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Polymerase Chain Reaction-Based Site-Directed Mutagenesis Using Magnetic Beads

Jacques Fieschi, Patricia Niccoli,* Christophe Camilla,† Patrick Chames, Martine Chartier, and Daniel Baty[1]

*Laboratoire d'Ingénierie et Dynamique des Systèmes Membranaires, Institut de Biologie Structurale et de Microbiologie, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France; *U38–INSERM, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France; and †Immunotech SA, 130 Avenue Jean de Lattre de Tassigny, BP177, 13276 Marseille Cedex 9, France*

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Thyroglobulin double mutants with various substitutions were obtained with a new polymerase chain reaction-based mutagenesis technique. Maximum length of megaprimer and efficiency of mutagenesis were improved by purification of single-stranded DNA, using the avidin–biotin interaction. This method might allow the construction of large libraries of DNA, mutated at different sites. © 1996 Academic Press, Inc.

Several methods have been described for site-directed mutagenesis using PCR.[2] Among these procedures, the megaprimer PCR-based method (1) requires two flanking primers and a mutagenic primer. First, the mutagenic primer is used to synthesize a megaprimer with the corresponding antiparallel flanking primer. This megaprimer is then used with the third primer to produce a full-length product containing the mutation. However, usually with this technique very low yields of mutated full-length DNA fragments are obtained. We hypothesize that this method is not convenient for obtaining libraries of mutants using a degenerated primer, particularly when the mutagenic primer is far from the flanking primers. To increase the amount of DNA, a third amplification step may be necessary. Moreover, the yield of mutated DNA decreases when the length of the megaprimer increases and beyond 400 base pairs no mutants are obtained (2). The most effective method for introducing a single-point mutation is the overlap extension (3) which uses two flanking and two mutagenic primers. Unfortunately, this method is expensive and time-consuming when several point mutations are to be introduced.

Our aim was to develop a new method for introducing point mutations at different sites. For this purpose we used a novel procedure employing a 5′-end-biotinylated primer and a mutagenic primer. After a first PCR, the megaprimer is purified on avidin attached to magnetic beads. The DNA is then denatured with sodium hydroxide and used as single-stranded oligonucleotide to perform a second PCR with a reverse primer. The product of this latter PCR is the full-length mutated DNA. We applied this procedure to mutate tyrosine residues 5 and 130 located on a hormonogenic peptide of human thyroglobulin. Those two residues are supposed to be involved primarily in thyroid hormone synthesis (4). The classical double-stranded megaprimer method gave very low amounts of DNA after the second amplification step, whereas the method described here appears to increase drastically the yield of mutant DNA.

MATERIALS AND METHODS

Materials

Polymerase chain reactions were performed on a Perkin–Elmer Cetus Thermal Cycler. *Pwo* DNA polymerase and dNTPs were purchased from Boehringer-Mannheim and used as specified by the manufacturer. *Pwo* DNA polymerase was selected for its 3′ to 5′ proofreading exonuclease activity. This activity avoids the addition of nucleotides at the 3′ end of the megaprimer that occurs when the amplification is performed with a classical *Taq* DNA polymerase (5) and gives rise to a point mutation in the last step. Magnetic beads were Estapor

[1] To whom correspondence and reprint requests should be addressed at LIDSM, CBBM–CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France. Fax: (33) 91 71 21 24; e-mail: baty@ibsm.cnrs-mrs.fr.

[2] Abbrevations used: PCR, polymerase chain reaction; BFP, biotinylated forward primer; RP, reverse primer; bp, base pairs.

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M1 standard or Estapor EM1 encapsulated beads from Prolabo, both coated with avidin at Immunotech S.A. Both types of beads were kindly supplied by Immunotech S.A. and activated as specified by the manufacturer. The template was the double-stranded transfer vector pVL1393 (Invitrogen) containing the cDNA encoding the hormonogenic peptide inserted in the *Xba*I site of the polylinker. The biotinylated forward primer (BFP), 5′ biotin-TTTACTGTTTTCGTAACAGTTTTG 3′ anneals 95 bases upstream of the *Xba*I site in pVL1393. The reverse primer (RP), 5′ CAACAACGCACAGAATCTAG 3′, anneals 648 bases downstream from the *Xba*I cloning site in pVL1393. The first mutagenic primer (named *Bst*BI (Tyr5)) mutates the tyrosine 5 and introduces a silent *Bst*BI restriction site (underlined sequence), 5′ GGGCATCCACCTGNNBTTCGAAGATATTGGCCG 3′. The second mutagenic primer (named *Kpn*I (Tyr130)) modifies the tyrosine 130 and introduces a silent *Kpn*I restriction site (underlined sequence) 5′ CAGCTGGCGGGTACCNNBCACCTCCATCCCC 3′. We decided to change the triplet TAY coding for tyrosine residues to VNN to avoid the synthesis of wild-type DNA or the insertion of a stop codon. The general strategy is summarized in Fig. 1.

Method of Mutagenesis

Polymerase chain reactions were achieved by the procedure described below.

PCR1. Ten nanograms of template, 10 pmol of biotinylated forward primer and 10 pmol of mutagenic *Bst*BI (Tyr5) primer, 1.25 U *Pwo* DNA polymerase, 0.2 mM dNTPs, 10 mM Tris–HCl, pH 8.85, 25 mM KCl, 5 mM $(NH4)_2SO4$, 2 mM MgSO4 were mixed in a total volume of 50 μl. The *Pwo* polymerase was added last to avoid 3′ to 5′ degradation of primers. Thirty amplification cycles (94°C, 1 min; 50°C, 2 min; 72°C, 2 min) were performed, followed by 10 min at 72°C, and holding at 10°C.

Single-strand isolation. Biotinylated mutated double-stranded DNA (187 bp) was trapped by binding on M1 or EM1 magnetic beads coated with avidin. Different amounts of magnetic beads were incubated with the PCR mix for 1 h at room temperature. Beads were then pelleted and washed extensively with salt buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 M NaCl). Beads bound to mutated double-stranded DNA were resuspended in 20 μl of 0.2 M NaOH. After an incubation of 20 min at room temperature, beads were pelleted; the supernatant (nonbiotinylated, single-stranded DNA) was discarded and beads were washed in TE buffer.

PCR2. A second PCR was performed as described in PCR1 but beads carrying the mutated single-stranded megaprimer (187 bases) and the mutagenic *Kpn*I (Tyr130) primer were substituted for the initial biotinylated forward primer and the *Bst*BI (Tyr5) primer, respectively (Fig. 1). After PCR, the mutated DNA was isolated with fresh beads to isolate the mutated single-stranded primer (564 bases) as described above.

PCR3. A third PCR was performed as described in PCR2, using the mutated single-stranded megaprimer (564 bases) and the reverse primer to obtain the mutated double-stranded full-length DNA (1329 bp).

Analysis of Reaction Products

Double-stranded DNA was released completely from the beads in 30 min at 94°C in a thermal cycler (data not shown). After 30 cycles of amplification, beads were pelleted and 5 liters of supernatant was analyzed on 2% agarose gel. The insertion of a mutation was checked by digestion with the appropriate restriction site endonuclease. The diversity of mutated fragments was checked by cloning and sequencing: after PCR3 a last amplification was performed using nonbiotinylated flanking primers and *Taq* DNA polymerase in order to add an adenine at the 3′ end of the strands. The products were ligated without any prior treatment to the pGEM-T cloning vector (Promega). DNA from bacteria that gave white colonies was prepared by miniprep, digested with *Kpn*I or *Bst*BI, and sequenced.

RESULTS

Isolation of Megaprimer Using Magnetic Beads

Magnetic beads coated with avidin are currently used to prepare single-stranded templates for direct sequencing of PCR products. We hypothesized that this system might be used to produce single-stranded megaprimer for site-directed mutagenesis. Unfortunately, most DNA polymerases are inhibited irreversibly by potassium ferrate, as demonstrated for DNA polymerase I (6) and reverse transcriptase of the murine leukemia virus (7). To investigate a possible limit to the length of the megaprimer and the effects of the magnetic beads we prepared single mutants of Tyr130, the most remote tyrosine residue in the peptide. The megaprimer for this mutation is 564 bp in length; it is then beyond the limit described for double-stranded megaprimers. In a first step, we produced a double-stranded megaprimer with the biotinylated forward primer (BFP) and the *Kpn*I (Tyr130) mutagenic primer (Fig. 2, lane 2). This 564-bp double-stranded megaprimer was used with the reverse primer (RP) to amplify the template, but the expected product of 1329 bp in length was never obtained (Fig. 2, lane 3). Many attempts of PCR protocol modification by varying the amounts of template and primers were unsuccessful (not shown). The biotinylated single-strand of this megaprimer was isolated on different amounts of beads and used as a new primer, as described under Material and Methods.

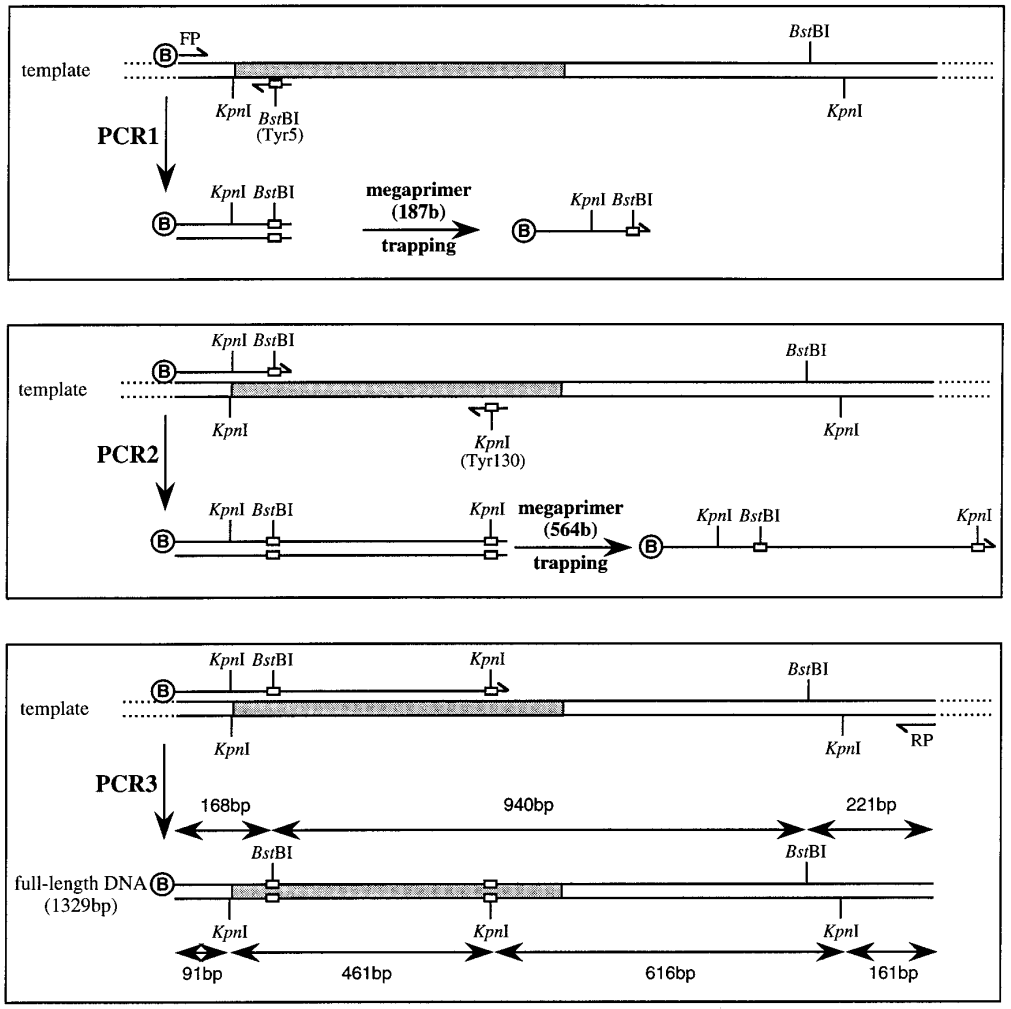

FIG. 1. Schematic representation of the method of mutagenesis. BFP is the biotinylated forward flanking primer; RP is the reverse flanking primer; *Bst*BI (Tyr5) and *Kpn*I (Tyr130) are the mutagenic primers introducing a single substitution and a restriction site. The *Xba*I fragment coding for the hormonogenic peptide of the human thyroglobulin is indicated by a stippled box. The *Bst*BI and *Kpn*I sites present in mutated and wild-type sequence are indicated.

Two types of beads were tested: M1 classical avidin-coated magnetic beads and identical beads, encapsulated into a polysaccharidic matrix, EM1, to avoid any contact between ferrate and the polymerase during the reaction. DNA was never detected in the supernatant when the mutagenesis was performed with M1 beads (Fig. 2, lanes 4 to 6). The occurrence of amplified DNA, remaining on the avidin, was checked after heat treatment of the beads. With EM1 beads large amounts of full-length DNA were present in the supernatant, but no DNA could be detected on the beads. The efficiency of the mutagenesis procedure was controlled by digestion of the full-length fragment with *Kpn*I.

This experiment shows that the enzyme *Pwo* DNA polymerase is inactive in the presence of M1 beads, probably due to the presence of ferrate (Fig. 2, lanes 4 to 6). By contrast, encapsulated beads allowed the

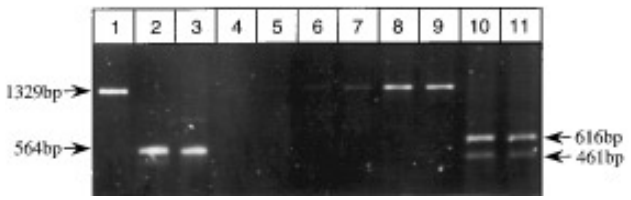

FIG. 2. PCR amplification of full-length Tyr130 single mutants with two types of beads used at different concentrations. Lane 1: full-length control DNA (1329 bp) obtained after amplification of the template DNA with the BFP and RP primers. Lane 2: 564-bp fragment obtained after amplification of the template with the BFP and *Kpn*I (Tyr130) primers. Lane 3: amplification of the template using the 564-bp double-stranded megaprimer obtained in lane 2 and the RP primer. Lanes 4 to 6: amplification of the template as in lane 3, except that the 564-base biotinylated single strand is trapped with 50, 100, and 300 μg of M1 beads, respectively. Lanes 7 to 9: as in lanes 4 to 6 but with EM1 beads. Lanes 10 and 11: the products, respectively, of lanes 8 and 9 digested with *Kpn*I.

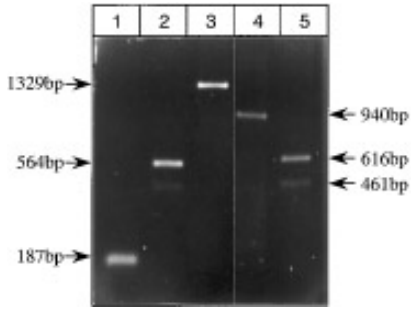

FIG. 3. Construction of Tyr5 and Tyr130 double mutants with successive PCRs. Lane 1: 187-bp fragment obtained with the BFP and *Bst*BI (Tyr5) primers. Lane 2: 564-bp fragment obtained with the trapped 187-base single-stranded megaprimer using EM1 beads and the *Kpn*I (Tyr130) primer. Lane 3: 1329-bp full-length DNA obtained with the trapped 564-base single-stranded megaprimer using EM1 beads and the RP primer. Lanes 4 and 5: 1329-bp fragment of the lane 3 digested with *Bst*BI and *Kpn*I, respectively.

amplification (Fig. 2, lanes 7 to 9). The digestion profile with *Kpn*I shows that most of the molecules bear the Tyr130 mutation (Fig. 2, lanes 10 and 11). These results show that with a single-stranded megaprimer it is possible to insert a mutation at least 560 bp from the biotinylated primer. Our results demonstrate that a large amount of full-length DNA was obtained with 100 or 300 μg of beads. All the experiments described below were performed with 100 μg of beads.

Construction of DNA Library Mutated at Different Sites

For the experiments shown in Fig. 3, we used this procedure to obtain double mutants of tyrosines 5 and 130. First, the biotinylated forward primer was used with the *Bst*BI (Tyr5) mutagenic primer to mutate tyrosine 5. The purified single-stranded megaprimer was then used as a new forward primer with the *Kpn*I (Tyr130) mutagenic primer to modify tyrosine 130. This new fragment was treated as described and used as forward primer in a third amplification with the reverse primer (Fig. 1). Aliquots were analyzed at each step and the full-length fragment was digested with *Bst*BI or *Kpn*I. These results show that it was possible to use three times the same oligonucleotide which was extended at each PCR step to introduce two mutations at different sites. Our results show that under these conditions the biotin–avidin bond is labile at high temperature. After cloning in pGEM-T, the plasmids from 21 bacteria that gave white colonies were digested with *Kpn*I and *Bst*BI. Several clones with both new sites were sequenced and gave different results indicating that they did not emerge from the same template (Fig. 4).

DISCUSSION

Our aim was to produce mutant DNA where classic procedures were unsuccessful because of the length of the megaprimer. Indeed, we used double-stranded megaprimers of 250 to 600 bp to introduce mutations in different genes without success (unpublished results). To obtain a mutation 560 bp downstream from the 5′ end of our DNA, we had to develop a new method. First, we used *Pwo* DNA polymerase because of its increased fidelity due to the presence of 3′ to 5′ exonuclease activity. When several rounds of PCR are necessary, this activity becomes extremely important to avoid misincorporation errors. Moreover, this enzyme does not add extra nucleotides to the 3′ ends of amplified double-stranded DNA. In many methods, the megaprimer is double-stranded so that after denaturation the single-strand can anneal with the template or with its complementary single-strand, thus decreasing the efficiency of the reaction. We used a biotinylated oligonucleotide to separate the strands on magnetic beads with avidin covalently attached to the surface. We showed that like many other polymerases, *Pwo* DNA polymerase is irreversibly inactivated in the presence of ferrate presumably released from the beads at high temperature. This difficulty was overcome with magnetic beads covered with a polysaccharidic capsule in order to prevent the release of ferrate. These beads were used directly in the PCR reaction mixture with no need for a step to release the single-stranded megaprimer from avidin. Nevertheless, after 30 cycles, all amplified DNA is released from the beads but it can be rebound to new beads for a subsequent denaturation step. We managed to obtain double mutants of tyrosines 5 and 130 of the peptide and introduced different amino acids at each position. This method allowed us to use a megaprimer 560 bp long.

A possible copurification of the megaprimer with residual biotinylated oligonucleotides might limit the rate of mutagenesis. To overcome that difficulty, we produced the megaprimer with different amounts of primer and chose a concentration where the biotinylated primer was limiting. One might use a different ratio with less biotinylated primer or even digest re-

Subclones analyzed	21
Mutant subclones at position 5	18 (86%)
Mutant subclones at position130	14 (67%)
Double mutant subclones	12 (57%)

Mutation at position 5	Mutation at position 130
His	His
Ala	Thr
Arg	Pro
Pro	Glu
Val	Ile
Thr	Asn
Ala	Gly
Gly	Gln

FIG. 4. Determination of mutagenesis efficiency. After ligation of the mutated full-length DNA in pGEM-T, 21 mutants were analyzed by restriction digestion for the presence of the mutations. Eight clones were sequenced to determine the amino acid substitutions.

maining primers with exonuclease I before purification on beads.

Active sites of proteins are formed generally when the folding brings together amino acids from remote polypeptidic regions; the study of protein function requires therefore the construction of mutants at different sites. The procedure described here may be useful for constructing libraries of mutants for the study of the role of specific residues of polytopic membrane proteins or for modifying the specificity of antibodies after structural studies and molecular modeling.

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