PCR with End Trimming and Cassette Ligation: A Rapid Method to Clone Exon–Intron Boundaries and a 5′-Upstream Sequence of Genomic DNA Based on a cDNA Sequence

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We described a method for PCR amplification of unknown flanking genomic DNA fragments. This method is a combination of PCR with "end-trimming method" and "cassettes and cassette-primers method". In this method, genomic DNA was digested with three different groups of restriction enzymes. DNA in group 1 was digested with *Bam*HI, *Bgl*II, *Fba*I, or *Mbo*I. DNA in group 2 was digested with *Bln*I, *Nhe*I, *Spe*I, or *Xba*I. DNA in group 3 was digested with *Sal*I or *Xho*I. Digested DNA in each group was end-trimmed with Klenow fragment of DNA polymerase I in the presence of only one dNTP; dGTP, dCTP, and dTTP for group 1, 2, and 3, respectively. The synthesized cassettes, C1, C2, and C3, had 5′ protruding sequences of 5′-ATC-3′, 5′-TAG-3′, and 5′-CGA-3′, respectively. Each compatible cassette was ligated to the end-trimmed DNAs in group 1–3, respectively. Nested PCR was then performed using an end-trimmed and cassette-ligated DNA as a template. Primers annealing to known sequences and cassettes were used for the nested PCR. The amplified DNA fragments were electrophoresed on a polyacrylamide gel and purified. The sequences of the DNA fragments were determined after cloning into pBluescript.

The PCR method, which permits specific in vitro amplification of DNA fragments, has found many applications in molecular biology. However, the PCR amplification of unknown flanking DNA fragments is difficult, because PCR requires two target-specific primers annealing to both ends of the DNA fragment to be amplified. To overcome this limitation, several methods have been developed.[1–10,13–15]

Inverse PCR[1] involves restriction enzyme digestion and circularization by intrastrand end-to-end ligation. The following PCR using primers directing toward an unknown sequence amplifies the flanking sequence. Panhandle PCR[2] and targeted inverted repeat amplification[3] involve restriction enzyme digestion of genomic DNA and ligation of a complementary oligonucleotide to the known core sequence. Denaturation and reannealing form a stem–loop within complementary strands with a 3′ recessed end, followed by filling with template-directed polymerization or ligation of an oligomer. The presence of an inverted repeat at two sites permits PCR amplification of the unknown flanking DNA. Anchored PCR[4] involves attachment of a homopolymer tail to the first-strand cDNA by terminal deoxynucleotidyl transferase that undergoes template-independent polymerization. PCR is performed using one primer annealing to a known sequence and another primer annealing to a homopolymer tail. In ligation-anchored PCR,[5] T4 RNA ligase is used to attach a designed DNA oligomer instead of forming a homopolymer strand to the first-stand cDNA.

An end-trimming method[6,7] amplifies a cDNA fragment using one compatible primer to a known sequence and another oligo(dT) primer. The PCR product is end-trimmed by T4 DNA polymerase in the presence of only one dNTP to create a sticky end to a corresponding vector. Cassettes and cassette-primers method,[7] cassette-ligation mediated PCR,[8] capture PCR,[9] and vectorette PCR,[10] involve restriction enzyme digestion of cDNAs or genomic DNAs and their ligation to oligonucleotides or vector sequences to create universal primer-annealing sites. PCR, in these methods, utilizes one specific compatible primer to a known sequence and another universal primer that anneals to the universal sequence. Roberts et al. have successfully defined the exon structure of the entire human dystrophin gene, which consists of 79 exons, using vectorette PCR and direct sequencing.[11,12] PCR in other methods makes use of nonspecific or partially specific primers. Targeted gene walking[13] makes use of sets of specific and nonspecific primers to amplify an unknown flanking DNA sequence. A

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single primer was shown to be useful in cloning genomic sequences adjacent to a known sequence.[14] Restriction-site PCR to amplify an unknown flanking sequence to a known locus was performed using a combination of a known sequence-specific primer and another primer specific to a restriction enzyme recognition sequence.[15]

We have described previously two methods of end-trimming method and cassettes and cassette-primers method to amplify adjacent cDNA fragments by PCR.[6,7] In this paper a new method for PCR amplification of unknown flanking DNA fragments is based on a combination of end-trimming method and cassettes and cassette-primers method, which we named "PCR with end trimming and cassette ligation (ETCL–PCR)." Using this method we successfully amplified DNAs of exon–intron boundaries and a promoter region of the rat amidophosphoribosyltransferase (ATase) gene.

MATERIALS AND METHODS

DNA Isolation

The rat genomic DNA was prepared from the liver of a 7-week-old male Wister rat by the standard method.[16]

Synthesis of Oligomers

We have synthesized oligomers, which are listed in Table 1, by an Applied Biosystems 392 DNA/RNA synthesizer (Foster City, CA). oRB613 is a main cassette oligomer (MC oligomer) and has two restriction enzyme recognition sites of *Sal*I and *Not*I. oRB611, oRB659, and oRB658 are adaptor cassette oligomers (AC oligomers). oRB183 and oRB185, of which sequences are the same as the rat ATase cDNA,[7] are primers annealing to the known sequence of the rat ATase gene hopefully in one coding exon, and oRB404 and oRB656 are primers annealing to cassettes.

Constructing Cassettes

Cassettes 1–3 (C1–C3) were constructed by annealing an MC oligomer of oRB613 to AC oligomers of oRB611, oRB659, or oRB658, respectively (Fig. 1). Ten nanomoles of oRB613 was mixed with 10 nmoles each of oRB611, oRB659, and oRB658 in a total volume of 100 μl. These mixtures were heat-denatured at 90°C for 3 min and cooled on ice for 5 min. Then, mixed oligomers annealed to each other and formed a double-stranded cassette. C1–C3 had the 5′ protruding sequences of 5′-ATC-3′, 5′-TAG-3′, and 5′-CGA-3′, respectively.

End-trimming and Cassette-ligation Method

The flowchart of PCR with end trimming and cassette ligation is shown in Figure 2. The case of *Bln*I digestion is depicted as an example. All 10 enzymes used in this study are listed in Table 2.

Step 1: Restriction Enzyme Digestion

The rat genomic DNA (0.5–1 μg) was divided into three groups. DNA in group 1 was digested with 5–10 units of *Bam*HI [New England BioLabs (NEB), Beverly, MA], *Bgl*II (Takara, Kyoto, Japan), *Fba*I (Takara), or *Mbo*I (Takara) in a buffer recommended by the manufacturer in a total volume of 30 μl at 37°C for overnight. In the same way, DNA in group 2 was digested with 5–10 units of *Bln*I (Takara), *Nhe*I (NEB), *Spe*I [Boehringer Mannheim GmbH, (BMG), Mannheim, Germany], or *Xba*I (BMG), and DNA in group 3 was digested with 5–10 units of *Sal*I (NEB) or *Xho*I (BMG).

Step 2: End Trimming

To the restriction enzyme-digested DNA in groups 1–3 in 30 μl, 1 μl of 5 mM dGTP, dCTP, or dTTP was added, respectively. Then, 2 units of Klenow fragment of DNA polymerase I (Toyobo, Osaka, Japan) in 0.5 μl was added to DNA in each group and these mixtures were incubated at 30°C for 15 min. Thus, restriction enzyme-digested ends of DNA in each group were end-trimmed only with G, C, or T, respectively. Klenow fragment of DNA polymerase I was inactivated by heat treatment at 75°C for 10 min, followed by ethanol precipitation.

Step 3: Cassette Ligation

Ethanol-precipitated DNA in each group was ligated with 100 pmoles of C1–C3, respectively, using a DNA ligation kit (Takara) at 16°C, from several hours to overnight in a total volume of 10 μl. After the ligation reaction, DNA in each group was ethanol-precipitated and dissolved in 10 μl of deionized and autoclaved water (DA water).

Step 4: Initial PCR Amplification

The initial PCR amplification was performed using a pair of primers of oRB185, an outer primer designed to anneal to the rat ATase gene, and oRB404, an outer primer annealing to cassettes, and 1 μl of each end-trimmed and cassette-ligated DNA as a template in a total volume of 10 μl. Because the 5′ ends of cassettes have no phosphate, the cassettes were ligated only to end-trimmed DNAs at their 3′ ends. Therefore, only oRB183 could work as a primer in the first cycle of PCR, and oRB404 could work as a primer only from the second cycle of PCR. oRB611, oRB659, and oRB658 have a mismatched A at their 3′ ends. Thus, they could not work as a

TABLE 1 Sequences of Oligomers for Constructing Cassettes and PCR Amplifications

Names	Sequences	Notes
oRB613	5′-CCTCTTCGCTATTACGCCAGTCGAC GCGGCCGCAAATC-3′	main cassette oligomer
oRB611	5′-*ATC*GATTTGCGGCCA-3′	adaptor cassette oligomer for C1
oRB659	5′-*TAG*GATTTGCGGCCA-3′	adaptor cassette oligomer for C2
oRB658	5′-*CGA*GATTTGCGGCCA-3′	adaptor cassette oligomer for C3
oRB404	5′-CCTCTTCGCTATTACGCCAG-3′	outer primer annealing to cassette
oRB656	5′-AATAAGTCGACGCGGCCGCAAATC-3′	inner primer annealing to cassette
oRB183	5′-CATCTATGAGAACAATTCTTTTGCC-3′	outer primer annealing to the rat ATase gene
oRB185	5′-TTAAAGTTGTCAGACAACACGCCGA-3′	inner primer annealing to the rat ATase gene

The underlined letters indicate the sequence to create restriction enzyme recognition sites for *Sal*I and *Not*I. The italic letters show the 5′ protruding sequence complementary to the end-trimmed DNAs.

FIGURE 1 Schematic presentation of the construction of cassettes. The main cassette oligomer oRB613 was mixed with each of adaptor cassette oligomers oRB611, oRB659, and oRB658 in an equal amount in molar value. The mixtures were heat-denatured and annealed by cooling on ice. We named cassettes consisting of oRB611, oRB659, and oRB658 as C1, C2, and C3, respectively. C1 is a cassette for *Bam*HI-, *Bgl*II-, *Fba*I-, and *Mbo*I-digested and end-trimmed DNAs. C2 is a cassette for *Bln*I-, *Nhe*I-, *Spe*I-, and *Xba*I-digested and end-trimmed DNAs. C3 is a cassette for *Sal*I- and *Xho*I-digested and end-trimmed DNAs.

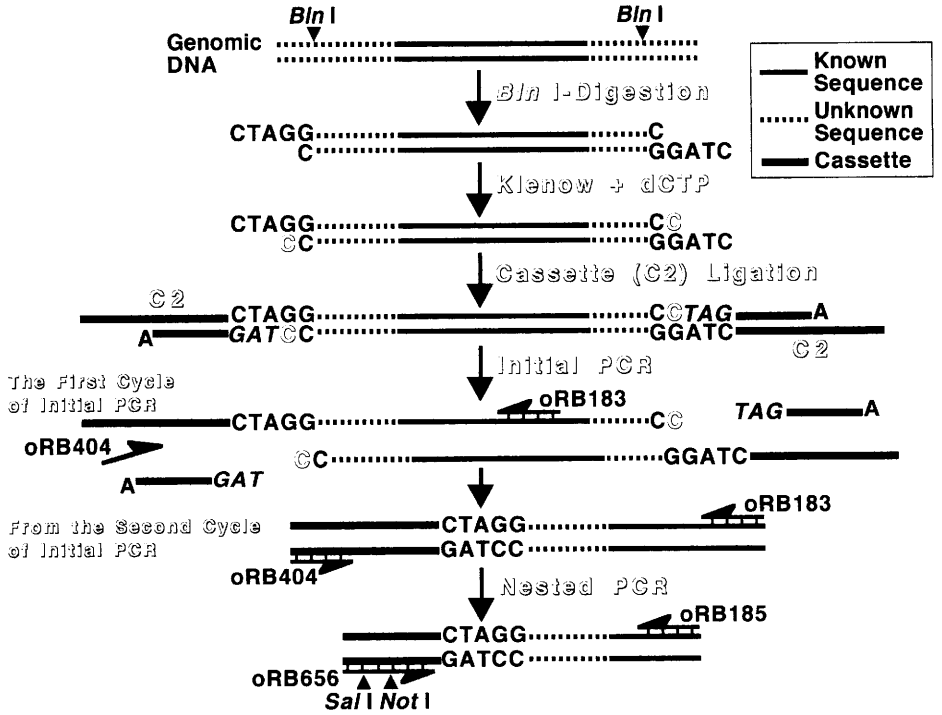

FIGURE 2 The flowchart of PCR with end trimming and cassette ligation in the case of *Bln*I-digestion. The rat genomic DNA was digested with *Bln*I and partially filled with dCTP using Klenow fragment of DNA polymerase I. C2 cassette was ligated to the *Bln*I-digested and end-trimmed DNA. The initial PCR was performed using oRB183, an outer primer annealing to the rat ATase gene, and oRB404, an outer primer annealing to cassette. oRB183 can work as a primer from the first cycle of the initial PCR, but oRB404 cannot work as a primer until the second cycle of the initial PCR, because the dephosphorylated 5′ end of C2 is not ligated to the genomic DNA. The initial PCR product, diluted 10×, was used as a template in the nested PCR. The nested PCR was carried out using oRB185, an inner primer annealing to the rat ATase gene, and oRB656, an inner primer annealing to cassette. oRB656 has two endonuclease recognition sites of *Sal*I and *Not*I. Solid lines, broken lines, and thick, bold lines indicate known sequence, unknown sequence, and cassette, respectively.

primer on the cassette sequence. The PCR products of the initial PCR were diluted 10 times by DA water and used as templates in the nested PCR.

Step 5: Nested PCR Amplification

The nested PCR was performed using a pair of primers oRB183, an inner primer annealing to the rat ATase gene, and oRB656, an inner primer designed to anneal to cassettes, in a total volume of 10 μl. The initial and nested PCR amplifications were performed in a mixture containing 1 μM each of two primers, 200 μM each of four deoxyribonucleotides, 0.025 U/μl of *Taq* DNA polymerase (Kurabo, Osaka, Japan) in buffer containing 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. Thirty cycles of the reaction at 95°C, 55°C, and 72°C for 1, 1, and 3 min, respectively, were carried out in a Program Temp Control System PC-700 (Astec, Fukuoka, Japan).

Electrophoresis and Detection of PCR Products

The PCR products of the second PCR were electrophoresed on an 8% polyacrylamide gel. The amplified DNA fragments were visualized by ethidium bromide staining and UV *trans*-illumination.

Isolation of PCR-amplified DNA Fragments

The DNA fragments amplified by the nested PCR were amplified again using the same set of primers and template at a scaled-up volume of 50 μl. To remove overhanging nucleotide added by *Taq* DNA polymerase at the 3′ end, 2 units of Klenow fragment of DNA polymerase I was added to the reaction mixture and incubated at 30°C for 20 min. Electrophoresis was performed on an 8% polyacrylamide gel, and DNAs were visualized with ethidium bromide staining and UV *trans*-illumination. The amplified DNA fragments were cut out and extracted by the "crush and soak" method with slight modification as described previously.[17] The recovered DNA was dissolved in 50 μl of DA water and quantitated using a DU-64 spectrophotometer (Beckman, Fullerton, CA).

TABLE 2 Combinations of Restriction Enzymes and Cassettes Applicable for PCR with End Trimming and Cassette Ligation

Restriction enzymes	Restriction sequences	Enzyme digestion	End trimming		Cassettes and their 5′ ends	
*Bam*HI	5′-GGATCC-3′ 3′-CCTAGG-5′	→ 5′-G 3′-CCTAG-5′	→ 5′-GG 3′-CCTAG-5′			
*Bgl*II	5′-AGATCT-3′ 3′-TCTAGA-5′	→ 5′-A 3′-TCTAG-5′	→ 5′-AG 3′-TCTAG-5′	+	5′-ATCG--3′ C---5′	C1
*Fba*I	5′-TGATCA-3′ 3′-ACTAGT-5′	→ 5′-T 3′-ACTAG-5′	→ 5′-TG 3′-ACTAG-5′			
*Mbo*I	5′--GATC--3′ 3′--CTAG--5′	→ 5′-- 3′--CTAG-5′	→ 5′--G 3′--CTAG-5′			
*Bln*I	5′-CCTAGG-3′ 3′-GGATCC-5′	→ 5′-C 3′-GGATC-5′	→ 5′-CC 3′-GGATC-5′			
*Nhe*I	5′-GCTAGC-3′ 3′-CGATCG-5′	→ 5′-G 3′-CGATC-5′	→ 5′-GC 3′-CGATC-5′	+	5′-TAGG--3′ C---5′	C2
*Spe*I	5′-ACTAGT-3′ 3′-TGATCA-5′	→ 5′-A 3′-TGATC-5′	→ 5′-AC 3′-TGATC-5′			
*Xba*I	5′-TCTAGA-3′ 3′-AGATCT-5′	→ 5′-T 3′-AGATC-5′	→ 5′-TC 3′-AGATC-5′			
*Sal*I	5′-GTCGAC-3′ 3′-CAGCTG-5′	→ 5′-G 3′-CAGCT-5′	→ 5′-GT 3′-CAGCT-5′	+	5′-CGAG--3′ C---5′	C3
*Xho*I	5′-CTCGAG-3′ 3′-GAGCTC-5′	→ 5′-C 3′-GAGCT-5′	→ 5′-CT 3′-GAGCT-5′			

Cloning of PCR Products

The amplified DNA fragment (0.01–0.02 μg) was digested with 5 units of *Not*I (BMG) in the buffer recommended by a manufacturer in a total volume of 10 μl. pBluescript II SK(+) (0.1 μg; Stratagene, La Jolla, CA) was digested with 5 units of *Not*I and *Eco*RV (Takara) in the buffer recommended by the manufacturer in a total volume of 10 μl. The restriction enzyme-digested DNA fragment and pBluescript were combined and ethanol-precipitated. The DNA fragment and pBluescript were ligated using a DNA ligation kit (Takara) in a total volume of 6 μl. The competent JM109 cell was transformed with recombinant pBluescript. Plasmid DNA was prepared by a small-scale alkaline lysis method.[18]

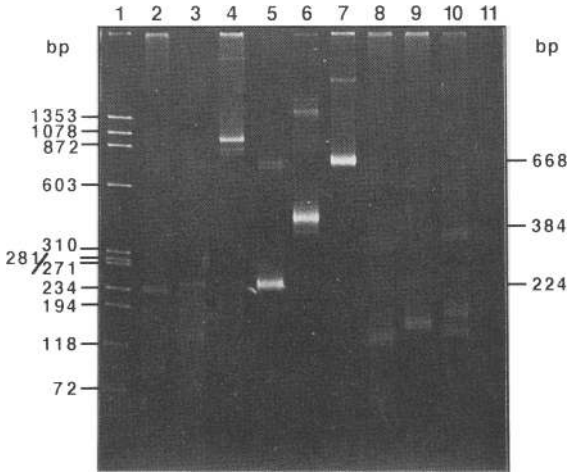

FIGURE 3 A gel image of the nested PCR products. The nested PCR was performed using oRB185 and oRB656, and its products were electrophoresed on an 8% polyacrylamide gel and visualized with ethidium bromide staining and UV *trans*-illumination. (Lane *1*) DNA size markers of *Hae*III-digested ϕx174; (lanes *2–11*) the nested PCR products starting from *Bam*HI-, *Bgl*II-, *Fba*I-, *Mbo*I-, *Bln*I-, *Nhe*I-, *Spe*I-, *Xba*I-, *Sal*I-, and *Xho*I-digested and end-trimmed DNAs, respectively.

Nucleotide Sequencing and Computer-Aided Analysis

Nucleotide sequence was determined using the *Taq* Dye Primer Cycle Sequencing Core Kit (401112) in the ABI 373A sequencer. Cloned pBluescript was used as a template. The nucleotide sequence identity between the rat ATase cDNA and part of newly cloned genomic DNA was analyzed using the SDC-GENETYX version 8.0 program (SDC, Tokyo, Japan).

RESULTS

As shown in Figure 1, constructed cassettes of C1–C3 have the 5′ protruding sequences of 5′-ATC-3′, 5′-TAG-3′, and 5′-CGA-3′, respectively. Because these cassettes have no phosphate at the 5′ end and their ends are not complementary to each other, they never ligate to themselves.

DNA in group 1 was digested with *Bam*HI, *Bgl*II, *Fba*I, or *Mbo*I and end-trimmed by partial filling with dGTP using Klenow fragment of DNA polymerase I. Their ends are complementary to C1 but not to themselves (Table 2). In the same way, DNA in group 2 was digested with *Bln*I, *Nhe*I, *Spe*I, or *Xba*I and partially filled with dCTP with their ends complementary to C2 (Table 2). The *Sal*I- or *Xho*I-digested and dTTP-filled DNAs in group 3 have complementary ends to C3 (Table 2).

Figure 3 shows the results of an amplified exon–intron boundary of the rat ATase gene by ETCL–PCR. The rat genomic DNA isolated from the liver was digested with *Bam*HI, *Bgl*II, *Fba*I, *Mbo*I, *Bln*I, *Nhe*I, *Spe*I, *Xba*I, *Sal*I, or *Xho*I. Each of restriction enzyme-digested DNAs was end-trimmed and cassette-ligated appropriately. The initial PCR amplification was performed with a pair of primers of oRB183 and oRB404 using end-trimmed and cassette-ligated DNA as a template. The nested PCR was performed with a pair of primers of oRB185 and oRB656 using an initial PCR product diluted 10 times as a template. Lanes 2–11 of Figure 3 show the results from *Bam*HI-, *Bgl*II-, *Fba*I-, *Mbo*I-, *Bln*I-, *Nhe*I-, *Spe*I-, *Xba*I-, *Sal*I-, and *Xho*I-digested DNA, respectively. Lanes 5–7 show promising bands.

The DNA fragments of lanes 6 and 7 of Figure 3 were amplified again in

scaled-up volume of 50 μl and isolated by the slightly modified crush and soak method. The isolated DNA fragments were cloned into *Not*I and *Eco*RV double-digested pBluescript, and the nucleotide sequences were determined (Figs. 4 and 5). As shown in Figure 4 (top), C2 was ligated to the *Bln*I-digested and dCTP-filled DNA fragment. C2, as shown in Figure 4 (bottom), was also ligated to the *Nhe*I-digested and dCTP-filled DNA fragment.

Figure 5 shows the alignment of the nucleotide sequence cloned by ETCL–PCR from the rat genomic DNA with that of the rat ATase cDNA. The sequence from 535 to 665 of the newly cloned DNA was identical with that of the rat ATase cDNA, whereas its 5′-end sequence differed from the sequence of the rat ATase cDNA. The starting boundary sequence in the unmatched sequence was AG, which was the consensus sequence of mRNA splicing acceptor site. This result suggested that an exon–intron boundary of the rat ATase gene was successfully cloned by ETCL–PCR.

Moreover, the newly cloned DNA contained the connected sequence of C2 and *Nhe*I recognition site and endonuclease recognition sites of *Bln*I and *Mbo*I. Therefore, it proved that the bands shown in lanes 5–7 of Figure 3 were specifically amplified by ETCL–PCR.

DISCUSSION

In this paper we have described a method of ETCL–PCR. Several other methods to PCR-amplify unknown flanking DNA fragments involve restriction enzyme digestion followed by a ligation reaction,[1–3, 7–10] but the generation of concatemers is a problem in those methods. To avoid concatemer formation, dephosphorylation by calf intestinal alkaline phosphatase or redigestion after ligation of oligomers was performed but a restriction enzyme recognition site was not completely reconstructed.[2,3] We used several tactics to prevent concatemer formation in ETCL–PCR. First, the end-trimmed DNA in our method does not ligate to itself, because genomic DNA is digested with a restriction endonuclease and partially filled with only one kind of dNTP using Klenow fragment of DNA polymerase I. Second, each synthesized cassette also does not self-ligate, because it has no phosphates at the 5′ ends and no complementary ends to itself. Because of these characteristics, the end-trimmed DNA is expected to exclusively ligate to a compatible cassette.

Another merit of ETCL–PCR is its specific amplification of the desired DNA fragment. Because the 5′ end of a cassette has no phosphate, a cassette ligates only to an end-trimmed DNA at the 3′ end of an MC oligomer of oRB613. In the initial PCR, a primer targeted to a known

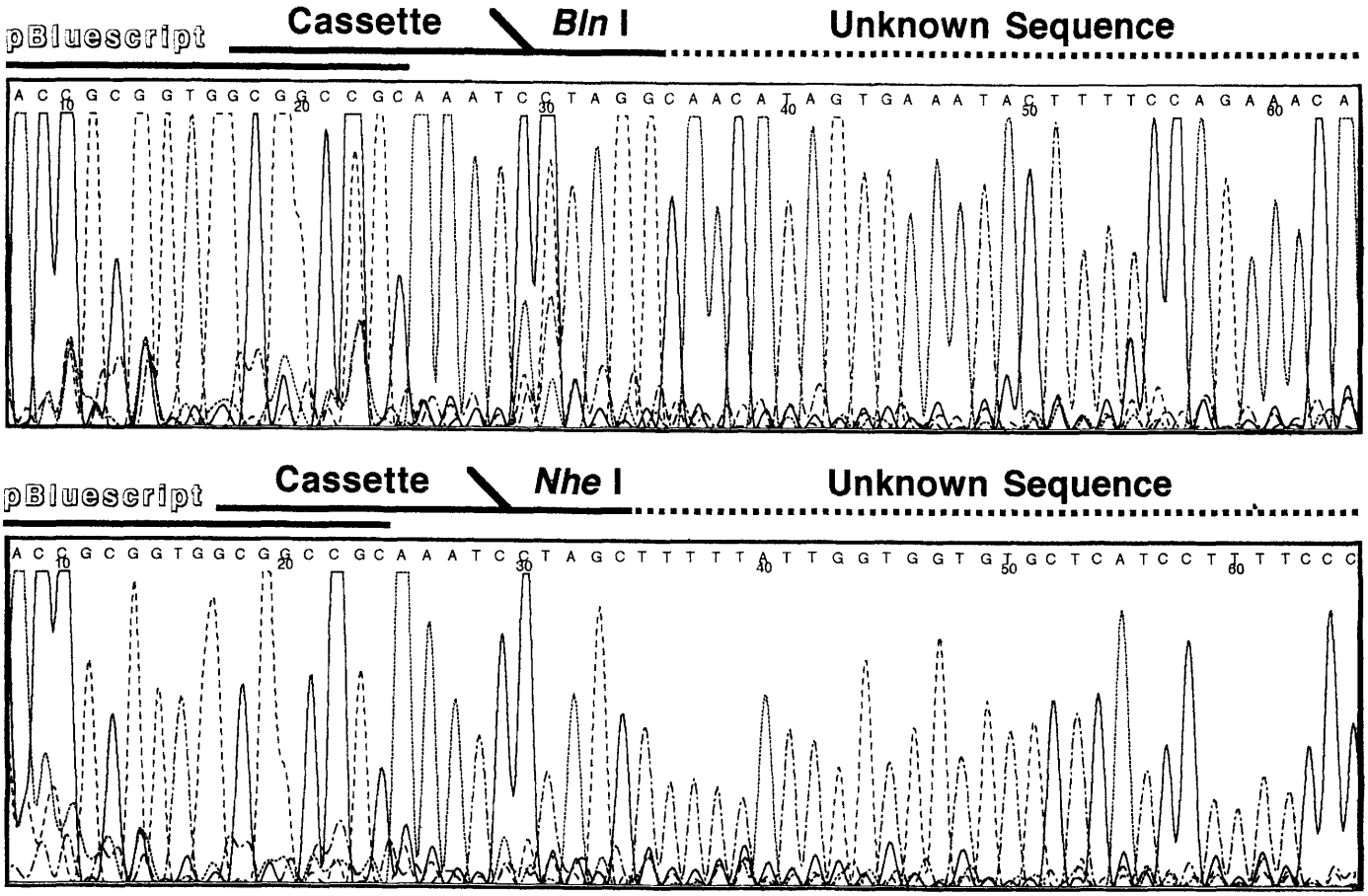

FIGURE 4 Nucleotide sequences of the DNA fragments amplified by ETCL–PCR. (*Top*) ETCL–PCR was started from the rat genomic DNA, which was *Bln*I-digested, end-trimmed with dCTP, and ligated to C2. The nested PCR product was cloned into pBluescript, and the nucleotide sequence analysis was performed. (*Bottom*) The rat genomic DNA, which was *Nhe*I-digested, end-trimmed with dCTP, and ligated to C2, was subjected to the initial and nested PCR and cloned into pBluescript. Nucleotide sequencing was performed using the recombinant pBluescript as a template.

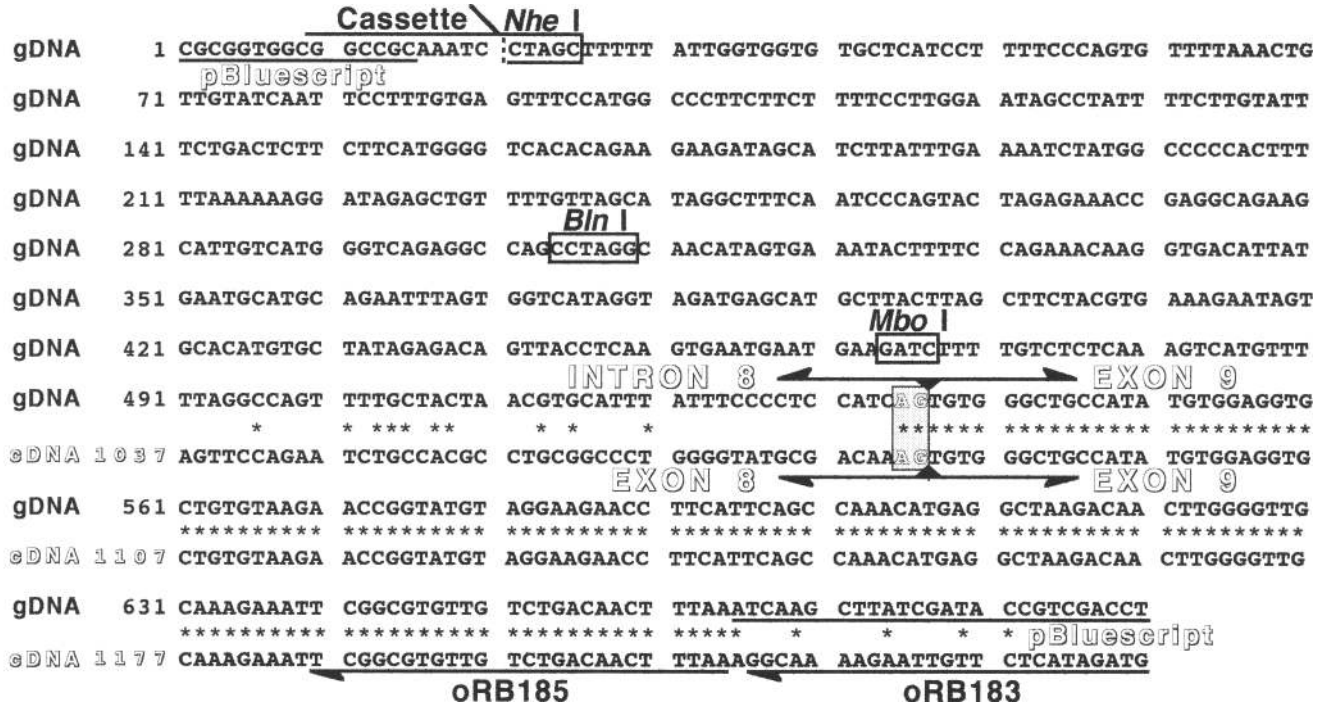

FIGURE 5 Alignment of the nucleotide sequence of newly cloned DNA by ETCL–PCR with that of the rat ATase cDNA. Asterisks (*) indicate the identical nucleotide between these two sequences. gDNA and cDNA indicate the newly cloned DNA from the rat genomic DNA and the rat ATase cDNA, respectively. The nucleotide sequence of the gDNA is numbered arbitrarily and that of the rat ATase cDNA is numbered as described by Iwahana et al.[7] The common DDBJ, EMBL, and GenBank accession number of the rat ATase cDNA is D10853.

sequence can work from the first cycle of PCR, whereas a complementary primer to a cassette can work only from the second cycle of PCR. Therefore, a DNA fragment, which elongates from a primer annealing to a known sequence, is the only specific fragment to be amplified. Another small tip is a mismatched A at the 3′ end of an AC oligomer that inhibits it from annealing to a cassette as a PCR primer. Because oRB656, a complementary primer to a cassette, has *Sal*I and *Not*I recognition sites, the PCR-amplified DNA fragments can easily be cloned into a plasmid such as pBluescript.

The average sizes of DNA fragments produced by 4- or 6-bp recognition restriction enzymes are theoretically estimated as $4^4 = 256$ bp or $4^6 = 4096$ bp, respectively. We used one 4-bp cutter and nine different 6-bp cutters (Table 2). Therefore, it is estimated that there is one 4-bp cutter recognition site in every 256 bp and there is one 6-bp cutter recognition site in every $4096/9 = 455$ bp, on average. Thus, the probability of amplifying a DNA fragment using ETCL–PCR with these 10 different restriction endonucleases is very high. The possibility that the designed primer overcrosses an exon–intron boundary could be overcome by using multiple primers.

Following our previous cloning of rat ATase cDNA,[7] ETCL–PCR was applied to amplify the intron sequences flanking the exons of the rat ATase gene. As shown in Figure 3, three DNA fragments of 668, 384, and 224 bp were clearly amplified by ETCL–PCR using primers oRB183 and oRB185, which annealed to the known rat ATase cDNA sequence. Nucleotide sequencing of these DNA fragments revealed that a cassette, C2, was ligated at the sites of *Bln*I and *Nhe*I as expected (Fig. 4). The alignment of nucleotide sequences of the amplified DNA fragments and the rat ATase cDNA showed that sequence mismatch starts from the sequence of AG, which is a consensus sequence of an acceptor site of mRNA splicing (Fig. 5). Using ETCL–PCR and ordinary PCR we successfully amplified 20 exon–intron boundaries and a promoter region of the rat ATase gene.[19] On the basis of the compiled result, the genomic DNA sequence of rat ATase consists of 11 exons and all exon–intron boundaries conform to the consensus splice junctions. oRB183 and oRB185 anneal to the sequence of exon 9, and the amplified DNA fragments contain the boundary of intron 8 and exon 9 (Fig. 5).

The ETCL–PCR is useful because (1) an end-trimmed DNA and a cassette do not self-ligate and (2) a DNA fragment, which elongates from a primer annealing to a known sequence, is the only specific fragment to be amplified; moreover, (3) changing the 5′ end sequence of AC oligomers makes it possible to apply ETCL–PCR to other restriction enzymes, which produce the 5′ end overhang, in addition to those stated in this report.

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