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Two Step Single Primer Mediated Polymerase Chain Reaction.[1] Application to Cloning of Putative Mouse, β-Galactoside α2,6-Sialyltransferase cDNA

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Abstract–Using the 2 step single primer mediated polymerase chain reaction(PCR), mouse β-galactoside α2,6-sialyltransferase cDNA was cloned. Single primer mediated PCR is a method to amplify a particular DNA fragment beyond its known sequence region. It employs only one primer for the reaction. Compared to other PCR methods to amplify an adjacent sequence of known DNA fragment, this method requires no enzymatic manipulation on template DNA and is applicable to a template on long DNA fragment. First, a short DNA fragment of the enzyme was obtained from mouse cDNA by the usual PCR method using degenerate primers synthesized according to a relatively conserved region in rat and human β-galactoside α2,6-sialyltransferase. Four primers were synthesized based on this sequence, then 2 step single primer mediated PCR were performed to obtain 5' and 3' flanking sequences of this short fragment resulting in 1.0 kb and 1.3 kb fragments being amplified respectively. The integrity of the two fragments was confirmed by an additional PCR using primers synthesized according to the joined sequence, which contained 1.2kb complete putative mouse β-galactoside α2,6-sialyltransferase coding region. The result showed that the specificity and consequently applicability of the single primer mediated PCR for amplifying a particular DNA fragment beyond known sequence region was remarkably improved by the successive 2nd reaction.

Single primer mediated PCR[1,2] is one of the methods used to amplify a particular DNA fragment beyond its known sequence region. It employs only one primer for the reaction. The other end of the planned primer site is generated by partial complementation of the same primer. Compared to other PCR methods to amplify an adjacent sequence of known DNA fragment, such as inverted PCR,[3] rapid amplification of cDNA ends (RACE),[4] anchored PCR,[5] and one-sided PCR,[6] this method has the advantage of requiring no enzymatic manipulation on template DNA and is applicable to long template DNA fragments. It is generally thought to be a rare phenomenon and to have very limited applicability.[1,2]

It is expected that if we combine 2 single primer mediated PCR sequentially, it will increase the specificity and the applicability of the method (Figure 1A). In this experiment, we applied it to a known 150 bp cDNA fragment of mouse β-galactoside α2,6-sialyltransferase to obtain the whole coding region of the cDNA.

β-Galactoside α2,6-sialyltransferase (EC 2.4.99.1) catalyzes the incorporation of sialic acids into terminal positions of glycoconjugate glycans with a NeuAc α2,6-Gal linkage. cDNA sequence from rat[7] and human,[8] along with genomic DNA sequence[9] and tissue specific alternative splicing[10] in rat have been described. To gain further insight into the function and regulation of this enzyme, we intended to obtain the cDNA sequence of the enzyme from mouse. Using this method, we obtained an unknown DNA sequence on both sides of this known cDNA fragment. The resultant 2.2 kb sequence contained the full coding region of mouse β-galactoside α2,6-sialyltransferase.

Materials and Methods

Total RNA was extracted from mouse liver and brain by the guanidium thiocyanate method and purified by ultracentrifugation through 5.7 M CsCl.[11] PolyA-RNA was purified by Oligotex-dT30 (Takara). cDNA was synthesized from polyA-RNA with oligo(dT) by AMV reverse transcriptase XL (Life Science).

A short cDNA fragment amplification

A relatively conserved region was selected from comparison between rat and human β-galactoside α2,6-sialyltransferase cDNA sequences. Degenerate primers were synthesized using 394 DNA synthesizer (Applied Biosystems), and PCR was carried out to obtain a 150bp fragment. The reaction mixture contained 0.1 μg oligo(dT)-cDNA, 40 pmole primer 1 (5'-**TGGGCCTTGGII(AC)AGGTGTGCTGTTG**-3'), 40 pmole of primer 2 (5'-**AGGCGAATGGTAGTTTTTG(AT)GCCCACAT**-C-3'), 200 μM each dNTP, 10 μl 10x Taq buffer

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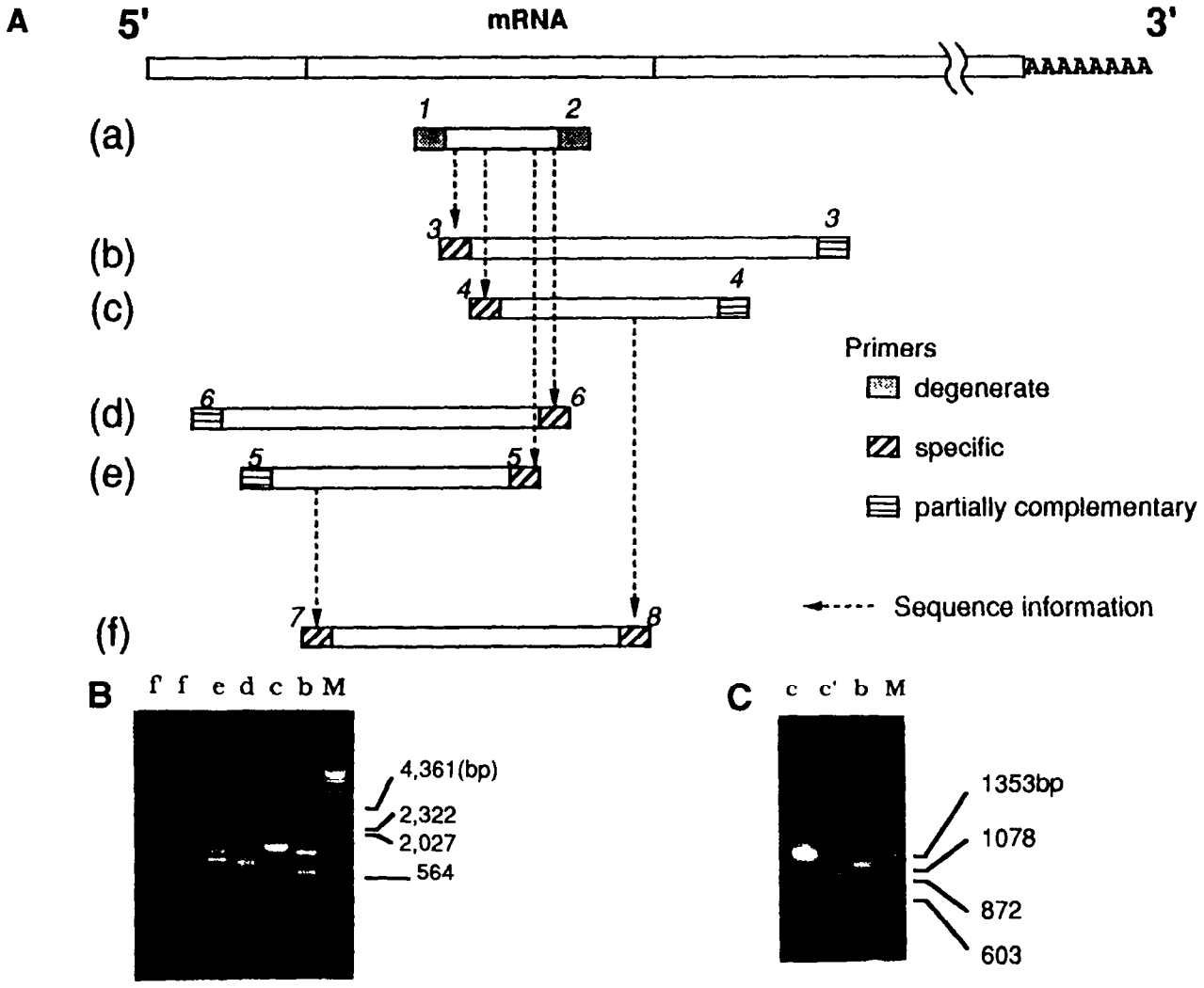

Figure 1. Single primer mediated PCR. (**A**) **a**: A short DNA fragment was amplified from mouse cDNA using primers 1 and 2, cloned into a plasmid and sequenced. **b**: For 3' side, 2 step single primer mediated PCR was carried out. First single primer mediated PCR was with oligo(dT)-cDNA, and primer 3. **c**: The second single primer mediated PCR for 3' side was carried out with primer 4 using 1 μl of the first PCR products (b) as a template. **d**: For 5' side, cDNA was synthesized from mouse liver polyA-RNA and primer 6 and was used as a template in the first single primer mediated PCR with primer 6. **e**: The second single primer mediated PCR for 5' side was carried out with primer 5 and 1 μl of the first PCR product (d) as a template. **f**: Primers 7 and 8 were synthesized based on 5' and 3' side fragments (c and e). PCR was performed with oligo(dT)-cDNA, primer 7 and primer 8. **B**. Lane b: First single primer mediated PCR for 3' side with primer 3. Lane c: Second single primer mediated PCR for 3' side with primer 4. Lane d: First single primer mediated PCR for 5' side with primer 5. Lane e. Second single primer mediated PCR for 5' side with primer 6. Lanes f, f': PCR confirmation of the integrity of the 5' and 3' fragment with primers 7 and 8. The templates were cDNA from liver (lane f) and brain (lane f'). Lanes b–f correspond to fragments b–f in Figure 1A. **C**. The effect of the enrichment of the template in the 2 step PCR. Lane b: The single primer mediated PCR product using primer 3 with oligo(dT)-cDNA. Lane c': The single primer mediated PCR product using primer 4 with oligo(dT)-cDNA as a template. The PCR condition was the same with the 2nd single primer mediated PCR for 5' side except that 0.1 μg oligo(dT)-cDNA was used as a template in place of first PCR product. Lane c: The second PCR product using primer 4 with 1 μl of first PCR product (lane b) as template. Lanes c and d are identical with Lanes c and d in Figure 1B respectively

(Promega, final concentration of $MgCl_2$ was 1.5 mM), 2.5U Taq DNA polymerase (Promega) in 100 µl. PCR condition was 94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 30 sec, at 40 cycles using a DNA thermal cycler (Perkin-Elmer Cetus). The fragment was separated in 3% agarose gel electrophoresis, visualized by ethidium bromide, recovered from the gel using Mermaid DNA extraction kit (Bio 101 Inc.), 5' ends were phosphorylated with T4 polynucleotide kinase prior to ligation into Sma I cut, dephosphorylated vector plasmid pUC119, and finally sequenced. DNA sequencing was carried out using Autocycle DNA sequencing kit and A.L.F. DNA sequencer (Pharmacia).

3' Side fragment amplification

A 2 step single primer mediated PCR was carried out as follows. First PCR was in 100µl containing 0.1µg of oligo(dT)-cDNA, and 40 pmole of primer 3 (Figure 1A, b). PCR conditions were 95 °C for 30 sec, 50 °C for 60 sec, 72 °C for 180 sec, at 40 cycles. The second PCR was carried out with primer 4 using 1 µl of the first PCR products as templates under the same conditions (Figure 1A, c). The 2nd PCR product was washed in Ultrafree C3 (Millipore) filter cup to remove primer and deoxyribonucleotides and cloned into a vector pUC19. Plasmids containing inserts were sequenced without further selection.

5' Side fragment amplification

cDNA was synthesized from 0.3 µg of mouse liver polyA-RNA and 0.1 pmole of specific primer (primer 6) in 20 µl at 42 °C for 2 h, and used as a template for the following 2 step single primer mediated PCR without 2nd strand synthesis. One microliter of this reaction mixture was used as cDNA template in the first PCR with 40 pmole of primer 6 (Figure 1A, d). PCR conditions were 95 °C for 30 sec, 55 °C for 60 sec, 72 °C for 120 sec, at 40 cycles. The second PCR was carried out with primer 5 and 1 µl of the first PCR product under the same conditions (Figure 1A, e). The product was cloned and sequenced as described above.

Confirmation of the integrity of 5' and 3' PCR product

Primers 7 and 8 were synthesized based on 5' and 3' side fragment sequences (Figure 1A, f). PCR was performed in 100 µl containing 0.1 µg oligo(dT)-cDNA from liver or brain, 40 pmole of primer 7 and 40 pmole of primer 8. The PCR conditions were 95 °C for 30 sec, 55 °C for 60 sec and 72 °C for 120 sec, at 40 cycles. The product was cloned and sequenced as described above.

Results

To obtain the DNA fragment coding the relatively conserved sequence of β-galactoside α2,6-sialyltransferase, PCR was carried out with degenerate primers and mouse cDNA as a template and several DNA fragments were amplified. One of these fragments which corresponded to the expected length (150 bp) was cloned into a plasmid vector. Several clones were found to have identical sequences which were highly homologous with the sequence of rat enzyme (Figure 1A, a).

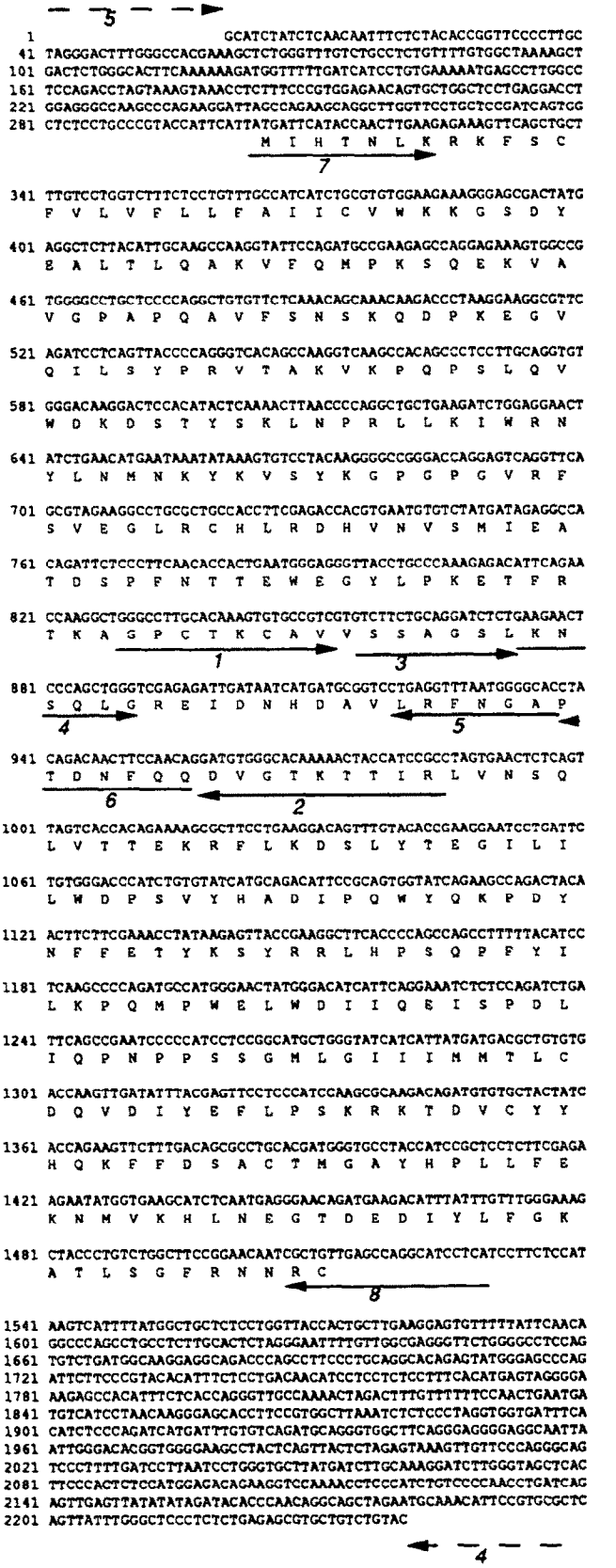

Figure 2. The nucleotide and predicted amino acid sequence of mouse β-galactoside α2,6-sialyltransferase. The translation initiation site was predicted by comparison of rat and human sequences. This sequence corresponds with combined sequence of fragments c and e in Figure 1A. Numbered arrows indicate primer sites. Partially complementary sites for primers 5 and 4 are -20 to -1 and 2241 to 2258 respectively, as indicated by dashed arrows. The sequences of these sites are not available from current experiments (accession number D16106)

Four primers (primers 3-6, Figures 1A and 2) were synthesized based on this sequence and 2 step PCRs were performed with single primer for both the 5' and 3' sides. In both 3' and 5' side experiments, first single primer mediated PCR products (Figure 1A, b and d) were heterogeneous (Figure 1B, lanes b and c) and were not cloned nor sequenced.

The second PCR for the 3' side amplified a 1.3 kb fragment (Figures 1A and B, c), which contained the sequence of primer 4 at both ends, a 0.6 kb sequence of C-terminal part of the enzyme and a 0.7 kb of 3' non-coding region judged from homology with rat (Figure 2). The second PCR for the 5' side amplified several DNA fragments (Figure 1B, lane e). All of them have the sequence of primer 5 at both ends of the fragments, and 1.0 kb, 0.9 kb and 0.6 kb fragments were found to have the sequence identical to the corresponding part of the 150 bp fragments. The 1.0 kb fragment had 0.7 kb of N-terminal part of the enzyme and 0.3 kb of 5' non-coding region (Figure 1A, e).

The integrity of the 5'-side 1.0 kb fragment and 3'-side 1.3 kb fragments was confirmed by additional PCR using primers 7 and 8 synthesized according to sequences on the 5' and 3' side fragments (Figure 1A, f). Fragments which contained the 1209 bp coding region of putative mouse β-galactoside α2,6-sialyltransferase were amplified from both liver and brain cDNA (Figure 1B, lanes f and f').

The 2.2kb joined fragment is not a full length cDNA, sequences outside of the fragment including partially complementary sites of primers 4 and 5 are still unknown, but it does contain the full coding region of β-galactoside α2,6-sialyltransferase, judged from comparison between rat and human enzyme (Figure 2).

The 0.9 kb fragment obtained by 2nd PCR for 5' side contains 17 bp of the sialyltransferase sequence flanking primer 5 at one end, and the adjacent sequence was homologous to intron 2 of the rat sialyltransferase gene.[9] This fragment seems amplified from hnRNA or genomic DNA. The 0.6 kb fragment has 0.5 kb of the sialyltransferase sequence and 80 bp of unidentified sequence. In our experiment, the integrity of this fragment and 3' fragment was not confirmed by PCR with primer 8 and primer 9. The latter was synthesized according to the unidentified sequence of the 0.6 kb fragment. The origin of this fragment remains to be investigated.

The effect of the 2 step PCR is shown in Figure 1C. The PCR products either with primer 3 or with primer 4 (Figure 1C, lanes b and c') contained many bands when carried out with mouse cDNA as a template, but two step PCR, in which first step PCR product (Figure 1C, lane b) was used as template for the second step PCR with primer 4, amplified a single fragment (Figure 1C, lane c).

Comparison with rat cDNA showed that the sequence was well conserved between the 2 species including non-coding region and at least one intron–exon junction (data not shown). Comparison of the amino acid sequences of the 3 species revealed that there was strong conservation in C-terminal catalytic domain,[12] and relatively weak conservation in N-terminal cytosolic and anchor domain.[13]

Discussion

Single primer mediated PCR is totally dependent on partial complementary binding on the secondary site. How often one can expect such a site within the PCR range of the authentic priming site is critical to the applicability of this method. As discussed below, the problem is not whether such sites exist within the range of the PCR but the interference of the nonspecific priming, especially when the annealing temperature becomes as low as 37 °C. Parks *et al.*[2] employed 34–37 °C as annealing temperature for the first 10 cycles followed by 20 highly stringent cycles and achieved amplification of 5 specific products out of 11 primers tested. Although their products were heterogeneous many of them were nonspecific.

In our experiments, the nucleotide sequences of incompletely matched sites are unknown, we cannot assess the extent of matching at partially complementary sites. Wang *et al.*[1] obtained single primer mediated PCR product of known sequence and reported 8 bases and 20 bases matching in 32 bases and 35 bases secondary priming site, respectively. Parks *et al.*[2] reported 14 bases and 10 bases matching in 20 bases and 27 bases secondary priming sites respectively. In both reports at least 2 bases at the 3' ends of the primers are completely matched. To calculate the probability of incomplete matching for a 20 bases primer, let us assume complete matching in 2 bases at 3' end and the matching of more than 9 bases in the remaining 18 bases is sufficient for the partial complimentary binding at low annealing temperature. The probability of the existence of such matching in a given 20 bases sequence is, assuming binomial distribution, as follows:

$$P = \left(\frac{1}{4}\right)^2 \sum_{k=9}^{18} \left(\frac{1}{4}\right)^k \left(\frac{3}{4}\right)^{18-k} {}_{18}C_k = 1.2\times10^{-3}$$

This means one of such a combination of sequences exists on average every 833 bases. This is well within the range of PCR. In our experiments we obtain heterogeneous product when single primer reaction was carried out with cDNA as template (Figures 1B and C, lanes b, d and c'). It is consistent with the calculation above which indicates that significant partial complimentary sites exist on almost all template DNA fragments.

To eliminate such problems and obtain specific product, we carried out 2 reactions sequentially (Figure 1A). Theoretically, two independent primers at one side of the desired DNA fragment carry the same amount of information with a pair of primers on each end of the DNA. It was previously thought that since single primer mediated PCR is a rare phenomenon, to do it 2 times sequentially seems almost impossible. However, as calculated above, it is not the case. Since the primer is perfectly matched at one side, (unlike other cases where both primers are incompletely matched such as degenerate primers made from amino acid sequence), the first single

primer mediated PCR should be carried out with high annealing temperature. It is not necessarily the true chain reaction, but could be a linear increase of single strand DNA extended only from authentic priming site. In the second single primer mediated PCR, if necessary, the annealing temperature may be lowered to facilitate partial complementary binding. In our experiment it was not necessary (50 °C for primer 4 and 55 °C for primer 5). The annealing temperature is not required to be extremely low, because the specific template is enriched by the first reaction. The idea of the two step reaction is to increase the chance of partial complementary binding not by the lower annealing temperature but by enriching the specific template by the first reaction.

The effect of the enriched template for increasing specific product is shown in Figures 1B and C. The second PCR products (lanes c and d) showed a dramatically reduced number of bands compared to first PCR products (lanes b, d and c'). Thus, both 5' and 3' side specific fragments were obtained by the 2 step single primer mediated PCR method.

The advantage of single primer mediated PCR over other methods such as RACE or inverse PCR is that it is applicable to a template on a long DNA fragment. In the other methods, the ends of the template DNA or a restriction site must exist within the PCR range (usually up to 2 kb). It is especially useful when amplifying 3' side of mRNA, because sometimes a 3' non-coding region extends more than 2 kb. On the other hand, if the expected length of the template is less than 1 kb, often the case in a 5' side of mRNA, RACE, anchored PCR or one-sided PCR is a better choice since it is not likely to have a suitable partial complementary binding site, and even if one exists, the obtained fragment will be shorter than that obtained by other methods. Another advantage of the method is its convenience, as it requires no enzymatic manipulation of template DNA as required in RACE or anchored PCR. When applying these methods (i.e. single primer mediated PCR, RACE, etc.) in parallel, one should keep it in mind that efficiency of the single primer mediated PCR is dramatically suppressed in the presence of other primers used in RACE or anchored PCR such as oligo(dT) or universal primers (data not shown).

In conclusion, we have established the rapid and convenient system—two step single primer mediated PCR—for getting the desired cDNA clone. This new method is particularly useful for cloning a gene which has very limited sequence information.

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