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Three-step PCR mutagenesis for 'linker scanning'

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ABSTRACT

'Linker scanning' has been used as an efficient method for systematically surveying a segment of DNA for functional elements by mutagenesis. A three-step PCR method was developed to simplify this process. In this method, a set of 'mutation primers' was made with 6 to 8 base substitutions in the center of the primers. In the first PCR reaction, these 'mutation primers' are paired with an 3′ primer from the opposite end of the analyzed sequences to form a 'ladder' of fragments containing the base pair substitutions. These are used as templates in the second PCR with the 3′ primer as the only primer to generate single stranded sequences, which are used as primers in the third PCR paired with an 5′ primer to complete the mutagenesis. We have tested the method in a mutation screen of the steroid sulfatase promoter. Its application to general site specific mutagenesis is discussed.

INTRODUCTION

'Linker scanning' is a systematic mutagenesis method developed by McKnight et al. (1) to study transcriptional control signals in a eukaryotic promoter. In this method, clusters of point mutations are introduced at discrete locations, scanning across a region of DNA known to contain the transcriptional control components of a gene. Individual mutants then are tested for the retention of transcriptional competence by a functional assay. The major advantage of this method is that it causes minimal change in the spacing between sequences. This is important because changes in distances between transcriptional regulatory sequences may affect their function. A disadvantage of the original linker scanning analysis is that it is tedious and time consuming. It requires the construction of two complementary libraries of deletion mutants generated by exonuclease III and S1 nuclease, linked to the same synthetic restriction enzyme recognition site. The end points of each 3′ and 5′ deletion mutant must be sequenced, and then the 'matching' 3′ and 5′ deletion mutants are combined at the synthetic restriction enzyme site in such a way that the linker occupies the space between the 3′ and 5′ termini to restore the full length of the original sequence. In the course of studying sequences in the steroid sulfatase promoter region, we developed a polymerase chain reaction-based mutation scanning method which is much simpler and faster than the method McKnight et al. proposed. In the new method, mutations can be pre-made with primers. By a three-step-PCR, mutant constructs can be efficiently generated.

MATERIALS AND METHODS

PCR primers were obtained from the DNA Synthesis Facility of the Howard Hughes Medical Institute/UCSF. Restriction enzymes and T4 DNA ligase were from New England Biolabs. Taq polymerase and 10×reaction buffer were from Promega. Agarose was obtained from BRL and NuSieve agarose was from FMC BioProducts. The Sequenase kit for dideoxy sequencing was purchased from United States Biochemical. The TA cloning kit was obtained from Invitrogen and a DNA Thermal Cycler from Perkin-Elmer Cetus was used for carrying out PCR reactions.

Plasmids and PCR primers

pSV0CAT was derived from pSV2CAT (2) by deleting the SV40 promoter sequence. Putative promoter sequences can be inserted into the HindIII site in front of the CAT gene to test promoter activity.

Plasmid P3 is a test plasmid which contains a portion of the steroid sulfatase 5′ sequence with a 390bp deletion between an upstream regulatory element and the putative promoter sequence, inserted into the HindIII site of pSV0CAT. Previous experiments have shown that the plasmid P3 has promoter activity in various cultured cells. (data not shown).

Mutation primers: Mutation primers are 24–25mers made with an 6-base alteration in the middle of the sequence as shown in Figure 1.

The 3′ primer has a HindIII site at its 5′ end: 5′-AGAAGC-TTGCTGGAGGCTTGGC- 3′.

The 5′ primer has an NdeI site at its 5′ end: 5′-AGCATA-TGTAGACTGCAATTTTGC- 3′.

Both restriction sites are used for the directional cloning of the PCR fragment into pSV0CAT.

PCR conditions

The first PCR reaction was carried out with 20 pmol of each mutation primer paired with 20 pmol of the 3′ primer with plasmid P3 as template, (which contains 110bp STS promoter plus upstream regulatory sequence), in a final volume of 25μl (Figure 2a). The reactions were carried out at 95°C for 50 seconds, 45°C for 1 minute, 72°C for 1 minute. After 40 cycles,

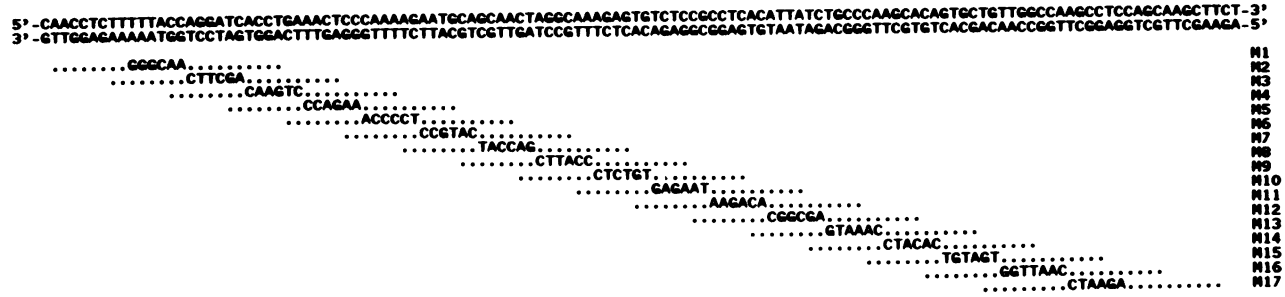

Figure 1. Mutation primer sequences. The top sequence is the 110bp promoter element of the human steroid sulfatase gene. M1 through M17 are mutation primers. The substituted nucleotides are shown and the unchanged nucleotides indicated by dots.

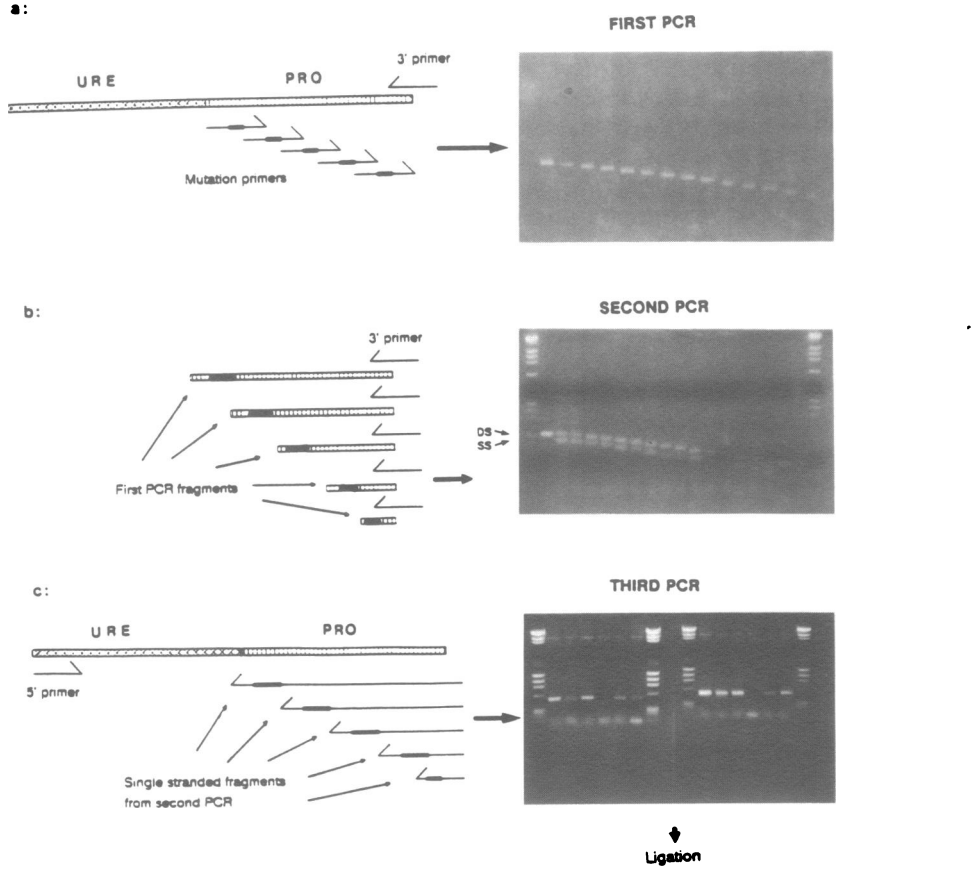

Figure 2. Outline of the procedure for generating mutation scanning plasmids. In each panel, the components in each PCR reaction are shown on the right, and the ethidium bromide stained gel of the resulting PCR reaction products is shown on the left. Panel a, the first PCR reaction: The template, mutation primers and the 3′ primer used in the first PCR reaction are shown. The heavy bars in the mutation primers indicate the positions of the 6 base substitutions. URE: upstream regulatory element. PRO: promoter sequence. Panel b, the second PCR reaction: The first PCR fragments, which have the nucleotide substitutions indicted as heavy bars, were used as templates. 3′ primer is added in the asymmetric PCR reactions. The first and the last lanes on the ethidium bromide stained gel are template DNAs which have not been subjected to PCR to demonstrate the position of the double stranded DNAs. DS: double stranded DNA. SS: single stranded DNA. Panel c, the third PCR reaction: The single stranded DNAs generated from the second PCR reactions were used as primers and paired with the 5′ primer. The heavy bars indicate the position of the mutations. These reactions generate full length URE/PRO fragments with different mutation substitutions.

5μl of this mixture was used in a subsequent asymmetric PCR in which only one primer, the 3′ primer was present (20 pmol) (Figure 2b). 30 cycles of amplification were then carried out, during which the annealing temperature was increased to 60°C. These reactions generated single stranded DNA's which were gel purified by Nusieve agarose and were used in the third PCR as primers paired with the 5′ primer. 40ng of plasmid P3 was used as the template and the annealing temperature was 45°C. After 40 cycles, the full length sequence of interest was generated but now each PCR fragment contained a 6bp mutation in the

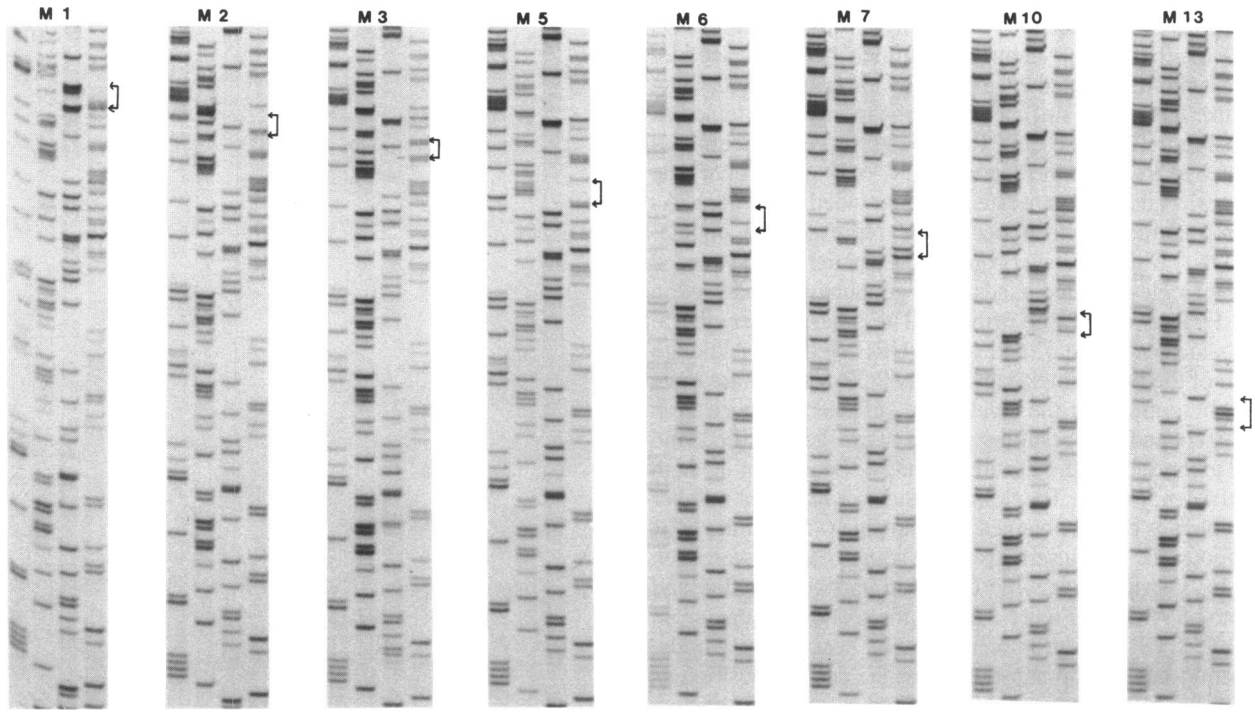

Figure 3. Dideoxy sequencing data of some of the mutant plasmids generated by the method described. The nucleotide positions were in the order A,G,C,T in each panel. The arrows bracket the positions of the mutagenized nucleotides.

promoter region and a HindIII site at the 3′ end as well as an NdeI site at the 5′ end of the fragment (Figure 2c). The amplified PCR products were eluted from an agarose gel as described by Heery et al. (3). Briefly, the agarose block with the correct PCR fragment was placed into a tube to which a hole has been punched at the bottom with a 23G needle and covered by glass wool. These tubes were placed inside a clean 1.5ml microcentrifuge tube, and centrifuged for 2 minutes at 10K RPM in a table top microcentrifuge. This was followed up by an additional 10 minute spin at a maximum speed of 14K RPM. The flowthough was collected and used directly for ligation.

Cloning and sequencing

The PCR products were cloned into the TA cloning vector from Invitrogen. The protocol for TA cloning was modified as follows: 10μl reaction buffer plus 2μl pCR™II were prepared according to the manufacturer's recommendations. This was divided into 5 tubes so that 2μl of reaction mixture were used per ligation plus 0.5μl of PCR product. After incubation at 12°C for 4 hours, the ligation mixtures were used to transform *E.coli* supplied by the company. Plasmid DNA was isolated from white colonies and digested with HindIII and NdeI. The inserts were recloned into pSV0CAT which was also digested with HindIII and NdeI. Clones were randomly picked and sequenced by the double-stranded sequencing method (USB). 90% of the clones made in this way contained an insert with the desired mutations.

RESULTS AND DISCUSSION

We have constructed a series of mutation scanning plasmids for studying the STS promoter region by using a three-step PCR method described above. The STS promoter consists of a 110bp sequence plus an upstream regulatory element (URE) separated by 390bp (Li et al., unpublished). The 110bp promoter sequence has little or no basal promoter activity without the URE. We have used a plasmid containing both the 110bp promoter and the URE sequence as a template to generate the mutations. The results

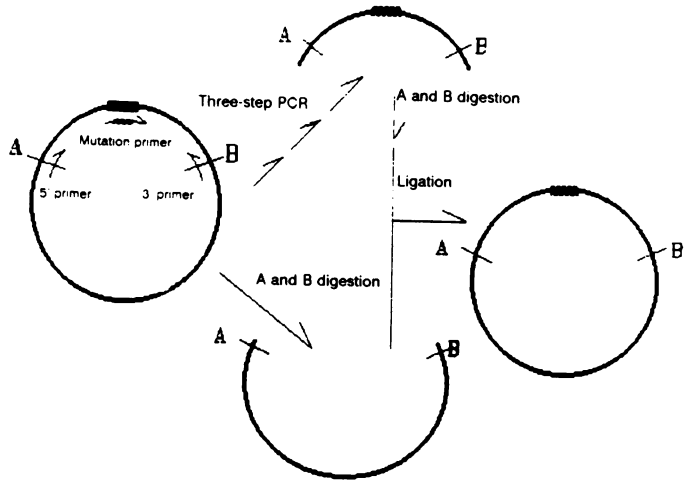

Figure 4. Outline of the strategy of using a three-step PCR to generate mutations in any plasmid sequence. A and B indicate restriction enzyme recognition sites that are unique in the plasmid. The black bar indicates the position at which a mutation is desired. After the three-step PCR, the engineered base pair substitutions were made as indicated by the shaded bars. The resulting PCR fragments and the parental plasmid segments can then be religated.

are summarized in Figure 2. The 3′ primer, which is at the 3′ end of the promoter sequence was paired with each mutation primer for the first PCR. Although the primers have 6 nucleotides differences with respect to the template, by lowering the annealing temperature, clean and sufficient amplification was obtained as shown in Figure 2a. These PCR products served as templates for the second PCR. In the second PCR, only one primer, the 3′ primer was added to generate single-stranded sequences. As shown in Figure 2b, the upper bands represent the double-stranded DNA while the lower bands represent the single-stranded DNA. The single-stranded bands were gel purified, and used in the third PCR as one of the primers. The other primer, the 5′ primer, is located at the 5′ end of the URE element. Although the length of the two primer pairs in this PCR reaction are quite different, by optimizing the conditions, sufficient amplification was obtained and the resulting bands were gel purified and subjected to the subsequent ligation reactions. Because of the high cloning efficiency of the TA cloning system, a sufficient number of white colonies can be obtained by just using 0.5 μl of the PCR amplification mix. The inserts from the white colonies were then released by restriction enzyme digestion and gel purified and ligated into pSV0CAT. Figure 3 shows the sequencing data from some of the STS URE/promoter mutation scanning plasmids. Each plasmid has an 6bp substitution at the desired position as indicated. This demonstrates that the method described is efficient and the mutants made this way are correct.

This method can also be used to make site specific mutations at any position of any sequence rapidly and efficiently. The strategy is depicted in Figure 4. The sequences around two adjacent unique restriction enzyme sites can used as the 3′ and 5′ primers. The mutation primer carries the desired nucleotide substitutions. After the three-step PCR reactions, the resulting mutagenized fragment can be digested by the two restriction enzymes and ligated back into its original position. It has been observed that Taq Polymerase may produce errors at a frequency of approximately one in 2×10^4 per nucleotide per cycle (4). In the method described, the sequences generated by the PCR reactions are restricted to short fragments whose sequences are easy to verify. The rest of the plasmid sequences are not subject to amplification by Taq polymerase and so unwanted errors can be minimized.

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