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Local mutagenesis within deletion loops of DNA heteroduplexes

(base substitutions/sodium bisulfite/plasmids)

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ABSTRACT An efficient method has been developed to generate base substitution mutations within deletion loops of DNA heteroduplexes. This method utilizes a heteroduplex formed between a deletion mutant cloned in a plasmid vector and its wild-type counterpart from which two restriction sites had been removed from the vector. The heteroduplex is exposed to sodium bisulfite to deaminate cytosine residues in the single-stranded loop, and the mutagenized plasmid DNA is used to transform a strain of bacteria lacking the enzyme uracil *N*-glycosylase. Pooled progeny DNA is digested with the two restriction enzymes, whose sites had been mutated in the wild-type plasmid, to eliminate the original deletion mutant DNA. Point mutants with C·G-to-T·A transitions are obtained at high frequency after a second transformation. To test the feasibility of the approach, the tetracycline resistance gene of pBR322 was chosen as the target sequence. It was found that the proportion of tetracycline-sensitive transformants increased as both the size of the heteroduplex loop and the time of incubation with the mutagen increased and this varied from 20% up to 70%. Nucleotide sequence analysis of several tetracycline-sensitive mutants confirmed that C-to-T transitions had been produced in the segment of DNA corresponding to the deletion loop.

Functional analysis of cloned genes or DNA regulatory elements often entails the *in vitro* construction of mutations by directed mutagenesis procedures (for review, see ref. 1). Regions of interest can be identified initially by enzymatic removal of nucleotides and by testing the resulting deletion mutants for biochemical or biological activity. Once a critical segment of DNA is localized by this means, finer analysis requires the construction of base substitution mutations within those sequences defined by the deletions. For this purpose an efficient and general method has been developed for generating base substitutions within deletion loops of heteroduplexes formed by pairing DNA from a deletion mutant with its full-length counterpart.

MATERIALS AND METHODS

Bacterial Strains. MM294 (*pro*$^-$, *endo*A$^-$, *thi*$^-$, *hsd*R$^-$, *hsd*M$^+$; ref. 2) was obtained from Brown Murr, BD1528 (*thy*A, *met*$^-$, *nad*BF, *ung*-1, *gal*$^-$, *sup*E, *sup*F, *hsd*R$^-$, *hsd*M$^+$) was obtained from Bruce Duncan, and GM48 (*thr*$^-$, *leu*$^-$, *thi*$^-$, *ton*A, *gal*-6, *lac*Y1, *lac*Z4 *dam*-3 *dcm*-6) was from Bernard Weiss.

Transformation. Competent cells from MM294 and BD1528 were prepared by using a modified $CaCl_2$ procedure (3, 4). With pBR322 and its derivatives, this procedure gave efficiencies of 1–2 × 10^7 transformants per μg of DNA with MM294 and 1–5 × 10^5 transformants per μg of DNA with BD1528. Transformants were plated onto L plates containing ampicillin (Ap; 100–250 μg/ml), and transformants were assayed for their tetracycline (Tc) resistance on L plates containing both Ap (100–250 μg/ml) and Tc (5 μg/ml). Partial resistance to Tc was scored when there was normal growth on Ap plates but less growth on Tc plates.

Preparation of Plasmid DNA and Construction of Mutants. Plasmid DNA was amplified overnight in the presence of chloramphenicol at 150 μg/ml (5) and was extracted by using a modified cleared lysate procedure (6) in which the stock lysozyme solution was 50 mg/ml and the lysing solution was altered to give final concentrations of 0.125% $NaDodSO_4$/0.25% Triton X-100/25 mM Tris·HCl/12.5 mM EDTA, pH 7.4. Centrifugation at 35,000 rpm at 4°C for 1 hr in a Beckman SW 41 Ti rotor removed cell debris and much of the chromosomal DNA. Plasmid DNA was purified by banding twice in CsCl/ethidium bromide gradients, and the ethidium bromide was removed by three isobutanol extractions and one chloroform/isoamyl alcohol, 24:1 (vol/vol), extraction. The DNA was concentrated by ethanol precipitation and dialyzed against 10 mM Tris·HCl/1 mM EDTA, pH 8. Rapid DNA preparations were made according to the method of Holmes and Quigley (7).

Deletion mutants were constructed in the Tc resistance gene of pBR322 (8). Plasmid DNA was digested to completion with *Sal* I and a variable number of nucleotides was removed by digestion at 0°C for 1–5 min with the exonuclease BAL-31 (Bethesda Research Laboratories), and DNA at 5 units/μg in 100 μl of 20 mM Tris·HCl, pH 8.0/12 mM $MgSO_4$/12 mM $CaCl_2$/200 mM NaCl/1 mM EDTA. Reactions were stopped by addition of EDTA to 20 mM and $NaDodSO_4$ to 1%; the reaction mixture was extracted with phenol and the DNA was precipitated with ethanol. After ligation to circles with T4 DNA ligase, digestion with *Sal* I, and transformation of MM294, Tc-sensitive (^{S}) transformants were screened for the presence of deletions by restriction enzyme analysis. DNA from three deletion mutants, pKP10, pKP11, pKP12, was prepared and their nucleotide sequences were determined (Table 1).

To eliminate the *Ava* I and *Eco*RI sites in pBR322, plasmid DNA was digested to completion with *Ava* I and the four 5′ single-stranded nucleotides were repaired by incubation of 0°C for 15 min with 1–2 units of *Micrococcus luteus* DNA polymerase I in the presence of the appropriate deoxynucleoside triphosphates at 20 μM each. The DNA was circularized with T4 DNA ligase, digested with *Ava* I, and Ap^R transformants were tested for the loss of the *Ava* I site. One, pKP25, was digested with *Eco*RI and that site was removed in a similar way; pKP30 is both *Eco*RI^R and *Ava* I^R and Ap^R and Tc^R.

Heteroduplex Formation and Mutagenesis. DNAs were linearized with *Pst* I (pKP10, pKP11, pKP12) or with *Bam*HI (pKP30). DNA (1 μg) from a deletion mutant was mixed with DNA (1 μg) from pKP30 in a volume of 1,155 μl of H_2O and was denatured by addition of 125 μl of 1 M NaOH and incu-

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Abbreviations: bp, base pair(s); ^{R}, resistant; ^{S}, sensitive; Ap, ampicillin; Tc, tetracycline.

Table 1. Properties and sequences of plasmids

Plasmid	Phenotype	Restriction sites	Extent of deletion, base pairs (bp)*
pKP30	Ap^R Tc^R	*Ava* I^R *Eco*RI^R	—
pKP10	Ap^R Tc^S	*Ava* I^S *Eco*RI^S	49 (653–701)
pKP11	Ap^R Tc^S	*Ava* I^S *Eco*RI^S	125 (584–708)
pKP12	Ap^R Tc^S	*Ava* I^S *Eco*RI^S	327 (476–802)

^{R}, resistant.
* Numbers in parentheses refer to nucleotide positions of pBR322.

bation for 15 min at room temperature. Annealing was accomplished by addition of 320 μl of neutralizing solution [1 M Tris·HCl, pH 7.2/1 M HCl, 2:1 (vol/vol); ref. 9] and incubation at 60°C for 2 hr. Transfer RNA was added to 20 μg/ml and the nucleic acids were precipitated by addition of 2.5 vol of 95% ethanol and overnight incubation at −20°C. The precipitates were collected by centrifugation, and the pellets were washed in 80% ethanol at room temperature, dried, and dissolved in 100 μl of 1 mM EDTA at pH 7.5. In the initial experiments the form II, heteroduplex molecules were purified by electrophoresis on 1.5% agarose gels and were extracted from the gel by the glass fiber filter method of Chen and Thomas (10), but in later experiments the mixture of linear and circular molecules was used directly for mutagenesis.

Sodium bisulfite mutagenesis was carried out according to published procedures (11, 12). The final concentration of sodium bisulfite was 2 M; control samples were incubated with 2 M NaCl. After the final dialysis step, the DNA was used to transform BD1528 cells.

DNA Sequence Analysis. Mutants were subjected to sequence analysis by using the method of Maxam and Gilbert (13) after labeling the 3′ ends of restriction fragments with *M. luteus* DNA polymerase I and the appropriate labeled [α^{32}P]dNTP and unlabeled dNTP. Cleavage products were resolved on 0.3-mm 8% and 20% polyacrylamide gels (14).

RESULTS AND DISCUSSION

To test the feasibility of producing point mutations in single-stranded heteroduplex loops, we chose to construct mutations in the Tc resistance gene of the bacterial plasmid pBR322, because phenotypic changes in this gene could be readily scored. The overall method is summarized in Fig. 1.

Heteroduplex molecules were prepared as described between pKP30 (*Ava* I^R *Eco*RI^R) and the deletion mutant derivatives (Table 1) of pBR322, pKP10 (49-bp deletion), pKP11 (125-bp deletion), and pKP12 (327-bp deletion). Heteroduplex formation was indicated by the appearance of molecules migrating at the position of form II molecules after agarose gel electrophoresis and was estimated to be between 20% and 40% of the total DNA. In the initial experiments form II molecules were purified from agarose gels (10). Subsequent experiments (see below) showed that gel purification was not necessary and the mixed population of circular and linear molecules was mutagenized directly.

Mutagenesis of the three purified heteroduplexes with 2 M sodium bisulfite was carried out as described (11, 12) for 1 hr and 2 hr. Control samples were incubated for 2 hr with 2 M NaCl. After dialysis the mutagenized and control DNA samples were used directly to transform BD1528 cells, a strain of *Escherichia coli* deficient in the enzyme uracil *N*-glycosylase (*ung*$^-$). Use of an *ung*$^-$ strain (BD1528) was found to be obligatory, because transformation of an *ung*$^+$ strain with bisulfite-treated heteroduplex DNA resulted in the loss of the heteroduplex loop and the only Tc^S mutants found contained the original deletion.

Replication of heteroduplex molecules after transformation

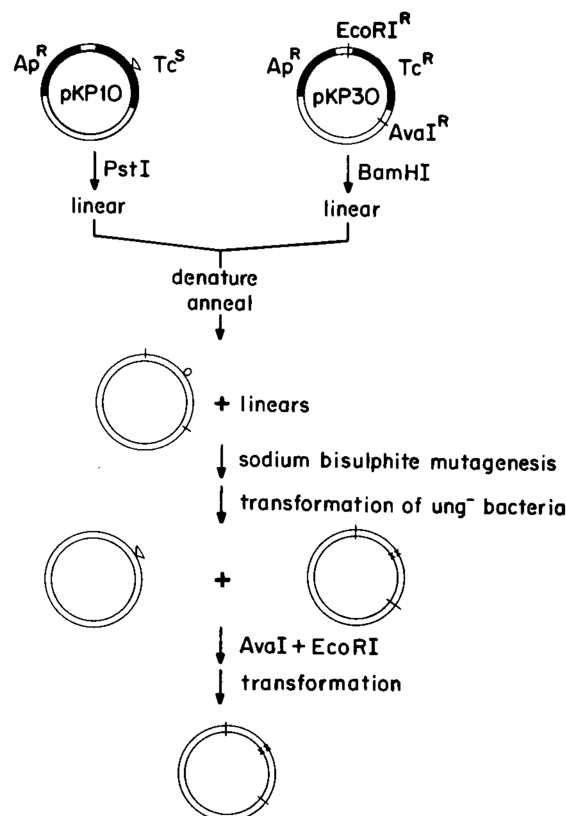

FIG. 1. Summary of the mutagenesis procedure. The plasmid pKP10 is Ap^R and carries a deletion rendering it Tc^S, whereas pKP30 is both Ap^R and Tc^R as well as being resistant to restriction enzymes *Eco*RI and *Ava* I.

should result in each cell containing the two original plasmid species, assuming that the deletion loop is not excised prior to replication. To enrich for the full-length plasmid, DNA prepared from pooled progeny BD1528 transformants was digested with *Ava* I and *Eco*RI prior to a second transformation of MM294 cells. Because the *Ava* I and *Eco*RI sites had been eliminated in the full-length molecule (pKP30), digestion with these enzymes selectively fragmented the deletion mutant DNA. Because linear molecules with noncompatible ends transform about 1/100th to 1/1,000th as efficiently as circular molecules (unpublished data), the yield of deletion mutant DNA was greatly decreased.

Individual Ap^R MM294 colonies resulting from the second round of transformation were tested for their Tc resistance and the results are summarized in Table 2. With no sodium bisulfite treatment and no enzyme digestion before the second transformation, clones containing full-length plasmids (Tc^R) occurred at frequencies varying from 36% with the 49-nucleotide loop down to 14% with the 327-nucleotide loop. If the DNA was digested with *Ava* I only, then the proportion of Tc^R transformants increased to >80% with all three loop sizes (data not shown). This value could be increased to >90% by digestion with both *Ava* I and *Eco*RI. In subsequent experiments in which heteroduplex molecules were not gel purified (Table 2), 98% of all Ap^R colonies arising after transformation with DNA digested with *Ava* I and *Eco*RI were Tc^R—i.e., only 2% carried the deletion in the Tc gene. The lower proportion of Tc^R transformants when the heteroduplex molecules were gel purified was most likely due to nicking of the single-stranded loop during recovery from the gel.

Sodium bisulfite mutagenesis increased the proportion of

Table 2. Phenotypes of transformants

Heteroduplex pKP30 with	Time of mutagenesis, min*	*Ava* I and *Eco*RI†	Tc sensitivity		
			Resistant	Partially resistant	Sensitive
Gel purified‡					
pKP10	0	–	36	0	64
49-bp deletion	0	+	91	0	9
	60	+	69	8	23
	120	+	60	12	28
pKP11	0	–	22	0	78
125-bp deletion	0	+	91	0	9
	60	+	64	13	23
	120	+	52	18	30
pKP12	0	–	14	0	86
327-bp deletion	0	+	92	0	8
	60	+	47	8	45
	120	+	21	6	73
Mutagenized directly§					
pKP11	0	–	26	0	74
125-bp deletion	0	+	98	0	2
	60	+	83	6	11
	120	+	63	13	24

*The times of incubation with 2 M sodium bisulfite were 60 and 120 min; 0 means that the DNA was incubated with 2 M NaCl.
†Indicates whether the DNA was digested prior to the second transformation.
‡The heteroduplexes were gel purified.
§The mixture of heteroduplexes and linears was mutagenized directly.

Tc^S colonies as both the time of exposure to the mutagen and the size of the deletion loop increased (Table 2). For example, with a loop of 327 nucleotides, 45% of the Ap^R transformants were made Tc^S with 1 hr of 2 M sodium bisulfite treatment, and this was increased to 73% after an additional hour of incubation. With a loop size of 49 nucleotides, ≈30% of the Ap^R transformants were rendered Tc^S after a 2-hr incubation with 2 M sodium bisulfite. In addition to the Tc^S phenotype, transformants that were partially resistant to Tc were found, as might be expected if the mutation decreased rather than eliminated the activity of the protein.

As an alternative approach to the enrichment of full-length molecules over deleted molecules, the effect of methylation in dam^+ *E. coli* on the relative recovery of full-length molecules was investigated (15). Unmethylated plasmid DNA was obtained by propagation in the dam^- strain GM48. Heteroduplexes were prepared between pKP30 and either methylated or unmethylated pKP11 (125-bp deletion). With no *Ava* I or *Eco*RI digestion prior to the second transformation, Tc resistance was found at a frequency of 37% with dam^+/dam^- heteroduplexes and at a frequency of 26% with dam^+/dam^+ molecules. If the DNA samples were first digested with *Ava* I and *Eco*RI, then the proportion of Tc^R transformants from the dam^+/dam^+ heteroduplex was increased to 98% (Table 2), whereas that from the dam^+/dam^- heteroduplex was 100%. Therefore, the use of the *dam* methylation system in this procedure resulted at best in only a slight improvement in the yield of full-length molecules when the deletion loop is 125 nucleotides in size. [The difference between dam^+/dam^+ (98%) and dam^+/dam^- (100%) suggests that correction of the mutations conferring *Ava* I and *Eco*RI resistance may have occurred after transformation with dam^+/dam^+ heteroduplexes, and perhaps it was this correction rather than incomplete digestion that accounted for the difference.]

To ascertain whether the Tc^S phenotypes were due to the production of base substitution mutations or due to the introduction of small deletions through bacterial correction mechanisms, several mutants were subjected to sequence analysis.

```
       Ala       LeuLeuLeuGlyCysPheLeuMetGlnGluSerHisLysGlyGluArgArgProMetProLeuArgAlaPheAsnProValSerSerPheArgTrpAlaArgGly
Mutant
       GCG(18 bp)CTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGC

  1                                                              ----------------------------------------------A--

  2                                                              -----------------------------------------------A-

  3                                                              -----A--------A--------------------------------A-

  4                                                              ---------TT-----------------T-------------T------

  5    ---       ---------A---A---------A----------------A-----------------------------------------------------A----------

  6    ---       ---T---------------------------T-------------------------------------------------------------------------

                    Leu   SerTyr       Ile      Leu      Glu        Pro   PheLeu            Leu            TrpTerm
```

FIG. 2. Sequences of constructed point mutants. The nucleotide sequence of the Tc resistance gene and the deduced amino acid sequence of its protein for the relevant region is shown. The dashed lines indicate the nucleotides exposed to the mutagen in the six mutants analyzed: 49 nucleotides in mutants 1–4 and 125 nucleotides for mutants 5 and 6. Mutants 1, 2, 5, and 6 were incubated with sodium bisulfite for 1 hr, whereas mutants 3 and 4 were incubated for 2 hr. Term, chain termination.

Fig. 2 shows the sequences of six mutants, four produced by mutagenizing the 49-nucleotide loop, and two produced by mutagenizing the 125-nucleotide loop. Mutants 1, 2, 5, and 6 were obtained after 1 hr of sodium bisulfite treatment; mutants 3 and 4 were obtained after 2 hr of treatment.

The results show that in all cases the changes found are those predicted by sodium bisulfite-induced C-to-T transitions in one or the other of the two strands, but C-to-T and G-to-A changes in the same molecule were not found. This result demonstrates that, as expected, each strand is available for mutagenesis. A comparison of the sequence changes in mutants 1 and 2 with mutants 3 and 4 indicates that the level of base substitution increases as the time of incubation is increased from 1 to 2 hr. Mutants 1 and 2 have only one change each, whereas mutants 3 and 4 have three changes and four changes, respectively. In the mutants thus far subjected to sequence analysis—the 6 shown in Fig. 2 and an additional 14 not shown—all of the base substitutions occurred within the heteroduplex loop. No changes were found up to 100 nucleotides on either side of the loop. Fig. 2 also shows that sodium bisulfite can react with cytosine residues within two and three nucleotides of the base of the loop.

The mutants shown in Fig. 2 were selected either for their Tc sensitivity or, in the case of mutant 6, its partial Tc resistance. Therefore, silent base substitutions would not have been observed and the actual level of base substitution should be higher than that predicted from the loss of antibiotic resistance. In recent experiments it has been feasible to screen for mutants by DNA sequence analysis.

In conclusion, it has been demonstrated that point mutations can be produced efficiently in deletion loops of plasmid heteroduplexes. The method is generally applicable to any cloned DNA segment, although the choice of restriction sites to be removed from the vector will be dictated by the sequence of the cloned fragment.

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