J. of the Chosun Natural Science
Vol. 1, No. 3 (2008) pp. 230 – 233

Extension of a 5'- or 3'-end Genomic DNA Sequence by a Single PCR Amplification

Taeck J. Jeon†

Abstract

A simple and rapid method is described for extending the 5'- or 3'-end genomic sequence of a known partial sequence by only a single round of PCR. This method involves digesting and ligating genomic and plasmid DNAs, and amplifying the 5'-upstream or 3'-end downstream sequence of the known DNA sequence, using two primers, one gene specific and the other plasmid specific. A single round of PCR amplification is sufficient to produce gene-specific bands detectable in gels. By using this approach, 5'-end genomic sequence of the D-amoeba *sams* gene was extended.

Key words : Genomic DNA, PCR, S-adenosylmethionine synthetase

1. Introduction

The PCR method has been extensively used for selectively amplifying discrete segments of DNA. Several PCR-based cloning methods have been devised for isolating genomic DNAs, such as inverse PCR (6), ligation-mediated PCR (5), rapid amplification of genomic ends (RAGE) (3), Biotin-RAGE PCR (1), and one-armed PCR (4). The degree of difficulty and the effectiveness of these procedures vary and they do not always result in successful cloning of genomic sequences.

The RAGE method has been used to extend 5'- or 3'-end sequence of genomic DNA based on known sequences (3). RAGE involves polyadenylation of digested genomic DNA followed by two rounds of amplification DNA by PCR using two sets of primers, a gene-specific and an anchored oligo-d(T) primer. However, for unknown reasons, it was unsuccessful to obtain 5'- and 3'-end sequences of S-adenosylmethionine synthetase (SAMS) genomic DNAs of amoebae and their symbionts by using the RAGE method, and a modified method was attempted in this study. Instead of polynucleotidating digested genomic DNA followed by two consecutive PCR amplification as done in the usual RAGE, the digested genomic DNA was, first, ligated with a plasmid DNA digested with the same restriction enzyme and then amplified by PCR using two primers, one gene specific and the other plasmid specific. Only one round of PCR amplification was sufficient to produce gene-specific bands detectable in gels. By using this approach, the 5'-end genomic sequence of an amoeba *sams* gene was successfully extended.

Department of Biology, College of Natural Sciences, Chosun University
†Corresponding author: tjeon@chosun.ac.kr
(Received : October 26, 2008, Accepted : December 6, 2008)

2. Materials and methods

2.1. Plasmid and genomic DNAs

The plasmid (pBSKII+) (Stratagene, USA) was digested with restriction enzymes (*Bam*H1 and *Eco*RI) for 2 hr at 37°C. In a preliminary study, several different enzymes that had only one restriction site in the plasmid and no restriction site in between the end of a known sequence and a designed primer were tested. Among those, *Bam*H1 and *Eco*RI produced the best results. So, the two restriction enzymes were used in further experiments. The digested plasmid DNA (40 μl) was dephosphorylated with calf intestinal alkaline phophatase (CIAP) by adding 5-μl CIAP 10X reaction buffer plus 5 μl of diluted CIAP (0.01 U/μl) and incubating for 30 min at 37°C to prevent recircularization and religation of linearized plasmid DNA. After additional incubation for 30 min with adding another aliquot of diluted CIAP, the reaction was stopped by the addition of

300 ml of CIAP stop buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA pH 7.5, 200 mM NaCl and 0.5% SDS), followed by extracting the plasmid DNA with an equal volume of phenol/chloroform/isoamyl alcohol. The dephosphorylated plasmid DNA was precipitated and resuspended in 30 μl of sterile distilled water (dH_2O).

The genomic DNA (1-2 μg) of *Amoeba proteus* was digested with the same restriction enzymes as were used in the digestion of the plasmid (*Bam*H1 or *Eco*RI). The sample was extracted with an equal volume of phenol/chloroform/isoamyl alcohol and precipitated with 2.5 volumes of 100% ethanol. The pellet was washed with 70% ethanol and resuspended in sterile dH_2O.

2.2. Ligation and amplification

Equal amounts of the digested plasmid and the digested genomic DNA were ligated with T4 DNA ligase (Promega) in 10 μl for 1.5 hr at room temperature. 5 μl of ligation mixture were used for PCR without further treatments.

Using two primers, one gene specific and the other plasmid specific, the ligated DNA was amplified. 5 μl of the ligated DNA as a template, 0.5 μM of gene-specific primer (forward or reverse), 0.5 μM of plasmid-specific primer (T7 primer; 5'-TAATACGACTCACTATAGGGCGA-3'), 200-μM dNTPs and 5 units of *Taq* DNA polymerase (Promega) were used in 50 μl of PCR reaction. PCR was carried out for 1 min at 94°C, 1 min at 55°C, and for 1.5 min at 72°C in 35 cycles. A longer extension period might be required for longer genomic fragments.

For extending 5'-end sequences, a reverse sequence located inside the known sequence and the T7 primer were used as a gene-specific and a plasmid-specific primer, respectively. In the case of 3'-end extension, a forward sequence located inside the known sequence might be used. For extending the D-amoeba *sams* gene (2), the SAM1 (5'-GTCCTCTGCTGACTGCTTCG-3') or SAM2 primer (5'-CTCAACATATTTGATACGATCGGG-3'), located at 38 -- 17 bp or 1197 -- 1174 bp in D-amoeba *sams*, as the reverse gene-specific primer, were used.

2.3. Analysis of the amplified DNA fragments

The amplified DNAs were analyzed by electrophoresis in agarose gel and eluted by using the GENE Clean Kit II (Bio101, CA). The eluted DNA fragments were subcloned into pGEM-T easy vector (Promega) and sequenced. To increase the possibility for selecting gene-specific DNA fragments, the eluted DNA was analyzed by further amplification with each primer under the same conditions as described above. When many fragments were found in a gel, eluted fragments were analyzed by Southern blotting using gene-specific probes.

3. Results and discussion

The partial D-amoeba *sams* gene previously obtained by screening an amoeba cDNA library (2) that lacked a portion of its 5'-end sequence was used as the basis for extending the 5'-end sequence. The gene-specific reverse SAM1 or SAM2 primer was used as a reverse gene-specific primer. By using the approach described in this study, the 5'-end of D-amoeba *sams* by 272-bp was extended. When the ligated mixture of D-amoeba genomic DNA and pBSKII+ after digesting both with *Bam*H1 was amplified with two primers, gene-specific reverse (SAM1 or 2) and plasmid-specific (T7), 1.5-kb and 300-bp fragments were obtained (Fig. 1, Lanes 1 and 2). These fragments were subcloned into the pGEM-T easy vector and sequenced. Both fragments contained a 272-bp upstream sequence not present in the D-amoeba *sams* template. The translation start site

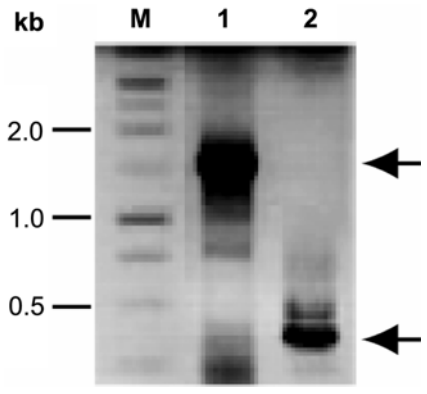

Fig. 1. Agarose gel showing PCR-amplified 5'-end fragments of D-amoeba *sams*. Ligated D-amoeba genomic DNA and pBSKII+ was amplified with gene-specific reverse SAM2 (Lane 1) or SAM1 (Lane 2) and plasmid-specific (T7) primers. Arrows indicate 1.5-κb (Lane 1) and 300-bp (Lane 2) fragments, respectively. Lane M, a 1-κb ladder used as the size marker.

was found at the 6th codon upstream from the first codon of the previously reported sequence. The same extension experiment was carried out with *Hind*III and *Cla*I, but there was no amplified band in the gel (data not shown). Analysis of the cloned DNA showed that there was no restriction site for *Hind*III or *Cla*I in the extended sequence.

A simple and rapid method for extending 5'- and 3'-ends of known DNA sequences without an extensive manipulation of DNA was described. Traditional procedures for cloning genomic sequences based on the cDNA sequence information involve the screening of lambda phage-generated libraries (7). Although effective, these methods consume much time, effort, and expenses, usually requiring the use of radioisotopes. The strategies and methodologies for gene isolation have been significantly improved by recently developed techniques, such as the introduction of PCR-based methods. However, such PCR-based methods including inverse PCR (6), ligation-mediated PCR (5), Biotin-RAGE PCR (1), and one-armed PCR (4) would be either limited to cDNA or require extensive manipulation of a template prior to amplification. Recently, Cormack and Somssich (3) devised RAGE as a simple method to clone upstream or downstream genomic sequences. In RAGE, the treatment of digested genomic DNA with terminal transferase produces polyadenylation of 3'-ends in both sides of the double-stranded DNA. Therefore, all of the DNA polyadenylated at 3'-ends may be nonspecifically amplified by oligo-d(T) primers only in subsequent PCR rather than by both oligo-d(T) and gene-specific primers, and hence two consecutive rounds of PCR amplification are needed in RAGE.

The method described in this study is simpler than others in that only one round of PCR is sufficient to produce identifiable gene-specific bands. It involves digestion and ligation of genomic and plasmid DNAs, and extension of the 5'-upstream or 3'-downstream end of a known sequence using two primers, one gene specific and the other plasmid specific. Furthermore, only a small amount of genomic DNA is sufficient for use in the procedure. Also, any cloning vector in the laboratory can be used, and a large amount of a digested plasmid can easily be generated and stored for future use. Another advantage is that all materials are common and readily available, such as ordinary restriction enzymes, *Taq* DNA polymerase and T4 DNA ligase.

In the present method, nonspecific fragments may occur during the amplification of plasmids, that are produced by ligation of plasmids themselves. However, religation of plasmids is blocked by dephosphorylation of digested plasmids. Thus, unlike in RAGE, the possibility for nonspecific amplification of all fragments with one of the two primers used in our method would be negligible. This may be one of the reasons why only one round of PCR amplification is enough to produce sufficient amount of gene-specific products by this method.

One concern about present method might be possible generation of false fragments during PCR by one or both of the primers used. However, the potential problem caused by the amplification with one primer can easily be excluded by comparing PCR products with those obtained from PCR using both primers. Also, the potential problem caused by amplification with both primers can be overcome by checking by Southern blotting using gene-specific probes. Although most false fragments can be excluded by the above-mentioned tests, it is still possible that unrelated genomic fragments might be inserted in between the plasmid and the target gene. Then the resulting PCR fragment might have some correct nucleotide sequences in addition to incorrect sequences. This problem could be overcome by checking whether the sequenced fragment has one or more restriction sites for the enzymes used in digesting genomic and plasmid DNAs. If the sequenced fragment is a correct one without unrelated genomic sequences, there would be a restriction site for the enzyme only at the end of the extended sequence.

References

[1] Bloomquist, B. T., R. C. Johnson, and R.E. Mains, "Rapid isolation of flanking genomic DNA using Biotin-RACE, a variation of single-sided polymerase chain reaction", DNA Cell Biol., Vol. 11, p. 791-797, 1992.

[2] Choi, J. Y., T. W. Lee, K. W. Jeon, and T. I. Ahn, "Evidence for symbiont-induced alteration of a host's gene expression: Irreversible loss of SAM synthetase from *Amoeba proteus*", J. Euk. Microbiol., Vol. 44, p. 412-419, 1997.

[3] Cormack, Robert S. and Imre E. Somssich, "Rapid amplification of genomic ends (RAGE) as a simple

method to clone flanking genomic DNA", Gene, Vol. 194, p. 273-276, 1997.

[4] Macrae, A.D. and S. Brenner, "One armed PCR (OA-PCR): amplification of genomic DNA from a single primer domain", Genomics, Vol. 24, p. 176-178, 1994.

[5] Mueller, P.R. and B. Wold, "Ligation-mediated PCR: applications to genomic footprinting", Methods, Vol. 2, p. 20-31, 1991.

[6] Ochman, H., A.S. Gerber, and D.L. Hartl, "Genetic applications of an inverse polymerase chain reaction", Genetics, Vol. 120, p. 621-623, 1988.

[7] Sambrook, J., E.F. Fritsch, and T. Maniatis, "Molecular cloning: A laboratory manual 2_{nd} ed.", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., 1989.