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Construction of mutant and chimeric genes using the polymerase chain reaction

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ABSTRACT

In the polymerase chain reaction (PCR) the specific amplification of a small segment of DNA within a complex DNA sample is effected by repeated cycles of DNA denaturation and enzymatic synthesis primed by two oligonucleotides complementary to regions within opposite strands of the DNA. In this report a simple and efficient method is described in which PCR methodology is used to introduce specific mutations into a double stranded DNA molecule. In this procedure a supercoiled plasmid DNA serves as template for a PCR in which a primer bearing the mutated sequence is incorporated into the amplified product. The presence of convenient restriction sites in the mutagenic primer and in the amplified DNA permit direct replacement of a wild type DNA segment with the mutated segment by treating the PCR mixture with the appropriate restriction endonucleases followed by DNA ligase. Using this procedure, a single amino acid replacement, a 16 amino acid deletion and a replacement of four amino acids with a twelve amino acid segment from another membrane protein were introduced into the amino terminal signal segment of rat hepatic cytochrome P450b (P450IIB1).

INTRODUCTION

Deletion, insertion and point mutations can be constructed in vitro by oligonucleotide-mediated, site-directed mutagenesis procedures in which appropriate oligonucleotides containing the mutation are used to prime DNA synthesis on a single stranded DNA template (1). In most cases, the wild type gene, present in single stranded form as a result of its prior cloning into a single stranded phage cloning vector, serves as template. Subsequent analysis of the phenotype of each mutation usually requires recloning into an appropriate expression vector.

In the original procedures used for site-directed mutagenesis, the efficiency of obtaining mutants was quite low because of the high frequency of plaques resulting from parental DNA molecules used as template. Recently these procedures were improved by incorporating steps that lead to specific destruction of parental DNA molecules in the transfected cells (2-4).

We have sought to introduce specific mutations into the amino-terminal twenty amino acid residues of rat hepatic microsomal P450b (P450IIB1). This region of the molecule serves as a combined insertion-halt transfer signal that mediates the cotranslational insertion of the polypeptide into the endoplasmic reticulum membrane

but blocks translocation of downstream sequences and serves to anchor the protein in the membrane (5). The segment contains 16 consecutive hydrophobic residues immediately after the four amino terminal residues of the polypeptide.

For these studies, we have developed an exceptionally simple procedure of general applicability that uses the PCR to mutate and amplify a specific portion of a gene and facilitates oligonucleotide-mediated, site-directed mutagenesis and the construction of chimeric genes. In the PCR procedure (6,7) which has recently been simplified and improved (8), two synthetic oligonucleotides that are complementary to regions within opposite strands of a double stranded DNA molecule are used to prime the geometric multiplication of the DNA segment extending between the primers. This involves repeated cycles of DNA denaturation, annealing of primers and DNA synthesis effected by a thermostable DNA polymerase. If the primers bear base substitutions, insertions or deletions or contain linked sequences encoding a portion of a different protein, one would expect that the amplified product would retain the sequence modifications incorporated into the primers. In the examples presented in this work, the oligonucleotide primers and the amplified DNA segment also contain convenient restriction sites that are unique in the recombinant plasmid used as template. Consequently, the amplified fragment, after endonuclease digestion, is easily cloned into the initial plasmid to replace the corresponding wild type segment so that mutants are obtained with a very high frequency. Simple modifications of the procedure should permit mutations to be introduced several hundreds of base pairs from a convenient restriction site, as well as the construction of in-frame fusions of extensive coding regions of different cDNAs.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Restriction endonucleases and modifying enzymes (T_4 ligase, etc.) were purchased from New England Biolabs (Beverly, MA.) and Taq DNA polymerase from Perkin-Elmer/Cetus Instruments (Norwalk, CT.).

Oligonucleotide primers were synthesized on an Applied Biosystem Model 380A automated DNA synthesizer (Foster City, CA.) and purified by gel electrophoresis as described by the manufacturer. Four different primers were used:

Primer A, 24mer: 5' CTGCATGAAGGAATTGAGGAGGCC 3'

Primer B, 45mer: 5' GCTTGATATCCAACTATCTCATCCACCATGCGCCCCAG TATCTTG 3'

Primer C, 42mer: 5' CTCATCCACCATGGAGCCCAGTAGGGGACACCCAAAGT CCCG 3'

Primer D, 61mer: 5' CAGGACCATGGCCAAGAGGAAATTTTCTGGCTTAGAAA TCACTATCTTGCTCCTCCTTGCT 3'

In the last three primers, the triplet representing the initiation codon in the mutated gene is underlined.

Recombinant Plasmids

The recombinant plasmid pSP450 (5,9) contains the entire coding region of P450b cDNA in the plasmid vector pSP64. A unique NcoI site (CCATGG) is present at position -2 to +4 encompassing the P450 initiation codon and a unique PstI site is located approximately 135 bp downstream (10). The plasmid was modified by the insertion, at the NcoI site, of a synthetic 39bp double stranded oligonucleotide that had cohesive NcoI sites at both ends and internal EcoRI, EcoRV, and HindIII sites that are convenient for transferring the insert into other vectors. The modified plasmid, pSR-P450, was used as the template for the polymerase chain reaction to produce the three different types of mutants described below. To complete the construction of the deletion mutant we first prepared a plasmid (pGEM-P450) in which the NcoI site in pSR-P450 upstream of that encompassing the initiation codon was removed. This was accomplished by excising the cDNA insert in pSR-P450 with EcoRI and cloning it into the EcoRI site of a modified pGEM3 plasmid which had been treated to remove the PstI site in its multiple cloning site. pGEM-P450 again contains unique NcoI and Pst sites in the P450 cDNA insert and could be used as a recipient of a PCR-amplified DNA segment after removal of its original NcoI-PstI fragment.

Restriction endonuclease digestions, DNA ligations and bacterial transformations were carried out as described (11).

Polymerase Chain Reaction

The reaction mixtures (100ul) were: 10mM Tris HCl, pH 7.3; 50mM KCl; 2mM $MgCl_2$; 80nM each deoxynucleotide triphosphate, and contained 1 ug of primer (corresponding to 130 pmol of primer A, approximately 70 pmol of primers B or C, or 50 pmol of primer D), DNA [0.1 ug (.034pmol) or 1.0 ug (.34 pmol) of pSR-P450] and Taq polymerase as indicated. The reactions were carried out for 30 cycles using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT.) although fewer cycles would certainly have been adequate. Each cycle included a heat denaturation step at 94°C for 1 min, followed by annealing of the primers to the DNA at 55°C for 2.5 min and DNA chain extension with Taq polymerase for 2.5 min at 72°C. The reaction mixtures were overlaid with a drop of light mineral oil, and, after the incubations were completed, were extracted once with chloroform prior to the digestion with restriction endonucleases.

DNA Sequencing

Double stranded DNA was sequenced by the dideoxy chain termination method (12) using a GEM SeqK/RT sequencing Kit (Promega Biotech; Madison, Wisconsin) and the SP6 universal primer according to the manufacturer's instructions.

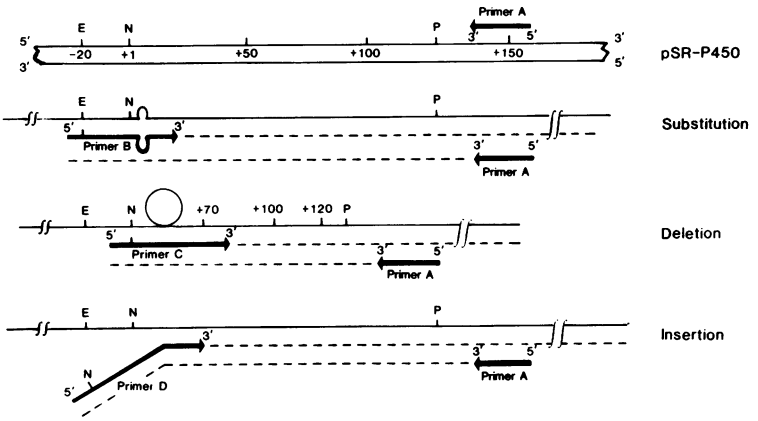

Figure 1: Scheme for the construction of mutants. Three different mutagenic primers (primer B, C, D) complementary to the P450b cDNA noncoding strand and a common second primer (primer A), complementary to the coding strand were used to generate the three different mutations using pSR-P450 as template as indicated. The sequences of the primers are given in Materials and Methods. Residue 1 corresponds to the first residue in the coding region. E = EcoRV; N = NcoI; P = PstI. Cleavage of the amplified DNA and the template with PstI and either NcoI or EcoRV followed by incubation with ligase leads to insertion of the amplified mutated fragment in place of the corresponding initial fragment.

RESULTS

Experimental Design

Three different types of mutations were introduced within the P450b signal. The first mutation was a conversion of codon 2, GAG, for glutamic acid, to CGC, for arginine. The second was a deletion of the hydrophobic core of the signal, residues 5 to 20 of the polypeptide. The third mutation involved a replacement of the first four residues of the P450 with the first twelve residues of the intestinal pro-sucrase-isomaltase, (13).

Our strategy (Fig. 1) was to use an oligonucleotide primer (primer A) complementary to residues 147 to 171 in the coding strand of the P450 cDNA sequence and a series of mutagenic primers that anneal to the noncoding strand of the cDNA near the junction of the 5' non-coding region and the coding region, to prime the PCR. This would specifically amplify the 5' portion of the cDNA while incorporating the mutated sequences into the amplified product. The amplified DNA could then be cleaved with PstI and either NcoI or EcoRV and the resultant fragment cloned back into the parental plasmid that had been cleaved with the same pair of restriction enzymes.

To generate the single amino acid change, an oligonucleotide 45 residues in length (primer B) extending from -27 to + 18 was used as a primer for the PCR. This sequence spans the EcoRV site and contains the triplet CGC at positions +4 to +6

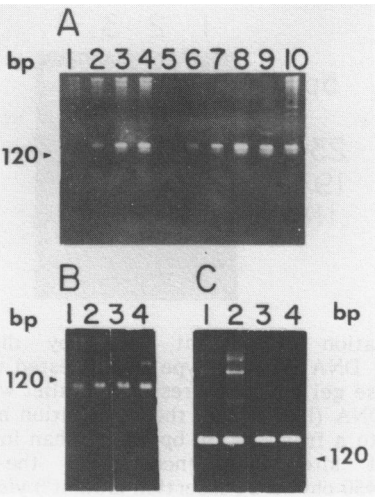

Figure 2: Agarose gel electrophoresis of PCR amplification products. (A) To introduce the single amino acid substitution, primers A and B were used with either 1.0 ug of pSR-P450 DNA (lanes 1-4, 10) or 0.1 ug DNA (lanes 5-9) and either 0 (lanes 1 and 5), 1 (lanes 2 and 6) 2 (lanes 3 and 7), 5 (lanes 4 and 8) or 10 (lanes 9 and 10) units of Taq DNA polymerase. (B) Primers A and C were used to effect the deletion of 16 amino acid residues in the PCR. The reaction mixtures contained either 1 ug of DNA (lanes 1 and 2) or 0.1 ug DNA (lanes 3 and 4) and either 5 units (lanes 1 and 3) or 10 units (lanes 2 and 4) of Taq DNA polymerase. (C) The PCR was carried out as in (B) using primers A and D to effect the replacement of the first four amino acids of P450 with the first 12 residues of prosucrase-isomaltase. The DNA markers used were a HaeIII digest of ϕX174 RF DNA (Pharmacia, Piscataway, N.J.).

instead of GAG. To effect deletion of residues 13 to 60 in the cDNA, an oligonucleotide 42 residues long (primer C) complementary to residues -10 to +12 and 61 to 80 of the cDNA sequence was used as primer. The third mutation was constructed by taking advantage of the fact that any sequence can be linked to the 5' end of a primer used in the PCR and that sequence will become incorporated into the amplified product (14). In this case, the primer (primer D) was complementary to residues 13 to 30 in the noncoding DNA strand and was preceded by a sequence that included the first 12 codons of the pro-sucrase-isomaltase, an NcoI site that encompasses the initiation codon, and five additional residues corresponding to the 5' untranslated sequence in P450b cDNA.

Optimization of Conditions for PCR amplificaton

In all three instances, the polymerase chain reaction was carried out for 30 cycles with either 0.1 ug or 1.0 ug of plasmid DNA and varying amounts of Taq polymerase. Aliquots of the reaction mixture were then analyzed by agarose gel electrophoresis (Fig. 2). In all cases, the major reaction product was of the expected size and, as previously observed (8), only at high enzyme to template ratios were

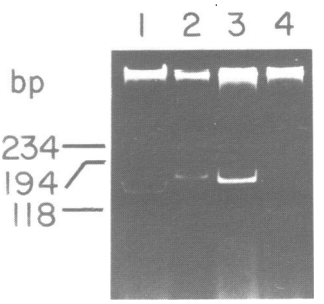

Figure 3: Characterization of mutant DNA by digestion with restriction endonucleases. A mutant DNA of each type was digested with NcoI and PstI and the digests analyzed by agarose gel electrophoresis in parallel with an NcoI–PstI digest of the parental, wild type DNA (lane 1). In the substitution mutation (lane 2), digestion with NcoI and PstI leads to a fragment 30 bp longer than in the wild type because of abolition of the NcoI site that encompasses the initiation codon. The pro–sucrase–isomaltase–P450 chimera ("insertion mutant") yields an NcoI–PstI fragment (lane 3) that is 24 nucleotides longer than the wild type fragment and the deletion–mutation yields a fragment 60 bp shorter than the parental DNA. The molecular weight markers indicated on the left were from a HaeIII digest of ΦX174RF.

additional higher molecular weight products detected. The ultimate yield of amplified product was similar regardless of the amount of template used.

Cloning of Amplified Fragments

In the case of the reaction mixtures used to generate both the single amino acid replacement and the sucrase–isomaltase–P450 chimera, a very simple procedure was used to insert the mutated amplified DNA into the parental plasmid so as to replace the original wild type DNA segment. The entire PCR mixture containing 1 ug of parental plasmid was digested with PstI and either NcoI (for the chimeric gene) or EcoRV (for the amino acid replacement). After phenol extraction and ethanol precipitation, the resultant products were treated with DNA ligase and then transfected into E. coli HB101. The amino acid replacement mutant could be distinguished from the parental wild type plasmid by virtue of the elimination of the NcoI site at the initiation codon in the mutant sequence (Fig. 3). Fifty percent of the clones analyzed carried the expected mutation by this criterion. DNA sequence analysis of the putative mutants verified the nature of the mutation (Fig. 4) and, equally important, established that no other sequence changes were introduced in the segment generated by the PCR (not shown). The pro–sucrase–isomaltase–P450 chimeric construct could also be tentatively identified by virtue of the increase in size of the NcoI–PstI fragment (Fig. 3) and the sequence between these two restriction sites was found to be exactly that expected (Fig. 4).

For the cloning of the deletion mutation, the amplified fragment was purified from the pooled PCR mixtures by gel electrophoresis after cleavage with PstI and NcoI

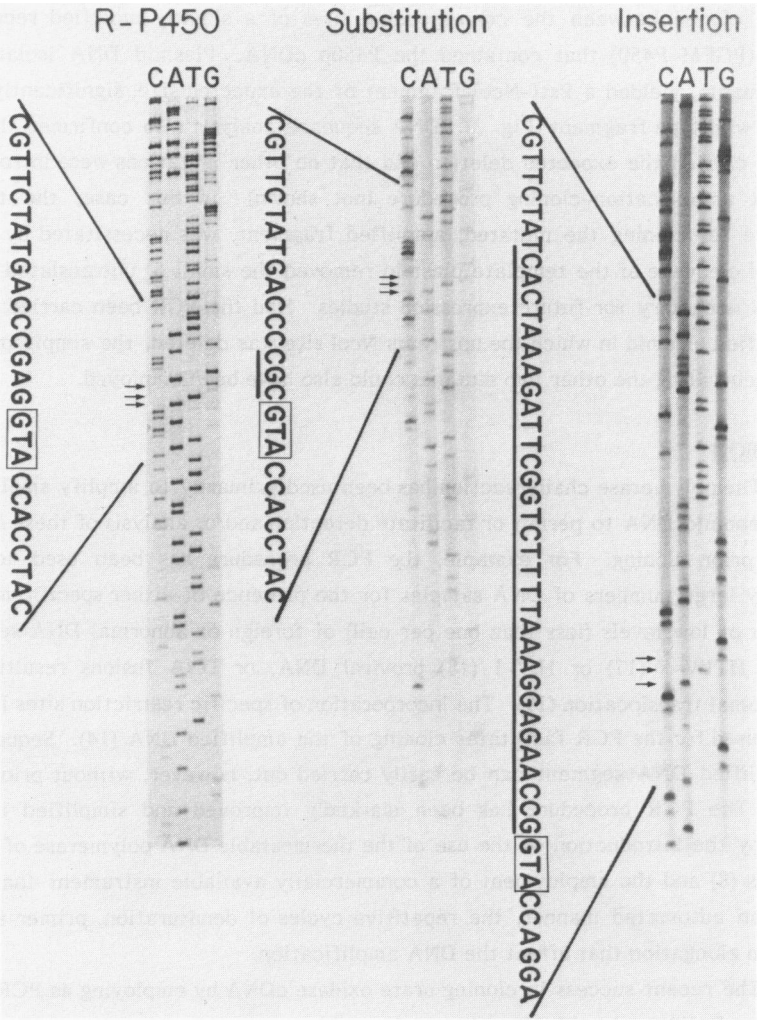

Figure 4: DNA sequence of substitution and insertion mutants. Only the region of interest is shown. In each sequence the initiation codon is enclosed in a box and the arrows at the left of each set of tracks point to the corresponding bands on the gel. The underlined residues correspond to the mutated bases introduced by the mutagenic primer. The bases just 3' to these residues in the substitution and insertion mutants, respectively, correspond to residues 7 and 13 of the wild type sequence. The sequence of the wild type P450 in the set of tracks on the left (R-P450) corresponds to that in the plasmid pSR-P450. Thus the residues just 5' to the ATG do not correspond to the natural 5' untranslated sequence but to residues in the 39 bp oligomer that was inserted at the NcoI site during the construction of this plasmid. On the other hand, the sequence upstream of the ATG in the insertion mutant does correspond to the natural 5' untranslated region since the 39 bp oligomer was deleted during the construction of this mutant.

and was cloned between the corresponding sites of a slightly modified recombinant plasmid (PGEM-P450) that contained the P450b cDNA. Plasmid DNA isolated from transformants, yielded a PstI-NcoI fragment of the expected size, significantly smaller than the wild type fragment (Fig. 3). DNA sequence analysis also confirmed that these plasmids carried the expected deletion and that no other mutations were introduced by the PCR amplification-cloning procedure (not shown). In this case, the two step procedure for cloning the mutated, amplified fragment, was necessitated by the fact that NcoI cleavage of the template plasmid removed the small 5' untranslated segment that was necessary for future expression studies. Had the PCR been carried out with the modified plasmid in which the upstream NcoI site was deleted, the simple procedure used in generating the other two mutants could also have been employed.

DISCUSSION

The polymerase chain reaction has been used primarily to amplify small regions within genomic DNA to permit or facilitate detection and/or analysis of these segments without prior cloning. For example, the PCR procedure has been used to screen relatively large numbers of DNA samples for the presence of either specific mutations (15,16) or of low levels (less than one per cell) of foreign or abnormal DNA sequences, such as HTLV-1 (17) or HIV-1 (18) proviral DNA, or DNA fusions resulting from chromosomal translocation (19). The incorporation of specific restriction sites into the primers used for the PCR facilitates cloning of the amplified DNA (14). Sequencing of the amplified DNA segment can be easily carried out, however, without prior cloning (20,21). The PCR procedure has been markedly improved and simplified in recent months by the introduction of the use of the thermostable DNA polymerase of Thermus aquaticus (8) and the employment of a commercially available instrument that carries out, in an automated manner, the repetitive cycles of denaturation, primer annealing and chain elongation that effect the DNA amplification.

The recent success in cloning urate oxidase cDNA by employing as PCR primers a mixture of oligonucleotides based on amino acid sequence data for the purified protein (22), represents a major innovation in the use of the PCR technique. From that work it was apparent that oligonucleotide primers with several mismatched bases could prime relatively effectively and effect the PCR-mediated amplification of a DNA segment while introducing the "mutated" nucleotides into the amplified segment. The purposeful generation of mutants by using specifically designed mutagenic primers, as reported in this paper, can be viewed as a logical extension of that finding. It was anticipated that even if the initial priming reaction with mismatched primers on the original template were very inefficient under the conditions used for the PCR, this would have little effect on the final outcome of the experiments since, in the subsequent cycles of synthesis, the primer would match perfectly with new template molecules that were

synthesized in vitro. Although the applicability of the PCR procedure to the generation of mutations in vitro had been previously suggested (7), such an application was reported in the literature only after submission of this manuscript (23).

The procedure used here for mutagenesis is very efficient because the PCR amplification results in a high ratio of mutant to wild type fragments. An added advantage is the feasability of direct cloning of the amplified fragment without its prior purification by simply subjecting the entire PCR reaction mixture to digestion with the appropriate endonucleases followed by incubation with DNA ligase. Point mutations, deletions and replacements of small gene segments can easily be constructed and, since the starting material is double stranded DNA, the mutations can often be inserted directly into the vector used for expression studies. Replacements of a small segment of the target DNA with a segment of another gene, as exemplified by our construction of the sucrase–isomaltase–P450 chimera, requires a shorter primer than in the classical site directed mutagenesis procedure since the primer must be complementary to the template only on one side of the insertion. Finally, plasmid DNA prepared by standard "miniprep" procedures (11) is of adequate quality to serve as substrate for the mutagenesis procedure.

The mutations generated in this work were located sufficiently close to a unique restriction site in the target DNA so that the mutagenic primer was designed to contain that restriction site and thus facilitate cloning of the amplified DNA bearing the mutant sequences. It is clear, however, that site directed mutagenesis via the PCR technique has more general applicability. Thus, in a situation in which the mutation target is not immediately adjacent to a unique restriction site, but the parental wild type DNA does contain such sites several hundred base pairs away on both sides of the target site, it should be possible to generate the required mutants by a somewhat more elaborate multi–step procedure. This would involve blunt end ligation of a PCR–amplified mutated DNA segment that extends to one restriction site to a downstream or upstream fragment, also generated by the PCR, that extends to the other restriction site. In this case, the PCR is also used to effect the precise "excision" of the required DNA segments from the parental DNA without the use of restriction endonucleases. Indeed, utility of the PCR method for "excising" specific DNA segments from cloned DNAs as previously reported (8) is likely to find application in many different experimental procedures. When the procedure that employs the blunt end ligation is required, the correctly ligated molecules can be selected by a second PCR amplification using the "outside" primers in a manner similar to that employed by Higuchi et al. (23).

It should be noted, however, that it may be necessary, especially in site directed mutagenesis experiments, to sequence any DNA segments cloned after PCR amplification, to ascertain that no other unwanted mutations have been introduced into the gene being studied. In the three mutations produced in this work, in which only

small segments of DNA (120–150bp) were amplified, no such extraneous mutations were observed. However, recent studies (8, 23, 24) suggest that the error frequency in the PCR would generate, on the average, after a 30 cycle reaction, one base pair change in a 400 bp molecule. This indicates that when carrying out mutagenesis procedures such as the one proposed here it is preferable to amplify small DNA segments and to minimize the number of cycles employed in the PCR.

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