990–991 Nucleic Acids Research, 1996, Vol. 24, No. 5

Single-stranded DNA ligation by T4 RNA ligase for PCR cloning of 5′-noncoding fragments and coding sequence of a specific gene

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Received October 30, 1995; Revised and Accepted January 18, 1996

Construction and screening of DNA libraries for genes of interest represent the standard but tedious cloning procedures (1). The advent of PCR technology has greatly enhanced the efficiency of traditional gene isolation and cloning techniques. Various strategies have been developed for PCR amplification of an unknown DNA fragment that flanks one end of a known sequence which include inverse PCR (2), panhandle PCR (3,4), vectorette PCR (5,6), ligation PCR (7,8) and capture (oligo-cassette) PCR (9,10). However, the procedures involved in these techniques are generally time-consuming, high-cost, and suffer from technical difficulties such as inefficient or low-specific PCR amplification. We have devised a simple method for cloning 5′-noncoding regions as well as coding sequences of a gene in a straightforward manner without going through library construction and screening. This technique has been used to isolate a phenylalanine ammonia-lyase (PAL) gene including its 5′-noncoding region from loblolly pine (*Pinus taeda* L.). In our method, a gene-specific primer was first extended by PCR using genomic DNA as a template and single-stranded DNA extension products were ligated to an arbitrary oligonucleotide with T4 RNA ligase to create templates with 5′- and 3′-ends of known sequences. These templates were then re-amplified to make double-stranded DNAs for further cloning and sequencing (Fig. 1). Using this simple technique, ≤2.0 kb 5′-noncoding fragments for PAL gene have been cloned.

Genomic DNA of loblolly pine was isolated according to a modified CTAB method (11). Based on the sequence of our partial PAL cDNA clone, a primer (primer A in Fig. 1), 5′-CAGTTCAGTGGATCGCTGCC located near the 5′-end coding region of PAL cDNA clone, was synthesized (Integrated DNA Technologies). The pine genomic DNA was used as a template for extending the PAL gene-specific primer. The PCR was carried out in 100 µl reaction mixture containing 200 ng genomic DNA, 0.2 µM primer A, 200 µM of each dNTP in *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase (Fisher Scientific) with a Perkin-Elmer DNA thermal cycler 480. The reaction mixture was first denatured at 94°C for 3 min, followed by 40 cycles of denaturing at 94°C (1 min), annealing at 60°C (45 s) and extension at 72°C (2 min). After passing through a PCR select®-III column (5 Prime–3 Prime, Inc) to remove primers and polymerase, the primer extension products were precipitated with ethanol. The precipitate was dissolved in distilled water and mixed with T4 RNA ligation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 60 µg/ml BSA), 25 nM of 5′-phosphorylated oligodeoxyribonucleotide B (oligo B in Fig. 1) and 10 U T4 RNA ligase (Boehringer Mannheim) in a final volume of 20 µl. The oligo B, 5′-AGGGTGCCAACCTCTTCA-AG, is an arbitrary 20mer. The mixture was incubated at 22°C overnight. The following PCR was carried out in 50 µl reaction mixture containing 1 µl ligation mixture, 200 µM of each dNTP, 0.2 µM of primers A and C in *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase. Primer C, 5′-CTTGAAGAGGTTG-GCACCCT, is complementary to oligo B. PCR was carried out at 94°C for 1 min, 58°C for 45 s and 72°C for 2 min for 30 cycles, followed by a final extension at 72°C for 10 min. Ten microliters of the PCR products were taken for agarose gel electrophoresis and Southern blot hybridization to confirm the identity of the PCR products (Fig. 2). Then the PCR products (1–2 µl) were used directly for ligation to vector pCR™ II, using TA cloning® kit (Invitrogen) according to manufacturer's protocol. Plasmid clones containing recombinant DNAs were then analyzed by restriction enzyme digestion, gel electrophoresis, and sequencing. With this method, we have isolated a pine PAL gene with the complete coding region and ~2 kb of the 5′-noncoding region.

This simple method uses T4 RNA ligase to attach an arbitrary oligomer to the extended target DNA fragments for a subsequent PCR amplification to clone the target DNA for its 5′-noncoding fragments and coding sequence (Fig. 1). Using an oligonucleotide from the 3′-region of a gene as a primer (Primer A in Fig. 1) and genomic DNA as a template, a mixture of single-stranded DNA primer extension products can be readily obtained. Since many genes, especially those in animal genomes, contain introns, the design of primer A should avoid intron junctions to ensure the primer specificity. Thus, our technique should also be applicable to cloning those genes with numerous or extremely long introns in their coding or 5′-noncoding regions. In this case, it is advisable that several PCR amplification and T4 RNA ligase-driven ligation should be carried out in a walking fashion so that overlapping fragments can be obtained to cover the whole region.

The T4 RNA ligase-based cloning method was originally designed by Edwards *et al.* (12) for constructing cDNA libraries and adapted by others (13) for cloning cDNAs. However, it has not been applied to genomic DNAs. T4 RNA ligase has low substrate specificity toward DNAs as compared with its affinity to RNA molecules. When coupled with highly sensitive PCR amplifica-

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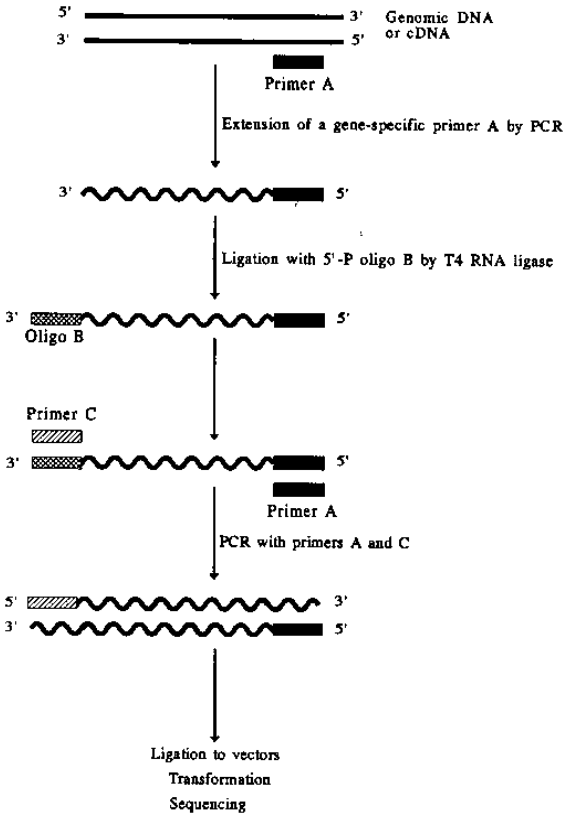

Figure 1. Strategy for single-stranded DNA ligation and PCR amplifications. Primer A is an oligonucleotide specific to the gene to be cloned. It is to be extended by PCRs using genomic DNA or cDNA as templates. Oligo B is an arbitrary, 5′-phosphorylated oligonucleotide. The single-stranded DNA from the primer extensions is ligated with oligo B by T4 RNA ligase. Primer C is an oligonucleotide complementary to oligo B. The PCRs using primers A/C and the ligation mixture as template amplify the target DNA fragments.

tion, the single-stranded DNA ligation mediated by T4 RNA ligase is not a limiting factor in our protocol to successfully clone the target gene. Furthermore, the efficiency of ligating DNA molecules with T4 RNA ligase can be increased by blocking 3′-hydroxyl group of the 5′-P-donor oligo B (14), addition of PEG and hexamine cobalt chloride (14) and longer incubation time.

The second round PCR was designed to amplify the target DNA and to obtain double-stranded gene fragments for cloning. In our experiment, three major products of 2.0, 1.1 and 0.8 kb in size were observed (lane 3 of Fig. 2A). Southern blot analysis shows that a ^{32}P-labeled probe of a 5′-portion of our pine PAL cDNA hybridizes to fragments corresponding to the three bands (Fig. 2B) mentioned above. We found that the sequences of these clones overlapped, extending from the coding region to the 5′-noncoding region. It illustrates the feasibility of our technique to clone large gene segments containing introns through walking along genomic DNA.

We have described a versatile strategy for gene cloning without going through laborious DNA library construction and screening, which provides an alternative tool to many other tedious cloning procedures. This method has the following advantages. First, genomic DNA or cDNA of any sources can be used directly to amplify the target gene without the need of library construction and screening, as long as a short sequence of the target gene is known. Secondly, by adjusting the operation conditions, especially the extension time for the PCR-driven primer extension, it is possible

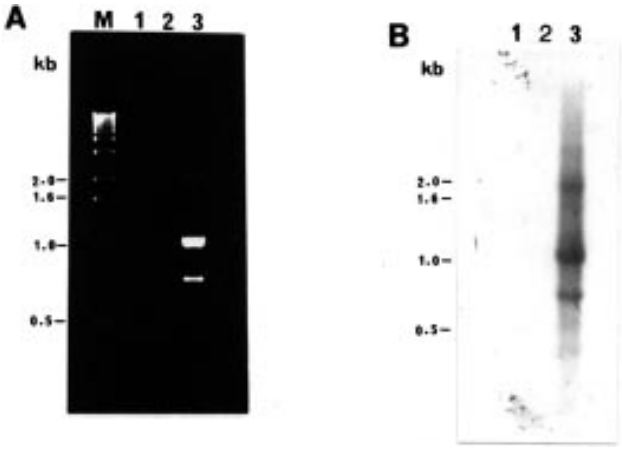

Figure 2. Agarose gel electrophoresis and Southern blot analysis of PCR products. Lane M is 1 kb DNA ladder (GIBCO BRL Life Technologies). Lane 1 is the reaction of primer A extension. Lane 2 is the negative control of PCRs with primers A/C (without template). Lane 3 is the amplified products of PCRs with primers A/C. (**A**) Ethidium bromide-stained gel. (**B**) Autoradiograph of hybridization of ^{32}P-PAL cDNA 5′-fragments with the PCR DNAs blotted onto Hybond^{TM}-N^{+} membrane (Amersham).

to obtain different sizes of the extended 5′-fragments to include promoter fragments. Thirdly, the arbitrary oligo B and its complementary fragment (Primer C; Fig. 1) can be repeatedly used for amplification of different genes, thus reducing the need of frequent primer synthesis. Oligo B can also be made to include restriction sites or tags to facilitate the subsequent manipulations, such as cloning, restriction analysis and sequencing. Attachment of a restriction site to the gene-specific primer (Primer A in Fig. 1) as well as inclusion of a restriction site in the arbitrary primers (Oligo B and Primer C in Fig. 1) would facilitate the ligation of large fragments to vectors. One disadvantage of this method and all other existing PCR-based techniques is the low fidelity of *Taq* DNA polymerase. However, this could be circumvented by choosing high fidelity polymerases and manipulating the reaction conditions.

ACKNOWLEDGEMENT

This work was supported in part by a grant (#IBN 9118386) from the National Science Foundation to V.L.C.

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