Benchmarks

Using the SMART[™] cDNA System to Map the Transcription Initiation Site

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Primer extension is a common method used to determine the transcription initiation site of a gene by determining the 5' end of the mRNA. A frequently encountered problem of this assay is that the reverse transcriptase pauses or terminates at various sites before reaching the 5' end of the mRNA, resulting in multiple extension products. Under these circumstances, the real transcription start site cannot be easily determined. This premature termination of the reverse transcriptase could be from various reasons such as the secondary structure of the mRNA. Because of this, primer extension assays require a primer quite close to the 5' end of the mRNA (preferably within 100 bases). In addition, when the gene of interest is expressed at low levels, a large amount of total RNA or even mRNA is needed

because of the limited sensitivity of this assay. These difficulties often make a simple primer extension assay into one of the most tedious and difficult assays to perform in the laboratory.

During our attempt to map the transcription initiation site of the zebrafish vascular endothelial growth factor gene (*vegf*), we encountered many of these difficulties. The zebrafish *vegf* mRNA is present at a very low level in adult total RNA, and there is a very long and extremely AT rich 5' untranslated region. We designed a few primers from the estimated 5' end of the mRNA (from Northern analysis data) and used them in primer extension assays. No extension products were obtained when avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) was used (data not shown). When SUPERSCRIPT II[™] reverse transcriptase (Life Technologies, Rockville, MD, USA) was used, multiple extension products appeared (data not shown). To solve this problem, we tried a PCR method based on the template-switching effect of the reverse transcriptase (the SMART[™] cDNA synthesis-coupled PCR method) to determine the

5' end of the mRNA (Clontech Laboratories, Palo Alto, CA, USA) (Figure 1) (1–3). The result was then confirmed by a primer extension assay using a primer located at about 70 bases downstream of the determined 5' end of the mRNA.

A gene-specific primer, GS1 (4 pmol; 5'-AAACTAAAAACACACACAAA-3'), was used to initiate the first-strand cDNA synthesis for 1 h at 42°C using 4 µg adult total RNA or 24-h embryonic total RNA as the template and SUPERSCRIPT II reverse transcriptase. When the reverse transcriptase reaches the 5' end of the mRNA, its terminal transferase activity added a few additional dCs to the 3' end of the first-strand cDNA (2,3). The SMART II oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'), which is included in the same reaction then paired with the tail Cs and caused a template switching of the reverse transcriptase. The reverse transcriptase then extends to the end of the SMART II oligonucleotide (Figure 1). Next, RNase H (Life Technologies) was used to digest the RNA in the DNA-RNA hybrid. Only the full-length, first-strand cDNA molecules containing the ex-

Figure 1. The outline of the strategy of the SMART cDNA PCR amplification method used in this work. GS1–4 represent gene-specific primers 1–4.

Figure 2. Agarose gel of the *vegf*-specific PCR products from the secondary reactions described in Figure 1. Lanes 1 and 2, molecular weight markers; lanes 3 and 4, secondary PCR products from GS3 and SMART PCR primers from 4 µg total RNA isolated from adult zebrafish or 24-h post-fertilization embryos; lanes 5 and 6, Southern analysis of DNA from lanes 3 and 4 using labeled GS4 as probe.

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single clean extended product, while SUPERSCRIPT II reverse transcriptase generated two extended products. The dominant band from SUPERSCRIPT II reverse transcriptase is identical to the ThermoScript reverse transcriptase band (Figure 4A, lanes 1 and 2). When

the ThermoScript primer extension products were run alongside a sequencing reaction using the same primer and genomic DNA clones, the transcription initiation site was mapped to AT nucleotides (UA in the mRNA). This T is identical to the T determined by the

SMART PCR method above (compare Figure 3B and Figure 4B).

It is important to mention that several primers located within 100 bases of the 5' end of the mRNA did not work well in primer extension assays in our hands. We had to screen multiple primers to find a suitable primer for the primer-extension assay of our gene. This is another example of the possible complexity of performing primer extension assays.

The second discrete band generated by SUPERSCRIPT II reverse transcriptase is about 15 bases longer than the dominant band (Figure 4A, lane 2). This additional extension product by SUPERSCRIPT II reverse transcriptase may reflect a second transcription initiation site of the *veg*f gene in zebrafish. However, both the SMART cDNA synthesis PCR (SUPERSCRIPT II reverse transcriptase used in first-strand cDNA synthesis) as well as primer-extension using ThermoScript reverse transcriptase did not detect this site. In addition, we noticed that SUPERSCRIPT II reverse transcriptase yielded many more primer-extended products compared with ThermoScript reverse transcriptase (Figure 4A). This seems to reflect the different level of activity of the two reverse transcriptases. While ThermoScript is derived from AMV reverse transcriptase, SUPERSCRIPT II is derived from Moloney murine leukemia virus (MMLV) reverse transcriptase.

Figure 4. Determination of the transcription initiation site by primer extension. (A) Primer extension using ThermoScript reverse transcriptase (lane 1) and SUPERSCRIPT II reverse transcriptase (lane 2) using primer GS4. The bold arrow on the right indicates the dominant primer extension product of SUPERSCRIPT II reverse transcriptase and ThermoScript reverse transcriptase. The thin arrow indicates the second primer extension product by SUPERSCRIPT II reverse transcriptase. (B) The primer-extended product from ThermoScript reverse transcriptase was run alongside the genomic DNA clones sequenced using a gene-specific primer. The arrow and arrowhead on the right indicate the primer-extended products.

Figure 5. Strong promoter activity of DNA fragment containing upstream and flanking regions of the transcription initiation site. (A) Control zebrafish embryo microinjected with P_0 -GFP DNA. P here indicates promoter, and 0 indicates zero promoter sequences. (B) GFP expression from zebrafish embryo microinjected with P_{veg} -GFP DNA. The embryos are about 24 h post-fertilization. P_{veg} here includes flanking DNA sequences from 815 bp upstream to 72 bp downstream of the transcription initiation site.

To prove the biological functionality of the DNA upstream of this transcription initiation site, an 887-bp genomic DNA fragment flanking this transcription start site was placed in front of the green fluorescent protein (GFP) reporter gene. When this DNA was microinjected into fertilized zebrafish eggs, it directed efficient GFP expression in the zebrafish embryos (Figure 5). This proves that the transcription initiation site determined above is correct.

It is important to mention that reverse transcriptase in primer extension assays may also be able to perform C tailing at the 5' end of the extended first cDNA. In this regard, when mapping the transcription initiation site by primer extension assays, the extended products could be a few nucleotides longer than the real 5'-end nucleotide of the mRNA. So it may not be precisely accurate when the transcription initiation site is determined using this method without directly sequencing the primer-extended products and comparing it to the genomic sequences. Considering this factor, the SMART cDNA synthesis PCR method used here should be as accurate as the primer extension assay in determining the 5' end of the mRNA. As in most gene regulation studies, knowing the approximate location of the transcription initiation site is sufficient to isolate the promoter-enhancer sequences of a gene. In this regard, both SMART cDNA PCR and primer extension assay should be accurate enough for these studies.

It is noted that when we used a modified oligo(dT) primer from Clontech Laboratories (5'-AAGCAGTGGTAACAACGCAGAGATCT₍₃₀₎N₍₋₁₎N-3') to synthesize the first-strand cDNA and then amplified the cDNA product using gene-specific (GS1 or GS2) primer, we could not obtain any *veg*f-specific PCR products directly. Instead, a DNA smear of various sizes was obtained, making it impossible to analyze the PCR products directly (data not shown). Southern analysis of the PCR-amplified products also showed no *veg*f-positive DNAs (data not shown). So, using the gene-specific primer to initiate the first-strand cDNA synthesis seems to be pivotal in our hands for the SMART cDNA synthesis PCR method to be successful.

Recently, the SMART cDNA synthesis PCR was modified to reduce the background nonspecific PCR products (3). In this work, we used the earlier SMART cDNA synthesis PCR method. This may explain the multiple bands in Figure 2, lane 3. Whether it is possible to obtain gene-specific PCR product of a low expression gene using the newly optimized SMART PCR method and modified oligo(dT) to initiate the first-strand cDNA synthesis is not known.

In conclusion, the SMART cDNA synthesis PCR method can be used as an alternative method to accurately determine the transcription start site of a gene. Compared to primer extension, the SMART cDNA synthesis PCR method is an easier assay to perform. This method also provides the advantage of not having to find primers very close to the 5' end of the mRNA. A primer quite far away from the 5' end can also be used to map the 5' end of the mRNA accurately (950 bases in this case). This can be quite useful when working with novel genes and the location of the transcription initiation site has not been estimated using other methods. Additionally, the amplification nature of PCR can increase the gene-specific cDNA many orders of magnitude. This makes the procedure less difficult even when the gene of interest is expressed at a very low level in the cells. Furthermore, it is a general practice that a series of RT-PCR or other assays be performed to confirm the primer extension assay determined transcription initiation site to be the correct one. Using the SMART PCR method, these steps can be omitted.

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