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Genetics

Segment-directed mutagenesis: Construction *in vitro* of point mutations limited to a small predetermined region of a circular DNA molecule

[site-specific mutagenesis/β-lactamase/*recA* protein/displacement-loop (D-loop) formation]

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ABSTRACT A general method for efficiently mutagenizing a predetermined segment of a closed circular duplex DNA molecule was used to construct mutations in two specific regions of the β-lactamase (*bla*) gene carried by the small plasmid pBR322. The principle of segment-directed mutagenesis is the use of a single-stranded homologous DNA fragment to direct the nicking of circular duplex DNA within a segment defined by the DNA fragment in a two-step reaction. First, *Escherichia coli recA* protein is used to catalyze assimilation of the homologous single-stranded DNA, producing a displacement loop ("D-loop") in the circular DNA. Second, a small amount of the single-strand-specific S1 nuclease is used to nick the displaced DNA. The segment-directed nicks are converted to small gaps, which are then mutagenized specifically with sodium bisulfite. A short (128-base pair) restriction endonuclease fragment from the center of the *bla* gene was used to direct mutagenesis with the result that 7.5% of the recovered plasmids were bla^- mutants and 49/51 of these mutants, mapped genetically, were found to lie in a deletion interval whose endpoints approximate those of the restriction fragment. Similar results were obtained when another short fragment covering the beginning of the gene was used; many of these mutations map in the region coding the "signal" sequence thought to be involved in secretion of β-lactamase.

Isolation of point mutations in a particular gene or regulatory site by random mutagenesis depends upon methods that will detect the rare mutant among a large background of nonmutants. When the mutant phenotype is unknown or difficult to discriminate from the wild type, acquisition of mutant alleles is often simply not feasible. Even when the gene has first been purified by recombinant DNA methods, considerable difficulties remain: the gene is often not expressed in the cloned state, and assays for gene function may be too laborious to carry out on more than a few candidates.

In the hope of improving the specificity and efficiency with which genes can be altered, several *in vitro* mutagenesis methods have been developed that generate base changes at predetermined sites on a DNA molecule (1–5). Two of these methods permit the construction of base substitutions in a small predetermined region of a duplex DNA molecule without the synthesis of unique oligonucleotides: mutagenesis by incorporation of nucleotide analogues (1) and mutagenesis of small single-stranded gaps with sodium bisulfite (2). Both of these methods attain their site specificity by the deliberate placement of a single-strand interruption ("nick") in the region to be mutagenized. Site-specific nicking has been accomplished by using restriction endonucleases, which, under the appropriate conditions, nick rather than cleave their targets (2, 6). Nicks can be used directly as a site for mutagenesis; alternatively, the site for mutagenesis can be moved a short distance to one side by limited nick-translation using DNA polymerase I of *Escherichia coli* (7). Nevertheless, the number of sites accessible to mutagenesis via restriction enzyme-catalyzed nicking is limited by the relative paucity of usable restriction targets.

In this paper we describe a two-step enzymatic method that efficiently introduces nicks into any segment of a small circular DNA molecule, given only that the DNA of that segment can be isolated as a short single-stranded fragment. When coupled with local mutagenesis using the bisulfite procedure, this segment-specific nicking procedure has allowed us to generate point mutations in two predetermined regions of the β-lactamase gene carried by the small plasmid pBR322.

MATERIALS AND METHODS

Strains. The highly transformable *E. coli* strain HB101 (thr^- leu^- pro^- $recA^-$ thi^- $hsdR^-$ $hsdM^-$ $supE^-$) and the small plasmid pBR322 (which confers resistance to ampicillin and tetracycline) were obtained from H. Boyer. The transformable *Salmonella typhimurium* strain DB4566 ($hspL^-$ $hspS^-$ *proC9 his-2253 dhuA1 purF145 galE503*) was constructed by P22 transduction of strain TA3426 (same genotype plus *bio-203*::Tn*10*; obtained from G. Ames) to biotin independence and tetracycline sensitivity.

P22Ap31*pfr1* is a derivative of P22 that contains the structural gene for β-lactamase (the *bla* gene); its origin is described elsewhere (8). Deletion mutants of the *bla* gene made on pBR322 were transferred to strain DB4566 by transformation and then onto P22Ap31*pfr1* by infecting these transformed derivatives and screening for bla^- recombinant phage. Deletion *del2* was generated *in vitro* by cleaving pBR322 DNA with the restriction endonucleases *Pvu* I and *Pst* I, each of which cleave the plasmid once in the *bla* gene 128 base pairs (bp) apart. The digested plasmid was treated with S1 nuclease to produce blunt ends and cyclized with phage T4 DNA ligase (9). The approximate size of the deletion was confirmed by electrophoresis through agarose gels. The deletions *del4391* and *del4393* (kindly provided by K. Talmadge) extend from the *Pst* I site to codons 4 and 25, respectively, of the β-lactamase coding sequence, as determined by DNA sequence analysis (K. Talmadge, personal communication). The relationships of these deletions and restriction sites are diagramed in Fig. 2 below.

Enzymes. Restriction endonucleases *Pvu* I, *Pst* I, *Taq* I, and *Hae* III and *E. coli* exonuclease III were purchased from New England BioLabs; *Micrococcus luteus* DNA polymerase I and S1 nuclease were from Miles. All units of enzyme activity are those of the manufacturers. The *recA* protein of *E. coli* was purified to homogeneity by using a published procedure (10).

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Abbreviations: bp, base pair(s); D-loop, displacement loop.

DNA Preparation. Monomeric covalently closed circular pBR322 DNA was prepared in a *recA*$^-$ strain (HB101/pBR322) by the standard method (11). It was further purified by acid phenol extraction (12) until contamination with nicked circular DNA was less than 1% as judged by electrophoresis through agarose gels. DNA restriction endonuclease fragments were separated by electrophoresis through 2% agarose and recovered by the method of Tabak and Flavell (13).

Preparation of Single-Stranded Fragments by Using Exonuclease III. Isolated duplex restriction endonuclease fragments were converted to single-stranded form with exonuclease III in a total volume of 25 μl containing: 1–2 μg of DNA fragment; 50 mM Tris·HCl, pH 8.0; 0.5 mM $MgCl_2$; 1 mM 2-mercaptoethanol; and gelatin at 100 μg/ml. This mixture was heated for 5 min at 45°C before adding approximately 5 units of enzyme. After a brief incubation at 45°C (2 min for the 128-bp *Pst* I-*Pvu* I fragment; 5 min for the 369-bp *Taq* I fragment) the reaction was terminated by adding an equal volume of 0.5 M NaCl/10 mM EDTA and heating to 65°C for 30 min. The DNA was then extracted with phenol, precipitated with ethanol, and dissolved in a small volume of 2 mM Tris·HCl, pH 8.0/0.5 mM EDTA. Before use in the *recA* reaction, the exonuclease III-treated DNA was denatured by immersion in a boiling water bath for 1 min.

Formation of Displacement Loops (D-loops) by Using *E. coli recA* Protein. D-loop formation reactions using *recA* protein were carried out at 37°C in polypropylene tubes, typically in a volume of 30 μl containing: 150 ng of supercoiled pBR322 plasmid DNA; 30–50 ng of exonuclease III-digested single-stranded homologous fragment; 20 mM Tris·HCl, pH 8.0; 10 mM $MgCl_2$; 1 mM dithiothreitol; and 1.3 mM ATP. At this fixed ratio of covalently closed plasmid to fragment, the amount of *recA* protein was varied in the range 20–100 pmol/30 μl in order to optimize the yield of D-loops as assayed by retardation of mobility during electrophoresis in agarose gels (see Fig. 3). Control reactions with no fragment DNA were run to assess the yield of D-loops and the extent of nonspecific nicking.

Nicking of D-Loops with S1 Nuclease. The minimal amount of S1 nuclease required to convert about 90% of D-looped DNA to the nicked-circular form was determined by diluting 1 vol of the D-loop reaction mixture directly into 9 vol of a solution (preheated at 45°C for 10 min) containing: 55 mM sodium cacodylate, pH 6.4; 1.1 mM $ZnSO_4$; 110 mM NaCl; 0.44% sodium dodecyl sulfate; and 20–500 units of S1 nuclease per 100 ng of closed circular DNA. After incubation for 2 hr at 45°C, the S1 was inactivated by making the solution 150 mM in Tris·HCl, pH 8.8, and 20 mM in EDTA and incubating at 45°C for an additional 30 min. DNA was then recovered by ethanol precipitation after addition of tRNA as carrier.

Local Mutagenesis *in Vitro*. Local mutagenesis was carried out essentially as described (2). The segment-directed nicks were converted to short gaps by subjecting the DNA to the 5′-3′ exonuclease activity of *M. luteus* DNA polymerase I exactly as before except that incubation was at 22°C instead of 11°C and 0.5 mM dATP was substituted for dTTP.

The deamination of the single-stranded C residues was carried out with 3 M sodium bisulfite (resulting in about 30% deamination) as described (2), as was the repair of the gap with DNA polymerase in the presence of all four deoxynucleoside triphosphates.

Purification of Nicked-Circular DNA by Chromatography on Acridine Yellow ED. To reduce the background of unmutagenized plasmid DNA, closed circular duplex DNA was removed by chromatography on acridine yellow ED beads (Boehringer Mannheim). The gap-repair reaction was stopped by adding EDTA to a final concentration of 15 mM. The reaction mixture was then diluted 1:20 into 10 mM sodium citrate, pH 6.0/0.5 mM EDTA/0.2 M NaCl. The diluted mixture was loaded onto a 0.2-ml column of the acridine beads. The nicked DNA was eluted with the same buffer containing 0.5 M NaCl and precipitated with ethanol in the presence of carrier tRNA.

Recovery of Mutants. The mutagenized pBR322 plasmid DNA was used to transform *E. coli* strain HB101 by the method of Dagert and Ehrlich (14). To assure recovery of independent mutants, the $CaCl_2$-treated cells were spread directly on nonselective LB agar (15) petri plates. The agar was immediately removed from the dish and laid on another dish containing one-third the volume of LB agar containing tetracycline at 80 μg/ml. This procedure allowed the cells time to recover from the calcium shock before application of the selection for drug resistance. Transformants (more than 500 per experiment) were picked and spotted on plates containing 20 μg of tetracycline per ml and plates containing 500 μg of ampicillin per ml.

Mapping of *bla* Mutations. Small preparations of each mutant plasmid DNA were used to transform *Salmonella* strain DB4566 (16). Mutations were mapped relative to point mutations and deletions carried on phage P22Ap31*pfr*1 by growing the *bla*$^-$ tester phage on the strain harboring the mutant plasmid and scoring the lysates for the appearance of recombinant phages capable of producing ampicillin-resistant lysogens. The plasmid-bearing *Salmonella* strains were plated in soft agar on λ agar plates (15) containing 0.2% glucose, 0.1% galactose, and 10 μg of tetracycline per ml. Spots of phage were applied to these plates and incubated overnight at 37°C. The spots were directly transferred (using a metal-pronged replicator device) onto plates containing 500 μg of ampicillin per ml that had been overlaid with 4×10^7 DB4566 cells in soft agar, incubated overnight, and scored for the presence of ampicillin-resistant lysogens. This test is reasonably sensitive, as judged from the fact that several classes of mutations in the same 130-bp interval are observed to recombine. Some map positions were verified by using high-resolution methods.

RESULTS AND DISCUSSION

Fig. 1 illustrates the principle of the segment-specific nicking procedure. Covalently closed circular DNA molecules and a homologous single-stranded fragment are incubated with the *recA* protein of *E. coli*. This enzyme, in the presence of Mg^{2+} and ATP, catalyzes the pairing of the single-stranded fragment

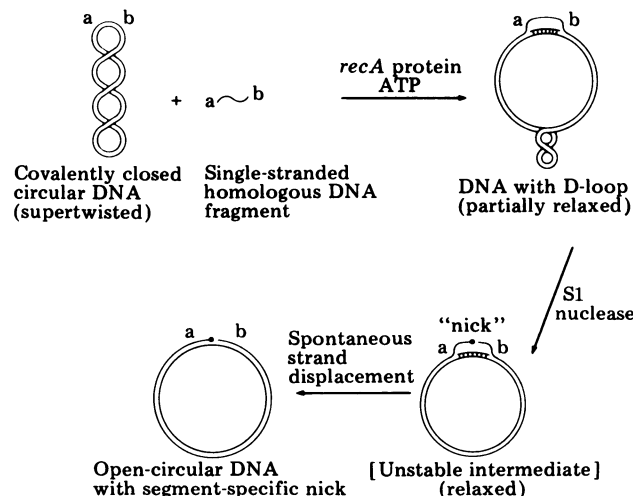

FIG. 1. Principal features of the segment-specific nicking procedure.

to the complementary sequence on the circular DNA (17, 18). One of the two strands of the covalently closed circular molecule is thereby displaced, forming an arc of single-stranded DNA called a "displacement loop," or "D-loop." Because one negative superhelical turn is removed for every 10 bp displaced, the D-loop structure is quite stable under the conditions employed here.

The next step of the procedure consists of the incubation of the mixture of D-loops and unreacted covalently closed circular molecules with a small amount of the single-strand-specific endonuclease S1 (19). The primary substrate for this enzyme is the displaced strand of the D-loop. The extent of the reaction with S1 is limited because the negative superhelical turns that stabilize the D-loop can be removed by rotation after the first S1 cleavage. After the loss of the superhelical turns, the D-loop decays in a fraction of a second (20) by spontaneous displacement of the single-stranded fragment, yielding a nicked-circular DNA that is not a substrate for the S1 enzyme.

The relaxed circular molecules that are produced by this procedure should, in principle, have a nick somewhere in the region defined by the single-stranded fragment, in the strand with the same sequence as the added single-stranded fragment. The use of separated strands rather than exonuclease III-digested DNA in making the D-loops will change the spectrum of nicking sites and therefore will change somewhat the set of C residues that may be mutagenized.

Construction of β-Lactamase Mutants Limited to a 128-bp Segment of the Structural Gene Defined by the *Pvu* I–*Pst* I Restriction Fragment. The structural gene for β-lactamase (the *bla* gene) carried on the small plasmid pBR322 was used as a substrate for segment-directed mutagenesis. A fine-structure genetic map of this gene has been constructed in this laboratory by using a derivative of the *Salmonella* phage P22 carrying the gene; the map has been correlated with the nucleotide sequence (refs. 21 and 22; unpublished data). A particularly accessible region of the gene is a 128-nucleotide segment bounded by restriction targets for the endonucleases *Pvu* I and *Pst* I, each of which cleave pBR322 only once. The *Pvu–Pst* segment makes up only 15% of the *bla* gene (Fig. 2), and a deletion corresponding to this segment was constructed as described in *Materials and Methods*.

The *Pvu* I–*Pst* I fragment was isolated and coverted to single-stranded form by digestion with exonuclease III. The single-stranded DNA was used to make D-loops from supercoiled pBR322 DNA by using the *recA* protein. D-loop formation was followed by electrophoresis through agarose gels subsequently stained with ethidium bromide; as shown in Fig. 3, the unreacted covalently closed circular DNA moves as a sharp band (lane a), while the D-loops move more slowly, forming a broad band just behind the unreacted DNA (lane e). This decrease in mobility is a consequence of the reduction in the number of negative superhelical turns that occurs upon pairing of the single-stranded fragment. The broadness of the band is due to the heterogeneity of the lengths of the single-stranded fragments, a consequence of the use of exonuclease III to make them single stranded. It should be noted that in cases in which the superhelical turns are all removed by the D-loop reaction (which will occur with single-stranded fragments greater than about 300 nucleotides in length), the D-loops will comigrate with nicked circular DNA, possibly necessitating a different assay.

The D-loops were then treated with various concentrations of the single-strand-specific endonuclease S1. As the S1 concentration is increased (Fig. 3, lanes e–h), the band corresponding to the D-loop structures disappears and the amount of nicked-circular DNA increases 10- to 20-fold. The constant intensity of the band corresponding to unreacted closed circular DNA indicates that there is little nicking of this species by S1, a conclusion supported by the low level of nicking in the control reactions (lanes a–d) in which no single-stranded fragment had been added to the *recA* reaction. In subsequent preparative reactions, an amount of S1 sufficient to nick approximately 90% of the D-loops (as judged from Fig. 3) was used.

An experiment was carried out to verify chemically that most of the nicks made in the last step by S1 nuclease had indeed been directed to the interval between the *Pvu* I and *Pst* I sites by the prior *recA* reaction with single-stranded *Pvu* I–*Pst* I fragment. The nicks were converted to small gaps by using *M. luteus* DNA polymerase and then were repaired by this enzyme in the presence of [α-^{32}P]dTTP. This results in the introduction of radioactive phosphorus at the site of each nick. Cleavage of the DNA thus labeled with an appropriate restriction enzyme allowed us to localize the radioactivity, and thus the location of the nick. As shown in Fig. 4 (lane b), the radioactivity is localized to *Hae* III fragment 6 (a 264-bp fragment spanning the *Pvu–Pst* interval, beginning 20 nucleotides before the *Pvu* I site and ending 120 bp beyond the *Pst* I site). There is much radioactivity near the bottom of the gel; this is also found in a control reaction in which the DNA was not cleaved with *Hae* III (lane a) and represents incorporation into the heterogeneous exonuclease III-treated single-stranded *Pvu–Pst* fragment. Also shown in Fig. 4 (lane c) is a similar experiment using pBR322 DNA nicked randomly with pancreatic DNase in the presence of ethidium bromide (24); as expected, the radioactivity is randomly distributed among the *Hae* III fragments.

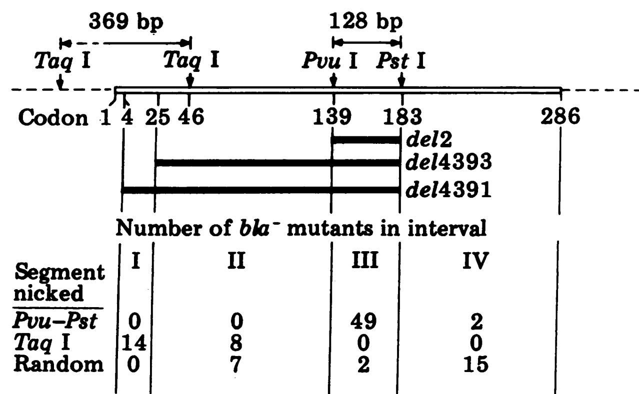

FIG. 2. Genetic map of the β-lactamase (*bla*) gene. The intervals are defined by the deletions as shown in the upper portion; the table at the bottom shows the distribution of *bla* mutations constructed *in vitro*.

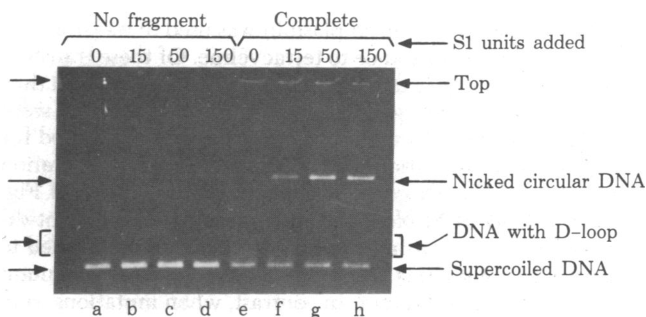

FIG. 3. Assay of the *recA* protein-catalyzed conversion of covalently closed circular duplex pBR322 DNA to D-looped structures and their nicking by S1 nuclease. The single-stranded fragment was the *Pvu* I–*Pst* I fragment digested with exonuclease III. Reaction conditions are given in *Materials and Methods*; 75 ng of circular DNA was applied to each lane.

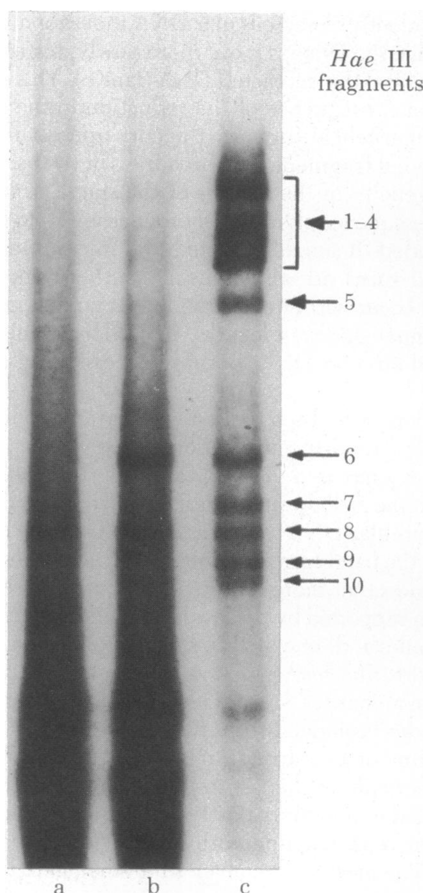

FIG. 4. Localization of the segment-directed nicks by gap repair in the presence of radioactive dTTP. Plasmid DNA (50 ng, gapped as described in *Materials and Methods*) was incubated in a 10-μl reaction mixture containing 70 mM Tris·HCl, pH 8.0; 7 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 50 μM each dATP, dGTP, dCTP; and 2 μCi of [α-^{32}P]dTTP (400 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) as follows: 10 min on ice, then addition of 0.2 unit of *M. luteus* DNA polymerase I, followed by 15 min further incubation at 0°C. The reaction was stopped by making the solution 15 mM in EDTA and heating to 65°C for 15 min. The labeled DNA was electrophoresed through a 4% acrylamide gel (23). Lane b contains DNA nicked by using the *Pvu* I-*Pst* I fragment, labeled as above, and cleaved with *Hae* III; lane a contains the same reaction material uncleaved; and lane c contains DNA randomly nicked with DNase I in the presence of ethidium bromide (24), labeled as above, and cleaved with *Hae* III.

A preparation of pBR322 molecules nicked in the *Pvu* I-*Pst* I interval was subjected to local mutagenesis with sodium bisulfite (2). The mutagenized plasmid was used to transform *E. coli*, selecting for resistance to tetracycline. Of these transformants 7.5% were found to be sensitive to ampicillin, and thus were mutant in the *bla* gene. These *bla*$^-$ mutant plasmids were transferred by transformation into *Salmonella* and tested for their ability to recombine with P22 phages carrying deletion derivatives of the *bla* gene. The results (at the bottom of Fig. 2) were that 49 of 51 *bla*$^-$ plasmids made by the segment-directed mutagenesis using the *Pvu* I-*Pst* I fragment failed to produce recombinants with the P22*bla*$^-$ deletion mutant lacking the same fragment. In contrast, when mutations were made by the same mutagenesis procedure on pBR322 molecules nicked at random with pancreatic DNase, only 2 of 24 *bla*$^-$ mutations failed to recombine with the same P22*bla*$^-$ deletion phage. These results confirm that the segment-directed nicking procedure indeed allows the efficient mutagenesis of a predetermined region in the target DNA.

High-resolution mapping of the mutants derived by segment-directed mutagenesis is not yet complete. However, we can distinguish at least four classes of mutations based upon recombination tests with a *bla*$^-$ frameshift mutation (*fs*7) that lies in the *Pvu-Pst* interval as well as upon the degree of residual resistance to ampicillin. These observations suggest that several distinct sites within the *Pvu-Pst* interval have been mutated by the segment-directed mutagenesis procedure. About half of the 49 *bla*$^-$ mutations in the *Pvu-Pst* interval revert to full resistance to ampicillin, indicating that these mutations are single lesions, at least with respect to any phenotypic effect. The observation that the other half of the mutants fail to revert is interesting (and different from the results with the *Taq* I fragment; see below). This result may reflect the fact that the *Pvu-Pst* interval is very rich in G·C bp (the substrate for the mutagenesis by bisulfite); at the level of cytosine deamination (about 30%) employed, a gap of six nucleotides (2) might well result in reaction of more than one C residue.

Construction of β-Lactamase Mutants Limited to the Beginning of the Gene by Using a *Taq* I Fragment. A second series of segment-directed mutagenesis experiments was carried out by using a *Taq* I restriction fragment 369 bp in length that includes the first 135 bp (i.e., the first 45 codons) of the β-lactamase structural gene. This segment includes the "signal" sequence region, consisting of the first 23 amino acid residues, which are removed during maturation and secretion of the protein (22, 25, 26). The results (bottom of Fig. 2) were that 14 of 22 mutations map in an interval completely covered by the *Taq* I fragment (interval I) and 8 map in an interval (interval II) partially covered by the *Taq* I fragment. Thus at least 65% (and possibly all) of the the mutations lie in the expected region.

The fourth codon of the β-lactamase gene is a CAA (glutamine) codon, which should be mutable to UAA (ochre) by bisulfite. In previous experiments, this ochre mutation (called *oc*U8) was isolated by mutagenesis of a P22*bla*$^+$ phage *in vivo*; its position was confirmed by mapping against the deletions of known sequence shown in Fig. 2 (unpublished data). All 14 mutants derived by segment-directed mutagenesis that mapped in interval I (which spans *oc*U8) were tested for recombination with a P22*bla*$^-$*oc*U8 phage. Four failed to recombine. One of these was transferred onto a P22 phage and the spectrum of suppression by amber and ochre suppressors was found to be identical to that of the original *oc*U8 mutation. In this case, it is possible to conclude with some assurance that the mutagenesis procedure resulted in the production of only one lesion affecting β-lactamase gene function.

Of the remaining 10 mutations in interval I, four subclasses could be distinguished by the level of residual resistance to ampicillin, again supporting the conclusion that several distinct sites have been mutated. In this case, however, 20 of 21 mutations tested revert to full ampicillin resistance, indicating that most of these mutations are single mutations.

A few noteworthy differences were observed in carrying out segment-directed mutagenesis with the *Taq* I fragment, all of which might be explained by the fact that the *Taq* I fragment, upon digestion with exonuclease III, should have an average length of approximately 185 bp as opposed to an estimated 64 bp for the *Pvu* I-*Pst* I fragment. First, 70–80% conversion of covalently closed circular DNA to D-looped products was observed (versus 40–60% with the *Pvu* I-*Pst* I fragment), suggesting that the reaction is somewhat more efficient with larger single-stranded fragments. Second, the D-looped structures formed a band that was retarded in mobility on agarose gels further than in the preceding experiment, a result consistent with the idea that the larger fragment should remove a larger

number of negative superhelical turns. Finally, the optimal concentration of S1 nuclease for nicking of the D-loops was approximately one-third that required in the experiment shown in Fig. 3, a value corresponding to the ratio of the expected lengths of the D-loops.

CONCLUSIONS

We have shown that one can efficiently direct mutations to a small segment of a closed circular DNA by using a small homologous single-stranded fragment. Both the efficiency and the specificity of the overall nicking reaction are high as judged by genetic and chemical analyses. The efficiency of the overall process seems high enough to permit the isolation of mutants even when no large-scale selection or screening methods are available.

Populations of circular molecules that have been nicked specifically in a small fraction of their length could be subjected to a number of *in vitro* manipulations besides mutagenesis with bisulfite. As mentioned above, incorporation of base analogues by limited action of DNA polymerase could be applied to make mutations. Another useful application is the formation of deletion mutations (9); a method for constructing deletions by direct and extensive S1 digestion of D-looped molecules (formed without catalysis) has recently been described (27).

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