3′ cycle-labeled oligonucleotides with predictable length for primer extension and transgene analysis

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Received August 9, 1996; Revised and Accepted October 29, 1996 DDBJ/EMBL/GenBank accession no. U13171

ABSTRACT

Efficient labeling of short oligos at their 3′-ends was achieved through polymerase chain reaction. The length of cycled-labeled oligos can be accurately predicted by omitting one or more dNTPs in the labeling step. Thus, labeled oligos can be simply column-purified, eliminating the need for tedious gel purification. We demonstrated the effectiveness of this technique in determining the transcription start site of aspen (Populus tremuloides Michx.) caffeic acid/5-hydroxyferulic acid transcription start site (TSS) of aspen (Populus tremuloides Michx.).

Efficient labeling of short oligos with α-32P-labeled radionucleotides at their 3′-ends was achieved through polymerase chain reaction (PCR) based on the working principle of cycle sequencing (1). By omitting one or more dNTPs during the cycle labeling step, 3′-ends of labeled oligos could be accurately defined which obviates the need for tedious gel purification. The use of α-32P radionucleotides in the labeling step also eliminates the exclusive requirement of γ-32P radionucleotide used in the 5′-end-labeling reactions. Moreover, radiolabeled primers can be synthesized directly from plasmid templates with this method. Using this technique, the labeled oligos can be used to hybridize to target DNA for primer extension through reverse transcription after a simple column purification to remove unincorporated nucleotides. Sequencing ladders can also be prepared using the same reagent/enzyme system with the same labeled oligos without purification. This further avoids the employment of additional enzymes, such as T7 DNA polymerase or sequenase. Using this protocol, the labeling was carried out using the T7 DNA polymerase and water for subsequent preparation of sequencing ladders according to the manufacturer’s protocol. The rest of the cycle-labeled oligos were column-purified using Chroma spin-10 column (Clontech). Three microliters of purified radiolabeled primer I (1.9 × 106 c.p.m.) were hybridized with total RNA isolated from developing xylem of aspen (5). The hybridization was carried out at 65°C for 1 h in a final volume of 20 µl containing 4 µl of 5× first strand buffer (GIBCO BRL). After brief centrifugation, the resulting primer–RNA hybrids were added to 20 µl extension solution containing 4 µl of 5× first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 2.5 µg/ml actinomycin D (GIBCO BRL) and 100 U of SuperScriptTM II (GIBCO BRL) and subjected to reverse transcription at 42°C for 1 h. The extension products were precipitated, vacuum-dried, and resuspended in 5 µl of 0.1 mM NaOH + 1 mM EDTA (6) with 5 µl of loading dye (sequencing stop solution + 10 mM NaOH). After denaturation, 2–4 µl of the products were electrophoresed on a 6% Long Ranger (FMC) sequencing gel in parallel with the sequencing ladders.

The cycle-labeling step incorporated 10 nt into the primer I, of which 7 nt were radioactive, resulting in a primer 35 bases long. Using this cycle-labeled oligo for primer extension, 5′ terminus of aspen OMT transcript could be effectively detected using as little as 5 µg of total RNA in only 3 h autoradiography (Fig. 1A). As seen in this figure, good resolution of sequencing ladders with a long readable length indicates the applicability of this technique to resolve even longer primer extension products. Another primer was thus employed to test the sensitivity of this technique further. This primer (primer II), 5′-CTGGCTAGTTGCTAGGCAAGAG (located ~50 bp downstream from primer I) and allows only 1 radionucleotide to be incorporated in the labeling step. The labeling reaction was carried out as described above, with the exception that only [α-32P]dATP along with dTTP and dGTP mix were added to the reaction, and 5 µl of purified labeled primer (3 × 105 c.p.m.) was used for primer extension. As seen in Figure 1B, these internally single-base labeled primers could also detect the OMT 5′ terminus after 3 h of autoradiography using total RNA of ≥20 µg. In this case, mapping of the 5′ terminus using a limited amount of RNA should also be achievable with longer exposure times. The 5′ end of the aspen OMT gene transcript was mapped at 124 and 175 nt away from primer I and primer II, respectively.

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OMT gene sequence (4). A putative TA TA box was found 31 bp giving a consistent result of adenine as the TSS according to the in developing xylem but poorly expressed in leaves (2). When the OMT cDNA (7). The endogenous OMT is preferentially expressed endogenous OMT gene from that of an introduced homologous analysis of transgenic aspen to differentiate the expression of not in leaves of wild-type (L) aspen, as shown in Figure 2.

Whereas, the TSS of the native OMT gene was mapped at 124 nt upstream from the same primer in xylem (X) of wild-type aspen. A very low level of signal at 124 nt could also be seen in wild-type and transgenic leaves (L, S3 and S7) due to the expression of introduced OMT gene in xylem of transgenic plants (S3 and S7) at 72 nt upstream from primer I, but not in leaves of wild-type (L) aspen, as shown in Figure 2. The same technique was also successfully applied in the primer extension analysis, 5' terminus of the transgene was mapped in leaves of two transgenic plants (S3 and S7) at 72 nt upstream from primer I, but not in leaves of wild-type (L) aspen, as shown in Figure 2. The labeling technique presented here offers a straightforward and easy-to-perform alternative to the existing ones. In our method, oligos can be efficiently labeled with predictable length and desired level of specificity. Since 3'-ends of radiolabeled oligos can be accurately defined, this protocol facilitates the use of plasmid DNA rather than the preparation of a specific PCR fragment as the template in the labeling reaction. The irritating problems in generating labeled oligos of varied lengths usually associated with other PCR-based techniques are also overcome, which further eliminates the need for gel purification. Although this study demonstrated the effectiveness of applying [α-32P]dNTP for oligo labelling, radio-nucleotides with lower emission energy isotopes, or non-radioactive nucleotides should also be adequate in this technique. The protocol could be further modified to use any thermostable DNA polymerase of choice in the primer labeling reaction, if cycle sequencing is not regularly performed in the lab. In this case, the preparation of sequencing ladders using the labeled oligos needs to be done according to any routine methods employed in the lab. This oligo labeling technique could also be applicable to other molecular biology studies, such as oligo hybridization and in vitro transcription.

ACKNOWLEDGEMENTS

This research was supported by the USDA Competitive grants (#92-37301-7598, #95-37103-2061) from the USDA National Research Initiative Competitive Grants Program to V.L.C. and G.K.P. and from CPBR-DOE Energy from Biomass Program to G.K.P. and V.L.C.

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