4530–4531 Nucleic Acids Research, 1995, Vol. 23, No. 21 © 1995 Oxford University Press

Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction

Arun K. Datta

Cytel Corporation, and the Department of Chemistry and Molecular Biology, Scripps Research Institute, 3525 John Hopkins Court, San Diego, CA 92121, USA

Received June 1, 1995; Revised and Accepted October 3, 1995

Among the procedures for site specific introduction of mutations (1) polymerase chain reaction (PCR) has been increasingly used because of its simplicity over other techniques. For example, Kunkel's method involves multiple steps and the yield of the single stranded DNA depends on the orientation of the insert as observed in our earlier work (2). PCR based mutagenesis, on the other hand, does not require any special treatment of the DNA; plasmid DNA can be used successfully. The technique originally introduced by Kammann *et al* (3) and later modified by others (4,5) is cost-effective among the PCR based methods since it uses only one mutant oligomer for each site-directed mutagenesis. This method involves two steps; the first step is performed using the mutant oligomer containing the desired mutation and the resulting product, termed 'megaprimer' (5), is then used in a second round of PCR. In the author's experience, the yield of final PCR product is often very poor supporting the observations made by other previous workers (6–8). This is probably due to the poor efficiency of priming of the megaprimer (300 oligomers or more) to the template. In this study, it is shown that initial five cycles of asymmetrical PCR using only the megaprimer greatly improves the yield of the final product.

Following the modifications described here the author has successfully generated numerous mutants of the enzyme Galβ1,4GlcNAc α2,6-sialyltransferase. This enzyme transfers sialic acid from its nucleotide sugar to various asparagine-linked glycoproteins (11). As summarized in Figure 1, the 1.6 kb cDNA (GenBank accession no. M18769) subcloned in pBluescript (12) was used as a template in a 100 µl reaction mixture containing 80 µM dNTPs, and 1× *Pfu* DNA polymerase buffer (Stratagene, CA). The forward primer commonly used in our various experiments was: GCTCTAGAATTCCAATCCTCAGTTACCACAG (sense 5′–3′, nt 214–236). For the mutant primer, typically ~22mer oligonucleotide is designed with the T_m of at least 60°C. Template DNA (10 ng) and 25–50 nmoles of each primer were used in the first step. The PCR was started with the addition of 2.5 U of *Pfu* DNA polymerase (Stratagene, CA) following the conditions: 94°C, 30 s; 56°C, 1 min; 73°C, 2 min, for 20 cycles (the number of cycles were kept at a minimum to avoid any undesired mutations). The analysis of this product using 1% agarose gel showed the generation of one major band, the double stranded megaprimer. This was purified to remove the forward primer which is essential in order to obtain desired mutant product with high efficiency. Because a single major product was obtained in the first step, purification using Geneclean (Bio101, CA) was sufficient without gel electrophoresis for complete removal of the forward primer. The purified megaprimer was then used in the second step. To maximize the yield of the final product, PCR was carried out for five cycles using only the megaprimer. Reaction mixture (100 µl) for the 2nd step contained ~50 ng of megaprimer (estimated from the agarose gel), 10 ng template and 80 µM dNTPs in the 1× *Pfu* DNA polymerase buffer. The reaction was started with the addition of 2.5 U of *Pfu* DNA polymerase following the conditions: 94°C, 1 min; 73°C, 3 min, for five cycles. While at 73°C, the tubes were taken out, and the reverse primer CCAGGAGAGGATCCATAAAATGAC (5′–3′; nt 1270–1247) was added. The tubes were gently vortexed, briefly centrifuged, placed in the thermocycler (Perkin Elmer 9600) and the reaction was continued following the conditions: 94°C, 1 min; 68°C (depending on the T_m of the reverse primer which in our experiment was 70°C), 1 min; 73°C, 3 min, for 20 cycles. Gel analysis of the 5–10 µl reaction mixture indicated the generation of one major band of 1.05 kb for the specific product [Fig. 1, (b)]. Using megaprimers of various sizes (ref. 9 and this study), it has been observed that the efficiency of the yield of the final product depends inversely on the size of the megaprimer produced in the first step. As shown in Figure 1C, the yield of the final product was comparatively better using a 450 bp fragment as the megaprimer. The final product was purified by agarose gel electrophoresis followed by Geneclean. For the cDNAs of P318A, S319A, S320A, C332A, E339A, and F340A (the nomenclature for the mutant sialyltransferases reflects the residue number for which alanine was substituted; e.g., for P318A, proline 318 is replaced by alanine, etc.), the gel purified product was digested with *Bst*BI (at nt 824) and *Bsp*E1 (at nt 1194), and the 370 bp fragment was purified and subcloned into similarly digested and purified larger fragment of spST-2 (13) following standard procedures (10). For subcloning D219A, V220A and S222A, *Apa* I (at nt 629) and *Bst*BI were used as mentioned earlier (9). The ligation mixture was directly used to transform competent cells of *E.coli* (strain GM2163) and 2–3 colonies were selected for sequencing. The mutations were confirmed by dideoxy double-stranded sequencing (14) of the entire fragment that has been subcloned, including the restriction sites used.

Previous to this study, there were several reports mentioning the difficulty of obtaining the final products using megaprimer method. While the megaprimer of various sizes could be obtained without difficulty, it was observed that the products of the second step of PCR using those megaprimers were little or none following the previous procedures (3–5) supporting the observations reported earlier (6–8). To increase the yield of the final products, Aiyer and Leis (7) introduced three steps of PCR using four primers. However, as shown in the present study, the two steps PCR using three primers as originally described (3) are effective in obtaining the final products in good yield provided the linear amplification introduced at the begining of the second step of PCR is followed. As shown in Figure 1C, without this modification, the final product obtained were heterogenous in size with little or no specific product. The reason of this efficient amplification following the linear amplification of the products is not very clear. Neverthless, using megaprimers of various sizes, it appears that the inefficient amplification using original megaprimer procedure

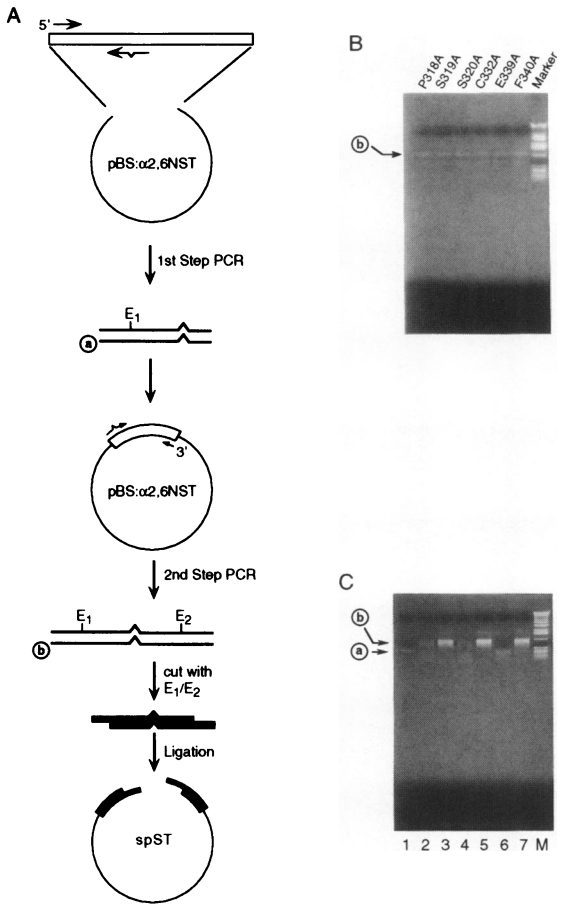

Figure 1. (**A**) Schematic diagram of the PCR strategy used in this study. In the first step, the mutant antisense oligomer and a common forward primer are used to amplify and incorporate the mutation in a desired site of the cDNA. The product is used as a megaprimer (a) in the second step. After five cycles of asymmetrical PCR using only the megaprimer, a reverse common primer is added and the reaction continued to obtain the final product (b). The final purified PCR product is subcloned using two unique restriction enzymes E_1 and E_2 following standard techniques (10). This strategy was successful to obtain number of mutants of Galβ1,4GlcNAc α2,6-sialyltransferase as shown in (**B**) and (**C**). The cDNA for the Galβ1,4GlcNAc α2,6-sialyltransferase subcloned in pBluescript KS+ plasmid (12) was used as a template. The mutagenic antisense oligonucleotides (the substituted nucleotides are underlined) used for construction of the corresponding cDNAs were (5′–3′): for P318A, GCCGGAGGATGCGGGATTTGGCTGA (nt. 963–939); for S319A, CCAGCATGCCGGAGGCTGGGGGATT (nt. 970–946); for S320A, CCAGCATGCCGGCGGATGGGGGATT (nt. 970–946); for C332A, CCTGGTCAGCCAGCGTCATC (nt. 1003–984); for E339A, GGGAGGAACGCGTAAATATCTA (nt. 1025–1004); for F340A, GGATGGGAGGGCCTCGTAAATA (nt. 1029–1008); for D219A TTTTGAGCCCACAGCCTGTTGGAAGTTGT (nt. 669–641); for V220A, GTTTTTGAGCCCGCATCCTGTTGG (nt. 671–648); and for S222A was: GGTAGTTTTTGCGCCCACATCCTGTTG (nt. 675–649). The two PCR steps and the subsequent subcloning of the final products were carried out as mentioned in the text. In (C) the final PCR product obtained was shown without (lanes 2, 4 and 6) and with (lane 3, 5 and 7) linear amplification at the begining of the second step using the megaprimers for D219A, V220A and S222A, respectively; lane 1, the purified megaprimer obtained in the first step using mutant oligomer for D219A. 1 kb DNA ladder (GIBCO-BRL) was used as a marker (M).

(3–5) might be due to the decreasing efficiency of the annealing as the sizes of the megaprimers increases, and the linear amplification probably helps to improve the efficiency of this annealing.

A modification of the megaprimer method has recently been described using parallel templates to increase the yield of the final products (8). This method requires subcloning of the cDNA of interest in two different vectors. Moreover, this procedure yielded mutants with 60% efficiency. On the other hand, following the procedures described here we have successfully obtained several mutants (see ref. 9 and this study) with about 100% efficiency. One critical step in this present technique is to purify the megaprimer from the forward primer after the first step of PCR. The 'carry over' of the forward primer in the second step of PCR may yield clones with wild type sequence. In this study, the author routinely sequenced three clones from each experiment and found all had desired mutation(s).

It may also be noted that, in the present study, the recombinant *Pfu* DNA polymerase has been used instead of *Taq* DNA polymerase. It is known that *Pfu* DNA polymerase has at least 12-fold higher fidelity of DNA synthesis than *Taq* DNA polymerase (Stratagene), thus increasing the mutagenesis efficiency. In fact, when examined, this author did not find any undesired mutation in sequencing a total of 12 kb of PCR products. Another disadvantage of using *Taq* DNA polymerase is that this enzyme often incorporates an extra non-templated nucleotide residue A at the 3′-end of the PCR product, thus making it difficult to obtain the desired protein product without a frameshift (8). Moreover, the PCR is easier to perform because no modification of Mg^{++} is necessarry while using the recombinant form of *Pfu* DNA polymerase, as opposed to *Taq* DNA polymerase.

In conclusion, the mutagenesis method described here is highly efficient to obtain desired final products in good yields using'megaprimer' of various sizes. The effectiveness of this method has made it possible for the author to study the structure–function relationship of the enzyme Galβ1,4GlcNAc α2,6-sialyltransferase using a large number of mutants.

ACKNOWLEDGEMENTS

The author would like to thank Dr James C. Paulson, Senior Vice President, Research and Development, Cytel Corporation, for support and encouragement; and Dr. Gary Hermanson for critical reading of this manuscript. This work was supported in part by United States Public Health Services Grant GM27904 (to J.C.P.).

REFERENCES

1 McPherson, M. J. (1991) *Directed Mutagenesis- A Practical Approach.* IRL Press, Oxford, UK.
2 Datta, A.K., and Lehrman, M.A. (1993) *J. Biol. Chem.* **268**, 12663–12668.
3 Kammann, M, Laufs, J., Schell, J., and Gronenborn. (1989) *Nucleic Acids Res.* **17**, 5404.
4 Landt, O., H.-P. Grunert and U. Hahn.(1990) *Gene* **96**, 125–128.
5 Sarkar, G., and S.S.Sommer. (1990) *BioTechniques* **8**, 404–407.
6 Barik, S., and Galinski, M.S. (1991) *BioTechniques* **10**, 489–490.
7 Aiyar, A., and Leis, J. (1993) *BioTechniques* **14**, 366–369.
8 Upender, M., Raj, L., and Weir, M. (1995) *BioTechniques* **18**, 29–31.
9 Datta, A.K., and Paulson. J.C. (1995) *J. Biol. Chem.* **270**, 1497–1500.
10 Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
11 Weinstein, J., Lee, E. U., McEntee, K., Lai, P., and Paulson, J. C. (1987) *J. Biol. Chem.* **262**, 17 735–17 743.
12 Lee, E.U., Roth, J., and Paulson, J.C. (1989) *J. Biol. Chem.* **264**, 13848–13855.
13 Colley, K.J., Lee, E.U., and Paulson, J.C. (1992) *J. Biol. Chem.* **267**, 7784–7793.
14 Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.