out for 45 s at 95°C (3 min for the first cycle), 2 min at 63°C (3 min for the first cycle) and 3 min at 74°C (10 min at 74°C for the last elongation step). Samples were then phenol extracted, ethanol precipitated and resuspended in 20 μl of 0.1× TE buffer. Drop dialysis was carried out for 1 h as previously described (13) against 60 ml of DEPC-treated water.

A 10 μl aliquot of each sample was then incubated for 2–3 h at 37°C in 40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 2 mM rNTPs, 10 mM NaCl, 1 U/μl RNasin and 1 U/μl T7 RNA polymerase (New England BioLabs), in a final volume of 50 μl. Following *in vitro* transcription, the samples were phenol extracted, ethanol precipitated and allowed to air dry. Samples were then reverse transcribed as described above except that in place of primer 1, primer 3 was used.

Half-nested PCR was performed as described above with primers 2 and 3. Samples (undiluted or diluted 1:10 in 0.1× TE

Figure 2. Outline of the TD.aRNA amplification method. Dashed line, RNA; solid line, DNA. The primers used and other details are described in the text. The thick black arrows show the direction of reverse transcription. Rectangles show the location of the universal linker sequence and primer 1; the clear portion of the rectangle representing primer 1 shows the location of the T7 promoter sequence.

buffer) were amplified using 23 or 26 cycles of 45 s at 95°C (3 min for the first cycle), 2 min at 54°C (3 min for the first cycle) and 3 min at 74°C, followed by a final elongation step at 74°C for 7 min. Following thermal cycling, samples were directly labeled by primer extension of ³²P-labeled primer 2 as described (23). Incubation with AmpliTaq polymerase was carried out for six cycles of 45 s at 95°C (2 min 45 s for the first cycle), 2 min at 54°C and 3 min at 74°C (8 min for the last elongation step). An equal volume of formamide loading dye was then added to 5 μl of each labeled sample and, following heat denaturation, the sample was analyzed by denaturing polyacrylamide gel electrophoresis (24) followed by use of a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

Figure 2 outlines the TD.aRNA amplification method. Total RNA is reverse transcribed with a primer containing the T7 promoter sequence at the 5'-end. TdT is then used to apply an average of three riboguanines to the 3'-end of the cDNA. A

Figure 3. *Snrpn* transcription start site detected in single neurons (see text for details). The strongest signals were obtained when samples were diluted 1:10 prior to the final PCR step (see lane 10). The distance from the 5'-end of primer 2 to the transcription start site (Fig. 1) was calculated by subtraction of the length of the universal linker (27 bp) from the band labeled TS (92 bp).

double-stranded universal linker is then added, with the 3'-terminus of the upper strand complementary to the three riboguanines. Following ligation of the lower strand of the linker to the terminal riboguanine, the cDNA is made double-stranded by three cycles of PCR, allowing it to serve subsequently as a template for *in vitro* transcription. The resulting antisense RNA is then subjected to RT-PCR, with a nested 3' primer in the PCR step (primer 2).

Results obtained by varying the conditions of the assay are shown in Figure 3. Lanes 1–8 and 10 show results obtained using single hippocampal neurons (the lanes marked M show size markers; lane 9 is a no RNA control). The experimental conditions for each sample are indicated below each lane. Conditions varied include the number of PCR cycles (lanes 1–4 versus lanes 5–8), the addition of a drop dialysis step prior to *in vitro* transcription (lanes 1, 2, 5 and 6 versus lanes 3, 4, 7 and 8) and the dilution of the sample prior to PCR amplification (lane 5 versus lane 10). Our attempts to use RT-TDPCR alone for single cell analysis failed (results not shown). As lane 10 demonstrates, a very strong signal is seen from single cells using 26 cycles of PCR, drop dialysis and the dilution step. The stuttering around the transcription start site is an expected result of the variation in the number of guanines added by TdT. The site labeled TS in Figure 3 is within 2 bp of the start site we have observed by 5'-RACE experiments and is the same site we observed when ThermoScript reverse transcriptase was used in place of M-MLV RT (results not shown). The importance of the dilution step is not understood, although it is probably diluting an inhibitor of the PCR reaction. Other experiments (unpublished data) suggest that the inhibitor is not dependent on the template concentration and is therefore likely to be a constant component of the reaction mix rather than the template itself.

Use of the protocol outlined here has allowed us to localize the major 5'-end of exon 1 of the *Snrpn* gene starting with total RNA from a single neuron. Interestingly, our unpublished data indicate that at least one upstream alternative first exon also exists, as has been found for human (4,25). The method we describe should be applicable to any gene, although the number of cycles at each of the two PCR steps may have to be increased for single cell analysis of less abundant RNAs.

Use of this technique should greatly enhance the sensitivity of primer extension-based procedures for the study of RNA structure and function. With some modification it may also be applicable to the creation of cDNA libraries from single cells, as the products of aRNA amplification are known to retain the size and complexity of the starting material (26,27).

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