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High-efficiency oligonucleotide-directed plasmid mutagenesis

(site-directed mutagenesis/phosphorothioate/plasmid pUC19)

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ABSTRACT **A number of single- and double-base substitutions have been introduced into either the polylinker region or the *lacZ* gene in the plasmid vector pUC19. The efficiencies of these changes upon transfection of TG-1 bacterial cells were generally 70–80%. A strategy has been devised by which the wild-type DNA can be selectively destroyed. It is primarily based on the resistance of phosphorothioate internucleotide linkages to some restriction enzymes. A mismatch oligonucleotide is introduced into a gapped region and the gap is filled using three deoxynucleoside 5′-triphosphates and one deoxynucleoside 5′-[α-thio]triphosphate. Reaction with a restriction enzyme that is unable to hydrolyze phosphorothioates ensures that the DNA containing the mismatch oligonucleotide is only nicked. Concomitantly, the DNA that does not contain the desired mutation is linearized. Subsequent reactions with an exonuclease and DNA polymerase I yield mutant homoduplex DNA for transfection.**

The change of the nucleotide sequence of a gene or a regulatory element by site-directed mutagenesis has become one of the most powerful tools available to the molecular biologist to study structure–function relationships (1–3). Several methods that involve a selection against wild-type sequence have been developed to obtain high mutational efficiencies by oligonucleotide-directed mutagenesis (4–7). These methods require the gene to be present in the form of single-stranded DNA (ssDNA). However, genes are usually cloned into double-stranded DNA (dsDNA) vectors. Therefore, in order to obtain high-efficiency mutagenesis the DNA sequences have to be subcloned into a single-stranded vector such as M13 (8) or a specialized vector whose DNA can be isolated in either the single- or the double-stranded form (9).

Alternatively, the polymerase chain reaction has been employed to prepare mutants. Methods have been published where only a section of a plasmid is amplified with a mutagenic primer and the product is then exchanged for the wild-type sequence as a "cassette" (10–12). In another procedure the whole plasmid is amplified as linear DNA, which is then isolated and religated (13). These procedures are subject to misincorporations catalyzed by *Thermus aquaticus* (*Taq*) DNA polymerase (14).

Oligonucleotide-directed mutagenesis has been performed directly on plasmids with varying degrees of success (15–17). These methods yield efficiencies generally between 1% and 15%. An efficiency of 55% has been reported for one method, but this is not of general applicability (18). One recent report describes a single experiment in which a mutation rate of 70% was obtained by using denatured double-stranded plasmid (19). Here we describe a method with up to 80% mutational efficiency. This method produces consistently high yields of mutants and should be generally applicable.

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MATERIALS AND METHODS

Enzymes and Nucleotides. Exonuclease III (100 units/μl) and the restriction endonucleases *Hin*dIII (20 units/μl), *Eco*RI (20 units/μl), and *Pst* I (20 units/μl) were supplied by New England Biolabs; phage T7 gene *6* exonuclease (30 or 100 units/μl) was purchased from United States Biochemical. Partially purified T5 gene *D15* exonuclease (1 mg/ml) was kindly provided by J. R. Sayers, (Max-Planck Institut für Experimentelle Medizin, Göttingen). ATP and dNTPs were from Boehringer Mannheim and were of the highest quality available. The S_P diastereomer of 2′-deoxyguanosine 5′-[α-thio]triphosphate (dGTP[αS]) was synthesized according to Ludwig and Eckstein (20) or purchased from Amersham. DNA polymerase I, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were prepared as described (21).

Oligonucleotides. The sequences of the mutagenic oligonucleotides are given below, with the mismatch bases underlined: ACO, 5′-d(GGTACCCGGGGATCCTCTAGAGTCG)-3′; BCO, 5′-d(CGACTCTAGAGGATCCCCGGGTACC)-3′; JAO, 5′-d(GGGTTTTCCTAGTCACG)-3′; M13SEQ, 5′-d(AGGGTTTTCCCAGTCACG)-3′; TGO, 5′-d(CGTGACTGGGAAAACCCT)-3′. These were prepared by the phosphoramidite method with an Applied Biosystems 380B DNA synthesizer. The phosphorylation and purification of the oligonucleotides have been described (22).

Preparation of Plasmid DNA. Plasmid DNA was purified from chloramphenicol-amplified culture as described by Miller (23) with the following changes. The RNase A was added along with the EDTA solution. After centrifugation of the cellular debris, the plasmid DNA was precipitated by incubation on ice for 3 hr with one-third volume of 30% (wt/vol) polyethylene glycol 6000 solution containing 1.5 M NaCl. After CsCl/ethidium bromide banding, the DNA was further purified by spin dialysis using a Centricon-30 microconcentrator (described below). Sequence analysis of the wild-type and mutant pUC19 plasmids was carried out by the procedure of Olsen and Eckstein (24).

Phenol Extraction. Solutions containing the plasmid DNA were extracted by vigorous mixing with 200 μl of buffer-equilibrated phenol, 200 μl of isoamyl alcohol, and finally 1 ml of water-saturated diethyl ester (25). Traces of ether were removed by heating the sample at 70°C for 10 min.

Spin Dialysis by Centricon Filtration. The exchange of buffer, removal of low molecular weight impurities, and concentration were achieved with a Centricon-30 filtration device (Amicon). The sample was placed in the unit after dilution with 2 ml of distilled water and centrifuged for 20 min at 3500 rpm in a Sorvall SS34 fixed-angle rotor or a Sepatech Medifuge tabletop centrifuge (Heraeus, D-G450 Haman, F.R.G.). This process was repeated twice more. The volume of the DNA solution after the last centrifugation was generally 50–60 μl.

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ccc, covalently closed circular; dNTP[αS], 2′-deoxynucleoside 5′-[α-thio]triphosphate.

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Agarose Gel Electrophoresis. DNA-containing samples were analyzed by 2% agarose gel electrophoresis in the presence of 2-mercaptoethanol and ethidium bromide (26).

Nicking of Plasmid DNA. Plasmid DNA (pUC19, 20 μg, 12 pmol) was nicked by incubation with *Hin*dIII (200 units) in a solution (240 μl) containing 40 mM Tris Cl (pH 7.4), 8 mM $MgCl_2$, 40 mM NaCl, and 10 μg of ethidium bromide. After incubation for 120 min at 30°C, 2 μl was removed for gel electrophoresis. Alternatively, the plasmid (20 μg) was nicked with *Eco*RI (600 units) in a reaction mixture (260 μl) containing 90 mM Tris Cl (pH 7.4), 90 mM NaCl, 370 μM $CoCl_2$, and 36 μg of ethidium bromide. After 16 hr at 16°C, 2 μl was removed for gel analysis. The nicked plasmid DNA was phenol-extracted, and the buffer was exchanged using a Centricon-30 microconcentrator.

First Gapping Reaction. (*i*) *Exonuclease III.* The nicked dsDNA was digested for 6 min at 37°C with exonuclease III (100 units) in a reaction mixture (95 μl) containing 110 mM NaCl, 10 mM Tris Cl (pH 8), 7 mM $MgCl_2$, and 7 mM dithiothreitol. (*ii*) *T5 gene D15 exonuclease.* Reactions with T5 exonuclease were performed under the same conditions as described for the exonuclease III gapping reaction except that 60 mM NaCl was used and the Tris buffer was replaced by 30 mM ethanolamine buffer (pH 9.3). After incubation, the enzymes were heat-denatured at 70°C for 10 min and the DNA was analyzed by taking 2 μl for gel electrophoresis.

Annealing. The NaCl concentration in the gapping reaction mixture was raised to 150 mM and 2 μl of 5′-phosphorylated mutant oligonucleotide (25 pmol, taken directly from a 4 A_{260}-units/ml phosphorylation reaction mixture) was added. The solution was incubated at 70°C for 10 min and then placed into a thermoblock at 56°C, which was then cooled to 25°C over 30 min.

Formation of the Mutant Heteroduplex. Polymerization of the primed, gapped template was performed by adjusting the annealing solution to 25 mM Tris Cl (pH 8), 70 mM NaCl, 5 mM dithiothreitol, 8 mM $MgCl_2$, 1.2 mM ATP, 280 μM each dATP, dCTP, dTTP, and dGTP[αS], 10 units of Klenow fragment, and 15 units of T4 DNA ligase in a total volume of 210 μl. The polymerization was carried out at 16°C for 16 hr; 5 μl was removed for analysis by gel electrophoresis and 2 μl was taken for transformation of component cells.

Reaction of Phosphorothioate-Containing Heteroduplex DNA with *Pst* I. The polymerization solution was extracted with phenol and spin-dialyzed in a Centricon-30 microconcentrator. This was followed by reaction with 70 units of *Pst* I in a solution (100 μl) containing 100 mM NaCl, 10 mM Tris Cl (pH 7.5), and 10 mM $MgCl_2$. After incubation at 37°C for 80 min and inactivation at 70°C for 10 min, 4 μl was removed for gel electrophoresis and 2 μl was taken for transformation.

Gapping of the *Pst* I-Nicked Plasmid. The nicking solution was extracted with phenol and the buffer was exchanged using a Centricon-30 microconcentrator. The solution was adjusted to contain 10 mM Tris Cl (pH 8), 60 mM NaCl, 7 mM $MgCl_2$, and 7 mM dithiothreitol in a volume of 100 μl. Gapping reactions catalyzed by exonuclease III or T5 exonuclease were carried out as described for the first gapping reaction. Digestion with T7 exonuclease was carried out for 30 min under identical conditions as described for T5 exonuclease, except that the ethanolamine buffer was replaced by 10 mM Tris Cl (pH 8). After incubation, the samples were heated at 70°C for 10 min and then placed into a thermoblock at 56°C, which was then cooled to 25°C over 30 min. Eight microliters was removed for gel electrophoresis and 2 μl was taken for transformation.

Formation of the Mutant Homoduplex by Repolymerization. The gapped-DNA solution was diluted to 220 μl by the addition of DNA polymerase I (10 units), four dNTPs, ATP, $MgCl_2$, Tris Cl (pH 8), dithiothreitol, and T4 DNA ligase as described above for formation of the mutant heteroduplex; after incubation either at 37°C for 3 hr or at 16°C overnight, 14 μl was removed for gel analysis and 2 μl was removed for transformation of competent cells.

Transformation and Plating of Plasmid DNA. *Escherichia coli* TG-1 competent cells prepared by the procedure of Chung *et al.* (27) were used for all transformations. Typically, 2 μl of control plasmid (wild-type pUC19 or pUC19 amber DNA, 10 ng/ml) or mutated plasmid (taken directly from the enzyme reaction mixture) was gently mixed with 100 μl of competent cells and placed on ice for 30 min. One control consisted only of 100 μl of competent cells (i.e., no DNA was added). After incubation, 2, 10, and 80 μl of transformed cells were spread on agar plates containing 2YT medium (38), ampicillin (75 μg/ml), isopropyl β-D-thiogalactoside (120 μM), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (120 μM). Cells were then placed at 30°C for 16 hr and the percent mutational efficiency was determined by counting blue versus colorless colonies.

RESULTS AND DISCUSSION

Rationale. The difficulty in carrying out highly efficient mutagenesis on plasmid DNA is the specific destruction of the unwanted wild-type sequence. We have taken advantage of the directionality of digestion of two exonucleases to prepare DNA with phosphorothioate groups incorporated into a specific region adjacent to a mismatch oligonucleotide. The mutant-containing strand is then resistant to certain restriction endonucleases due to the presence of these phosphorothioate groups (22). Thus, a reaction with one of these endonucleases cleaves the wild-type strand, providing the basis for selectivity and the theoretical possibility of 100% mutational efficiency. One sequence of manipulations is illustrated in Fig. 1.

Creation of Mutant Heteroduplex. For strand selectivity to occur, a ssDNA region where the mismatch oligonucleotide will anneal must be created. The first step is to produce a nick that is adjacent to the region of the desired mismatch in the plasmid. To do this we took advantage of the fact that many restriction endonucleases hydrolyze only one strand of DNA within their recognition sequence when incubated in the presence of ethidium bromide. This cleavage naturally is not strand-specific. Thus, when the nicking reaction is performed on plasmid DNA, a mixture of dsDNAs is obtained in which the nick is located in either the coding or the noncoding strand (Fig. 1*B*). This nick can be taken as the starting point for a gapping reaction with either a 3′→5′ or a 5′→3′ exonuclease. Two different gapped products are generated by the action of the exonuclease, depending on which strand possesses the nick. The product of the gapping reaction that leaves the strand complementary to the mutant oligonucleotide intact is considered "productively gapped" (Fig. 1*C*). This reaction must proceed past the region to be mutated so that the mutant oligonucleotide can be inserted into the gap. As it is complementary to only one of the strands, it will strand-selectively hybridize to it. The gap in both strands is filled by a polymerization reaction in which one of the normal dNTPs is replaced by a dNTP[αS], the choice of which depends on the restriction enzyme to be used in the next step (Fig. 1*D*). One of the products of the first polymerization is a heteroduplex DNA that contains the desired mutation in only one of the strands.

Mutant Strand Selectivity by Reaction with a Class I Restriction Endonuclease. The choice of restriction enzyme for this step is governed by two considerations. (*i*) It should be an enzyme that is inhibited by phosphorothioates (7, 22, 28, 29). (*ii*) The cleavage site, or possibly sites, for this enzyme must be located exclusively in that gapped region to which the oligonucleotide had been annealed. We have chosen the enzyme *Pst* I for the mutations created here.

This second restriction enzyme reaction ensures that any original plasmid DNA that has escaped the previous manipulations will be linearized (Fig. 1*E*). In addition, DNA molecules that do not carry phosphorothioates in the recognition site of this enzyme (*Pst* I in our case) will also be linearized. This, of

course, will be roughly 50% of the DNA population that arises from those molecules that were "unproductively gapped" and thus do not contain phosphorothioates at the *Pst* I site in either strand (Fig. 1*E*). Thus, the *Pst* I reaction eliminates all those molecules that carry the wild-type sequence.

The molecules that were "productively gapped" contain phosphorothioates in the mutant strand and phosphate linkages in the wild-type strand within the recognition sequence of *Pst* I. Thus this situation is identical to that generated in the original phosphorothioate-mutagenesis procedure for ssDNA (7, 26). The subsequent steps in the plasmid mutagenesis are therefore identical to that method.

Creation of the Mutant Homoduplex. The nick created by the reaction of *Pst* I is used as the starting point for the subsequent exonuclease reaction (Fig. 1*F*). This is followed by a repolymerization step to form the mutant homoduplex (Fig. 1*G*). This then completes the sequence of enzyme reactions, and the resulting DNA is used for transformation.

Electrophoretic Analysis. One of the advantages of this protocol is the ease with which the products of any given

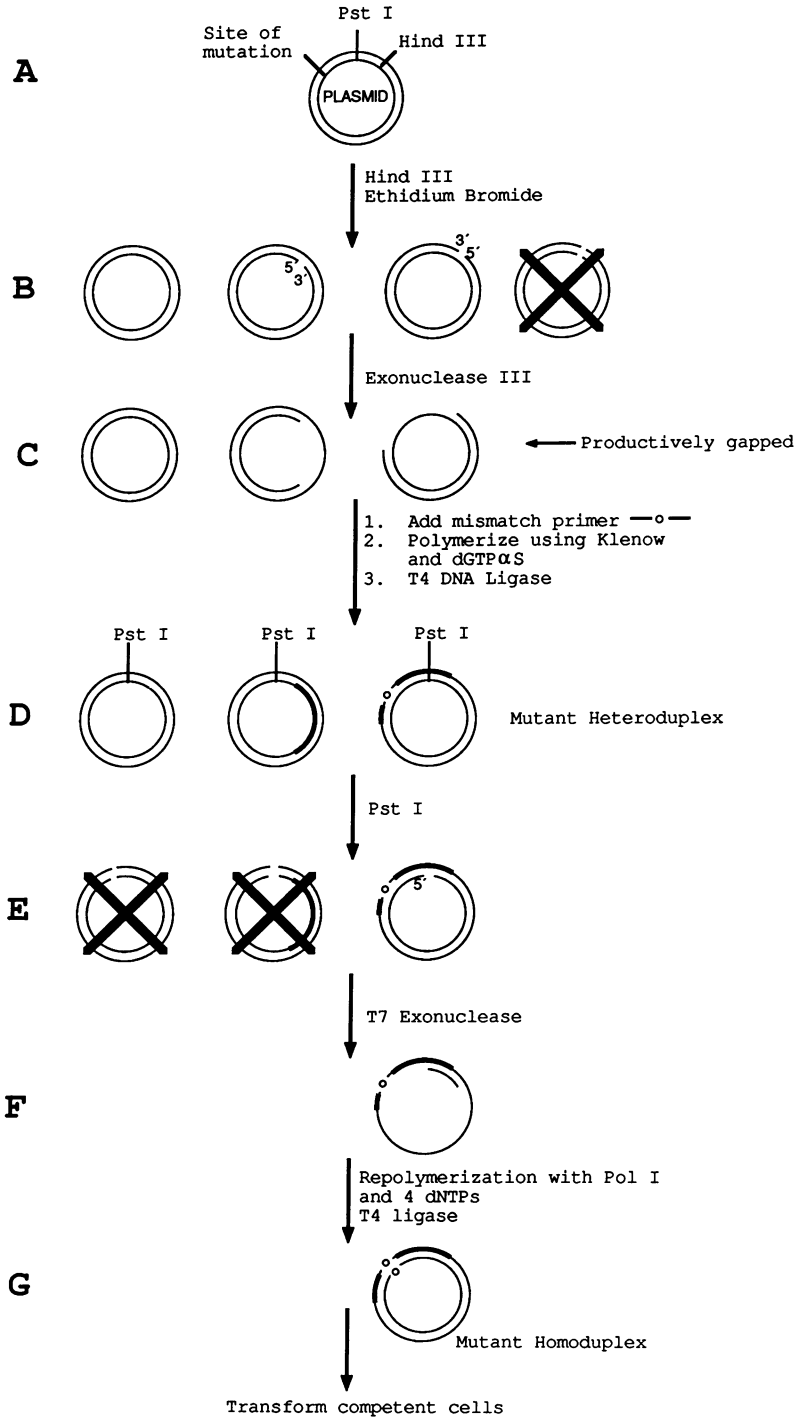

FIG. 1. Oligonucleotide-directed plasmid mutagenesis. (*A*) pUC19 covalently closed circular (ccc) dsDNA. (*B*) Products of *Hin*dIII reaction. (*C*) Products of exonuclease III reaction. (*D*) Mutant heteroduplex. (*E*) Products of *Pst* I nicking/linearization reaction. (*F*) Products of T7 exonuclease reaction. (*G*) Repolymerized mutant homoduplex. The symbol "o" represents the mutation within the mismatch oligonucleotide. Heavy lines indicate the area where phosphorothioates have been incorporated. Plasmid that has been linearized is crossed off because it is unable to transform. Pol I, DNA polymerase I.

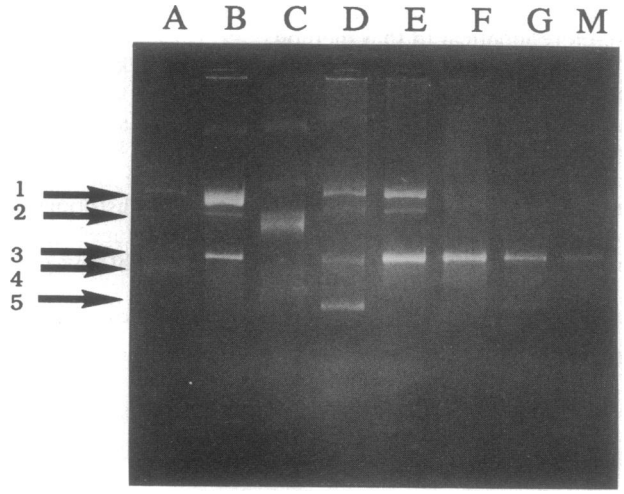

FIG. 2. Agarose gel electrophoresis of products after various reactions. The lettering of lanes corresponds to that in Fig. 1. Lane A: arrow 4, pUC19 ccc dsDNA, starting material. Lane B: arrow 1, nicked DNA; arrow 3, linear DNA; products of *Hin*dIII reaction. Lane C: arrow 2, gapped DNA, product after T5 exonuclease reaction. Lane D: arrow 5, mutant heteroduplex. Lane E: arrow 1, nicked DNA; arrow 3, linear DNA, products of *Pst* I reaction. Lane F: arrow 3, products of exonuclease III reaction. Lane G: arrow 5, repolymerized mutant homoduplex. Lane M: arrow 3, *Hin*dIII-linearized pUC19 marker. The DNA indicated by arrow 1 in lanes A, D, and E represents a small amount of concatemer DNA. Arrow 3 indicates linear DNA throughout.

reaction may be determined. The monitoring of the various enzymatic steps by agarose gel electrophoresis is shown in Fig. 2. Samples were taken after each stage of the process. The *Hin*dIII reaction produces predominantly nicked but also some linear DNA (lane B). As linear DNA is hydrolyzed by the reaction with T5 exonuclease, gapped DNA is the main product of this reaction (lane C). Repolymerization generates mainly ccc dsDNA, but some linear and some gapped DNA molecules also are present (lane D).

Probably the most important step is the nicking/linearization reaction catalyzed by the class I restriction endonuclease, *Pst* I in this case (lane E). It is important to be certain that after completion of this reaction, no ccc dsDNA remains. It is to be expected that this reaction will yield large amounts of linear DNA because of the destruction of the wild-type species. A distinct gapped species produced by the exonuclease III reaction (lane F) is not distinguishable, presumably because it comigrated with the linear DNA. Since exonuclease III is unable to hydrolyze DNA that has 3′ protrusions of four or more bases (30), the linearized DNA produced by the *Pst* I reaction is not digested by the exonuclease (lane F). The final repolymerization sometimes does not yield enough religated mutant homoduplex to be visualized on the gel (lane F). However, enough DNA is present for several transfections to yield a significant number of colonies.

Plasmid DNA. The quality of the plasmid DNA used as starting material is crucial for the success of the method. Poor results were obtained when the DNA preparation contained some small RNA fragments that still remained even after CsCl centrifugation. These fragments, resulting from the RNase treatment, can be seen by agarose gel electrophoresis when the band corresponding to the DNA is overloaded. The small RNA fragments can decrease the efficiency of priming by the mutant oligonucleotide either by competing for the same binding region on the DNA or by binding directly to the primer. To alleviate this problem, the small RNA fragments were removed by spin dialysis in a Centricon-30 microconcentrator. We have also experienced lower mutational efficiencies when an excessive amount of material having a lower mobility than ccc dsDNA in gel electrophoresis was present. These extra bands presumably are due to the presence of concatemers. We have found that amplification of the DNA with chloramphenicol seems to reduce the ratio of concatemer DNA to ccc dsDNA. Alternatively, the higher molecular weight DNA can be removed by using a Nucleogen anion-exchange HPLC column supplied by Macherey–Nagel (D-5160 Düren, F.R.G.).

Efficiency. Table 1 indicates the efficiencies obtained for various mutations by the use of several combinations of enzymes. The efficiencies are generally 70–80%. The lower efficiency observed in two cases (Table 1, lines 4 and 5) was probably due to impurities within the T5 exonuclease preparation. The mutational efficiency of the DNA that has gone through the second gapping reaction is already quite high, and hence the final repolymerization step could possibly be left out (Table 2). Theoretically, of course, the efficiency should not be increased upon filling of the gap, as the gapped DNA contains the same sequence information. It is conceivable that DNA polymerase I removes, in a nick-translation reaction, the sequence opposite the mutant oligonucleotide in molecules that have escaped the action of the T7 exonuclease.

Table 1. Summary of plasmid-mutagenesis results obtained with various combinations of restriction endonucleases and exonucleases

Enzyme combination*	pUC19 DNA†	Phenotype‡	Site of mutation§	No. of colonies analyzed (% mutant)	Mutant oligonucleotide
*Hin*dIII/exo III/*Pst* I/T7 exo	WT	BL → CL	*lacZ*	646 (80)	JAO
*Hin*dIII/exo III/*Pst* I/T5 exo	Amber	CL → BL	*lacZ*	656 (72)	M13SEQ
*Hin*dIII/exo III/Pst I/T7 exo	Amber	CL → BL	*lacZ*	304 (80)	M13SEQ
*Hin*dIII/T5 exo/*Pst* I/exo III	Amber	CL → BL	*lacZ*	208 (54)	TGO
*Hin*dIII/T5 exo/*Pst* I/T7 exo	Amber	CL → BL	*lacZ*	161 (39)	TGO
*Eco*RI/exo III/*Pst* I/T7 exo	Ochre	CL → BL	Polylinker	1305 (75)¶	BCO
*Eco*RI/exo III/*Pst* I/exo III	Ochre	CL → BL	Polylinker	1201 (73)¶	BCO

*exo, Exonuclease. The *Hin*dIII site is located at position 447 and the *Pst* I site at position 435 in pUC19.
†WT, wild type. Amber plasmid was constructed by mutagenesis using the conditions given for wild type; the DNA from three colorless colonies was sequenced (see *Materials and Methods*) and all three contained the desired mutation. Ochre plasmid was constructed by mutagenesis with unoptomized conditions used early in this work.
‡BL denotes blue colonies and CL denotes colorless colonies on plates containing isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside.
§The mutation in the plasmid-encoded *lacZ* gene is a transversion (at position 366) that changes a tryptophan codon (TGG) to an amber stop codon (TAG). The polylinker mutation is a double transversion (at positions 419 and 421 within the polylinker of pUC19) that changes a codon for an aspartic acid (GAT) to an ochre stop codon (TAA).
¶Average of two separate experiments.

Table 2. Percent mutational efficiency of plasmid mutagenesis at various intermediate stages of the procedure using the enzyme combination *Hin*dIII/exo III/*Pst* I/T7 exo (line 3, Table 1)

DNA (step in Fig. 1)	No. of colonies counted			% blue
	Blue	White	Total	
Heteroduplex (D)	5	168	173	3
Pst I-nicked (E)	36	242	278	13
T7 exonuclease-gapped (F)	26	19	45	58
Repolymerized (G)	242	62	304	80

Versatility of the Various Steps. We have limited this study to a combination of only a few enzymes, but it is clear that the method is not limited to these. As shown in Fig. 1, the plasmid DNA can be nicked in the presence of ethidium bromide with *Hin*dIII, but we have also used *Eco*RI to produce specifically nicked DNA (Table 1). We have found that the use of Co^{2+} instead of Mg^{2+} for the reaction with *Eco*RI (31) results in a greater proportion of nicked versus linear product. The conditions have been determined for the reaction with many restriction enzymes in the presence of ethidium bromide (32–36). Thus, many different restriction enzymes are available for the first nicking reaction as long as the linearization is prevented by the presence of ethidium bromide. Ideally there should be only one site for the first nicking enzyme on the plasmid.

The exonuclease used in the first gapping reaction should be one that operates in a nonprocessive manner, such as exonuclease III (21) or T5 gene *D15* exonuclease (ref. 37; J. R. Sayers and F.E., unpublished data), as this allows a control over the size of the gap introduced. As T7 exonuclease is very processive (21), it is not recommended for this step.

As mentioned above, the second restriction enzyme should be a class I enzyme as described by Taylor *et al.* (22). This list includes seven restriction endonucleases. Recent results have shown that polymerization with two dNTP[αS]s along with two dNTPs provides protection for a number of other restriction endonucleases (29). Other restriction endonucleases can also specifically nick phosphorothioate-containing dsDNA when the reaction is carried out in the presence of ethidium bromide (28).

The nonprocessive T5 exonuclease and exonuclease III are best suited for the introduction of gaps of a certain desired length. Therefore, we recommend the use of these enzymes for the second gapping reaction when the site of mutation is within several hundred base pairs from the class I nicking site. Alternatively, the highly processive T7 exonuclease can be used because it will gap the entire wild-type strand.

Conclusion. We have presented an efficient plasmid-mutagenesis protocol that allows the manipulation of genes to be performed in the vector in which they have been cloned. This method is based on enzymatic nicking and gapping of the plasmid to produce a limited stretch of ssDNA. It allows the introduction of an oligonucleotide containing the desired mutation. At this point the method becomes very similar to, and as simple as, the phosphorothioate-mutagenesis procedure for ssDNA developed earlier in this laboratory. At no stage during the process does the DNA require gel purification. Therefore, this protocol should yield equal mutational efficiencies for insertions and deletions, as was observed for the point mutations described here.

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