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Amplification of cDNA ends based on template-switching effect and step-out PCR

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ABSTRACT

A new method for amplifying cDNA ends is described which requires only first-strand cDNA synthesis and a single PCR to generate a correct product with very low or no background. The method can be successfully applied to total RNA as well as poly A+ RNA. The same first-strand cDNA can be used to amplify flanking sequences of any cDNA species present in the sample.

The PCR-suppression effect (PS-effect; 1,2) has promoted the development of several novel techniques for gene identification and investigation. The basic principle is that DNA molecules flanked by inverted terminal repeats ~40 bp long ('suppression sequences') cannot be amplified with a primer corresponding to the distal half of the repeat.

Here we propose a new convenient way of introducing suppression sequences, which we call step-out PCR (SO-PCR). A similar idea has recently been employed to overcome a very specific problem: primer-dimer amplification (3). In our view, it has a wider area of application: it can be utilized whenever there is a need to avoid amplification produced by a single primer. To achieve this, the primer that tends to produce background is substituted in PCR by two oligos: one long oligo with a 3′ part identical to the original primer and a 5′-heel at least 20 bp long, and a shorter oligo with only the sequence of the heel. The long oligo is added to the reaction at a low concentration (0.02 µM) and serves only to incorporate the heel sequence into DNA next to the site of original primer annealing. The short oligo functions as an actual PCR primer in subsequent cycles and is present in the reaction in a concentration of at least 0.1 µM. Thus, background DNA molecules which are to be amplified by the original primer alone become flanked by inverted terminal repeats (consisting of the original primer's sequence and the heel's sequence), while the PCR primer corresponds to the outer part of the repeat. For such molecules, the PCR suppression effect occurs. In cases such as the one described here, SO-PCR is the only method that avoids background amplification.

Recently, a procedure was described that makes it possible to add any oligonucleotide sequence to the 3′-terminus of first-strand cDNA during reverse transcription (4) with the help of the 'template-switching' effect. It has been observed that Moloney murine leukemia virus reverse transcriptase (MMLV RT) is able to add a few non-template nucleotides (mostly C) to the 3′-end of a newly synthesized cDNA strand upon reaching the 5′-end of the RNA template. When an oligonucleotide having oligo(rG) sequence on its 3′ end [so-called 'template-switch (TS) oligo'] is present in the RT reaction, it base-pairs with the attached deoxycytidine stretch. Reverse transcriptase then switches templates

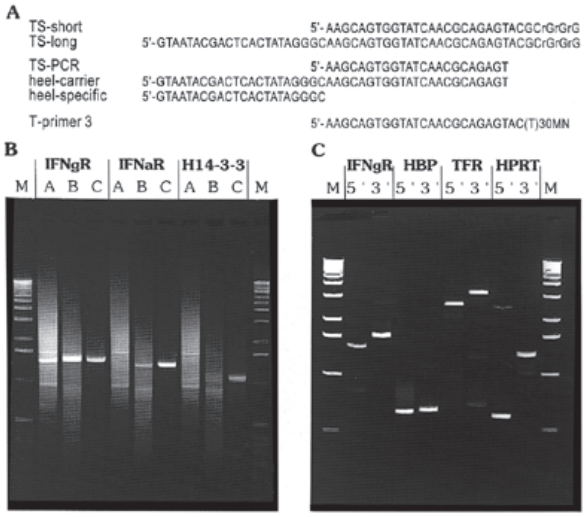

Figure 1. (**A**) Oligonucleotides used in this work. In T-primer 3, M is a mixture of A, G and C; N is a mixture of all four bases. (**B**) Development of 5′-step-out RACE protocol. First-strand cDNA was synthesized starting from human placenta polyA+ RNA. Different template-switching oligos and PCR primers were used in each experiment: lanes A, 'TS-short' and 'TS-PCR' primer; lanes B, 'TS-long' and 'heel-specific' primer; lanes C (the final variant of the protocol), 'TS-short' and step-out primer system including 'heel-carrier' and 'heel-specific' primers: IFNgR, interferon γ receptor; IFNaR, interferon α receptor; H14-3-3, H14-3-3 protein; M, 1 kb DNA ladder. (**C**) Products of 5′- and 3′-step-out RACE starting from human placenta total RNA: IFNgR, interferon γ receptor; HBP, 23 kDa highly basic protein; TFR, transferrin receptor; HPRT, hypoxanthine guanine phosphoribosyl transferase; M, 1 kb DNA ladder (Life Technologies).

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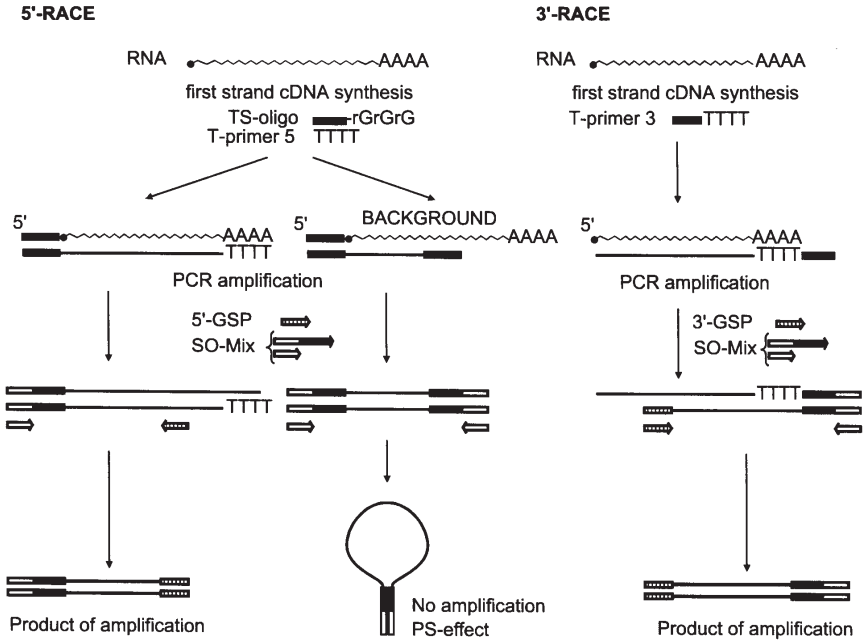

Figure 2. Schematic representation of 5′- and 3′-step-out RACE. Two separate RT-reactions were carried out for 5′-RACE and for 3′-RACE. Each contained 0.5 µg of poly A+ or 1 µg of total RNA, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM each dNTP and 200 U of MMLV reverse transcriptase (SuperScript II, Life Technologies), in a total volume of 10 µl. In addition, the 5′-RACE RT reaction contained 0.5 µM of T-primer '5' (oligo dT stretch 30 bp long) and 0.5 µM of template-switching oligo (TS-short, see Fig. 1A), and the 3′-RACE reaction contained 0.5 µM of T-primer 3 (see Fig. 1A). The reactions proceeded for 90 min at 42°C. Then they were diluted in water (20-fold when polyA+ RNA is used and 10-fold for total RNA), and 1 µl of these dilutions was put into 20 µl of PCR mixture. PCR mixtures for 5′- and 3′-reactions were the same except for the gene-specific primer. They contained 1× Advantage KlenTaq Polymerase Mix with provided buffer (Clontech), 200 µM dNTPs, 0.15 µM of gene-specific primer, 0.02 µM of 'heel-carrier' oligo and 0.15 µM of 'heel-specific' oligo (see Fig. 1A). Gene-specific primers were from commercially available amplimer sets (Clontech). In our study, we used touchdown PCR (6). The cycling profile was (Perkin-Elmer 480 thermocycler): 5 cycles for 30 s at 94°C; 2.5 min at 72°C; 5 cycles for 30 s at 94°C; 2.5 min at 70°C; 25–35 cycles (depending on the transcript's abundance) for 30 s at 94°C; 2.5 min at 68°C. All oligonucleotides were purified through polyacrylamide gel before use.

and continues replicating to the end of the oligo. Thus, the complementary TS-oligo sequence becomes attached to the 3′ terminus of the cDNA. This effect serves as a basis for obtaining amplified cDNA samples enriched for full-length sequences (4).

The obvious simplicity of the template-switching procedure makes it tempting to develop a technique for cDNA end amplification based on this mechanism. Straightforward attempts to amplify the 5′-end of a cDNA using a TS-PCR primer that corresponds to TS-short sequence (Fig. 1A) and gene-specific primer failed (Fig. 1B, lanes A) due to heavy background amplification produced by the TS-PCR primer alone. This is not surprising since during the RT reaction the TS-oligo is free to anneal not only at the oligo(C) stretch at the 3′-end of the cDNA, but, non-specifically, to anywhere in the RNA, acting as a primer for reverse transcription, which should cause heavy background if the TS-PCR primer is used in subsequent PCR. To suppress the amplification produced by the TS-PCR primer alone, an attempt was made to introduce the PS-effect: a longer TS-oligo was designed (Fig. 1A, TS-long), so that the 5′-primer could correspond to its 5′-half. Under these conditions, cDNA molecules originating from 'in-strand' annealing of the TS-oligo during the RT reaction should become flanked by inverted terminal repeats longer than the corresponding primer; therefore, their amplification should be suppressed. This modification improved results to a certain extent (Fig. 1B, lanes B), but the background was still unexpectedly high. We supposed that the reason for this is non-specific template switching which may take place when the middle region of TS-oligo hybridizes to a stretch built at the 3′ end of cDNA by reverse transcriptase. As a result, the 3′-half of the long TS-oligo sequence, which is responsible for the PS-effect, may be partially or completely absent in the resulting cDNA.

This problem was resolved by the use of SO-PCR (Fig 1B, lanes C). In the final protocol of 5′-end amplification (Fig. 2), the TS-oligo was left short (Fig. 1A, TS-short), but the subsequent PCR was performed using a pair of oligos corresponding to it, as described above for SO-PCR (Fig. 1A, heel-carrier and heel-specific primers). In this case, the integrity of suppression sequences is observed throughout the procedure. Only those molecules which received a complete copy of the TS-oligo at the 3′ terminus could be amplified, because under the high-stringency conditions the long heel-carrier primer is unable to anneal to an incomplete copy of TS-short oligo, and therefore no target is generated for subsequent PCR with the heel-specific primer.

Using the same principle, we have also developed a complement technique for 3′-end amplification, which also requires only the first-strand cDNA synthesis (Fig. 2). It can be noticed that the protocol for 3′-flank amplification is very similar to the one described by Frohman *et al.* (5) for 3′-RACE (rapid amplification of cDNA ends). The difference is in the use of SO-PCR to enhance the PS-effect, which leads to the pronounced decrease of background amplification. Besides, in our method the same first-strand cDNA can be used to obtain 3′-flanks of all cDNAs

present in the sample, while the method of Frohman *et al*. (5) requires separate cDNA synthesis for each transcript using a specially designed primer.

We have tested our technique on 13 mRNA species found in human placenta. The rarest of them, namely interferon α receptor and interleukin 10, comprised <0.001% of the particular cDNA sample (estimated by RT–PCR, data not shown), which corresponds to about one mRNA copy per cell. In all cases the correct PCR products (5′- or 3′-cDNA ends, confirmed by sequencing) were obtained in a single PCR reaction starting with an aliquot of first-strand cDNA. We have also demonstrated that total RNA can be successfully used in 5′- and 3′-step-out RACE (Fig. 1C).

The step-out RACE method is obviously the least technically complicated of all analogous techniques (2,5). Besides, in a comparative experiment we demonstrated that products of 5′ step-out RACE contained more complete 5′ cDNA ends than the analogous RACE products obtained by one of the commonly used techniques: Marathon method (2) (data not shown). This is not surprising, since the loss of several 5′-most bases in Marathon should be the inevitable consequence of the second-strand cDNA synthesis by nick-translation replacement. However, it should be noted that our method does not solve some general RACE problems originating from the RT reaction, such as under-representation of cDNAs corresponding to transcripts which either are very long or have a stable secondary structure.

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