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**Deletion loop mutagenesis: a novel method for the construction of point mutations using deletion mutants**

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**ABSTRACT**

Deletion loop mutagenesis is a new, general method for site-directed mutagenesis that allows point mutations to be introduced within a sequence of DNA defined by a previously isolated deletion mutant. Wild type and deletion mutant DNA are cloned into a bacterial plasmid and each is cleaved with a different single cut restriction enzyme. Heteroduplexes are formed between the two DNAs to produce circular molecules containing a nick in each strand and a single-stranded deletion loop. The deletion loops are mutagenised using sodium bisulphite and the DNA transfected directly into a uracil repair deficient strain of *Escherichia coli*. Up to half of the resultant clones contain DNA produced by replication of the wild-type length strand and bear mutations exclusively within the target area. An example is given in which a deletion mutant lacking 21 nucleotides from the region coding for SV40 large-T was used. Eight of the possible nine target cytosine residues were mutagenised. The method described is specific, efficient and simple.

**INTRODUCTION**

The methods of reverse genetics are currently being used to study the structure and function of numerous proteins and to define sequence elements controlling gene expression. These methods involve the introduction of mutations into cloned DNA molecules and the analysis of consequent changes in the biological properties of the genetically altered molecules. Deletion mutants have been used extensively to map coding regions (1) and to identify sequences that, for instance, affect the accuracy and levels of transcription of adjacent genes both *in vitro* and *in vivo* (2, 3, 4, 5). Deletions are, however, likely to alter not only primary nucleotide and amino acid sequences but also the shape of proteins and secondary structures that may be adopted by localised regions of DNA or RNA as part of a regulatory signal. The effect of point mutations must therefore also be studied in order to define precisely the components of regulatory signals and to probe the function of proteins in detail. Thus

the identification of important coding and regulatory sequences initially through the analysis of deletion mutants, followed by the generation of point mutations within the same sequences provides a powerful genetic approach to the study of protein function and gene expression.

Methods presently employed for the creation of point mutations use unique restriction endonuclease sites (6, 7, 8), synthetic oligonucleotide primers (9) or restriction fragments (10, 11, 12, 13) to direct the mutagenesis to a specific site. In practice, many of these methods require special reagents, favourable DNA sequences or extensive manipulations. The techniques for isolating deletion mutants (reviewed in (14) ) are generally not so demanding with regard to reagents, manipulations, screening procedures and the specificity of product required. We therefore sought a two-step approach to the generation of point mutations, which would have the added advantage that many deletion mutants that could be used as intermediates already exist and have been extensively characterised. One such two-step method for making point mutations has already been described (15) but it requires that a unique restriction site, usually inserted as a short segment of linker DNA, should span the deleted region. This method cannot therefore be used for a great number of existing deletion mutants nor in cases where it is essential to study the properties of the deletion mutant intermediate in the absence of added linkers.

The method reported here allows the introduction of single base changes within a region of DNA that is defined by the position of a deletion in an otherwise identical DNA sequence. The method is simple, efficient and imposes no sequence constraint on the substrate for mutagenesis. It therefore provides a rapid, general method for generating point mutations from existing deletion mutants.

### MATERIALS AND METHODS

#### Bacterial strains

ED8767 (supE supF hsd5 met<sup>-</sup> recA56). K58 (ung<sup>-</sup>) was kindly provided by Dr P.D. Baas (State University of Utrecht, The Netherlands) (16) The transfection efficiency of pBR322 related plasmids in K58 is about one tenth of that for ED8767.

#### Plasmids

The plasmid pPVU-0 contains SV40 early region sequences from nucleotide 2533 to 268 (in the modified numbering system of Fiers and colleagues (17) ) cloned between the Bam HI and Pvu II sites of pBR328.

pPVU-I is identical to pPVU-0 except for a deletion of 21 bp in the SV40 large-T coding between nucleotides 4480 and 4502. Both plasmids confer ampicillin resistance and were isolated from cultures of ED8767 by the alkaline extraction procedure (18) followed by centrifugation in ethidium bromide-caesium chloride gradients.

#### Enzymes

Restriction enzymes, Pvu II and Bam HI were from New England Biolabs; Hinf I was from Bethesda Research Laboratories and digestions with these enzymes were carried out in buffers recommended by the suppliers. Digestion of pPVU-0 and pPVU-1 with Pvu II and Bam HI, respectively, were performed using a 5-fold excess of enzyme. After phenol extraction and ethanol precipitation these digestions were repeated to reduce the amount of undigested plasmid to a minimum and the DNAs were again precipitated after phenol extraction.

#### Hybridisation Procedure

3.5µg of each linear plasmid were mixed together in 23µl water. 4.8 µl 1M NaOH was added and the mixture left at room temperature for 10 mins. Renaturation was accomplished by sequential addition of 280 µl water, 40 µl 0.5M Tris-HCl pH 8.0 and 48 µl 100mM HCl, prior to incubation at 63°C for 2 - 3 hrs.

#### Bisulphite Mutagenesis

The annealed products were ethanol precipitated twice, resuspended in a small volume of water and incubated in the dark under paraffin at 37°C in a freshly prepared solution of 3M sodium bisulphite pH 6.0, 2mM hydroquinone for 4 hrs. Subsequent dialyses were exactly as in (13), after which the DNA was ethanol precipitated and resuspended in 10mM Tris-HCl pH 8.0, 1mM EDTA.

#### Transfection procedure

Transfection of ED8767 or K58 was by the method of Dagert and Ehrlich (19). After heat-shock, bacteria were spread directly on non-selective LB-agar plates to which ampicillin in soft agar was added to a final concentration of 100 µg/ml after 4 - 6 hrs at 37°C. Independent colonies thus obtained were streaked once before analysis.

#### Analysis of plasmid DNA

Plasmid DNA was isolated from 5 ml overnight cultures by the alkaline extraction procedure (18) and was electrophoresed on non-denaturing polyacrylamide gels after digestion with Hinf I. Hinf I L-fragments of wild-type size were eluted from the gel, end-labelled with  $\gamma$ -<sup>32</sup>P ATP, strand-separated and sequenced by the chemical method of Maxam and Gilbert (20).

### RESULTS

#### Outline of the Method

The principle of deletion loop mutagenesis is illustrated in Figure 1. The wild-type DNA of interest is cloned in a bacterial plasmid and cut with a single site restriction enzyme. The deletion mutant DNA is cloned in an identical manner and cut with a different single cut restriction enzyme. Heteroduplexes are formed between the wild-type and deletion mutant DNAs. Because of the stagger in the restriction enzyme cleavage sites such heteroduplexes are circular whereas the homoduplexes that form simultaneously are linear. The heteroduplexes contain a single-stranded region of DNA in the wild-type strand corresponding to the sequence missing in the deletion mutant. The sequence within the deletion loop is therefore susceptible to chemical mutagenesis using sodium bisulphite, which selectively converts single-stranded cytosine residues to uracil. The modified heteroduplex is transfected directly into a strain of *E. coli* that lacks the uracil repair enzyme, uracil-N-glycosylase. Approximately half of the recovered plasmids are of wild-type length and the great majority of these contain one or more A:T base pairs in place of G:C base pairs in the target region.

The procedure has been used to mutagenise 8 out of the possible 9 sites in a 21bp region that forms part of the coding region for SV40 large-T and that is absent from the deletion mutant used in this study.

#### Formation of heteroduplex molecules

The plasmids, pPVU-0 and a derivative, pPVU-1 missing a 21bp sequence in the coding region of SV40 large-T were each cut at a unique but different site with the restriction enzymes, Pvu II and Bam HI respectively. The full-length linear products were mixed in equal proportions, denatured with alkali and allowed to re-hybridise. The annealed mixture was characterised by agarose gel electrophoresis (data not shown). The major product migrated with linear homoduplexes of the plasmids pPVU-0 and pPVU-1 but approximately one quarter of the annealed products co-migrated with the nicked circular forms (form II) of the plasmids and were presumed to be circular heteroduplexes formed between complementary strands derived from pPVU-0 and pPVU-1.

#### Transfection with heteroduplexes

The unfractionated mixture of homo- and heteroduplexes was used directly to transfect competent bacteria of the strain, ED8767. The number of transfectants formed per microgram of this DNA was thirty-fold greater

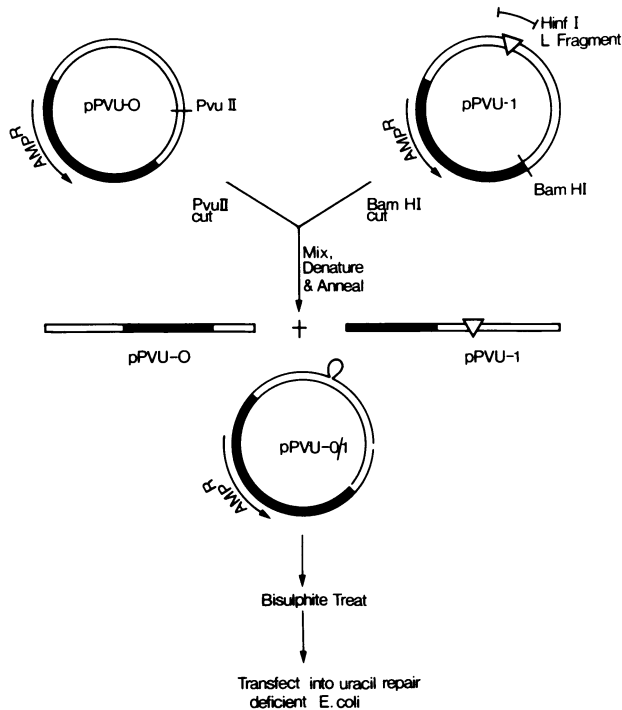


Figure 1. Principle of deletion loop mutagenesis.

than for the same mixture of linear plasmid DNA prior to denaturation and hybridisation (data not shown). This suggests that the majority of clones result from the transfection of heteroduplexes rather than homoduplexes. Plasmid DNA was isolated from these clones, digested with Hinf 1 and run on polyacrylamide gels to determine whether Hinf 1 L-fragments of 88bp length, characteristic of the deletion mutant plasmid pPVU-1, or of 109bp length, characteristic of the wild-type plasmid pPVU-0 were produced. Only one clone out of thirteen examined contained plasmids of both sizes. Eight of the remaining twelve clones contained exclusively plasmids of wild-type length. It thus appears that transfection with heteroduplexes allows the isolation of pure plasmid populations that are derived in roughly equal proportions from one or other of the two strands of the heteroduplex.

#### Transfection with bisulphite-treated heteroduplexes

When the same heteroduplex-containing mixture was treated with sodium bisulphite prior to transfection of ED8767, clones were again found

to contain largely (16 out of 17) only one size of plasmid. However, in all of these 16 clones the plasmid found was of deletion mutant length. Sequencing of the 109bp Hinf I L-fragment from the mixed plasmid clone revealed a wild-type distribution of G:C base pairs. It therefore appears that a strong selection is exerted in ED8767 against the amplification of wild-type length strands that contain uracil residues following bisulphite mutagenesis.

K58, a strain of *E. coli* deficient in the uracil repair enzyme, uracil-N-glucosidase (16) was therefore used as an alternative recipient for the transfection of bisulphite-treated heteroduplexes. In this case, 14 out of 34 clones analysed contained only wild-type length plasmid and one clone harboured both sizes of plasmid in approximately equal proportion (Figure 2).

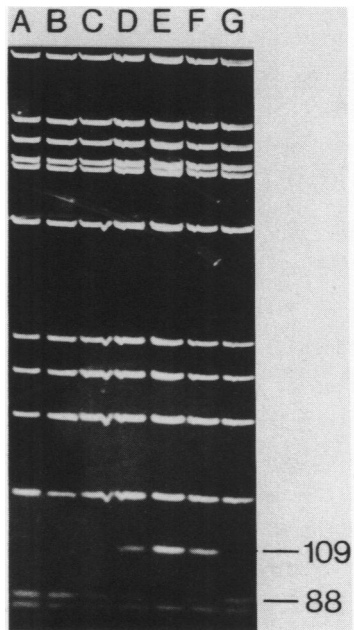


Figure 2. Hinf I restriction enzyme digest of plasmids derived from transfection of bisulphite treated heteroduplexes into K58. Plasmids isolated from 5 ml overnight cultures were digested with Hinf I and run on an 8% (1:50) polyacrylamide gel, subsequently stained with ethidium bromide (0.5 µg/ml). The plasmids run in lanes A, B, C and G are of deletion mutant length whereas those in lanes E and F are of wild-type length. Lane D shows the presence of an approximately equimolar mixture of the two sizes of plasmid.

Characterisation of mutant plasmids

The distribution of G:C base pairs within the 15 wild-type length Hinf I L-fragments isolated from K58 was determined using the chemical sequencing method. Three fragments displayed a wild-type sequence but 12 of the 15 had lost either G or C residues (in one strand sense), exclusively from the region of DNA exposed as a single-stranded deletion loop in the heteroduplex (Figures 3 and 4). The complete nucleotide sequence of the 109bp Hinf I fragment of one of the point mutants was determined to verify that the C residues lost had been converted to T

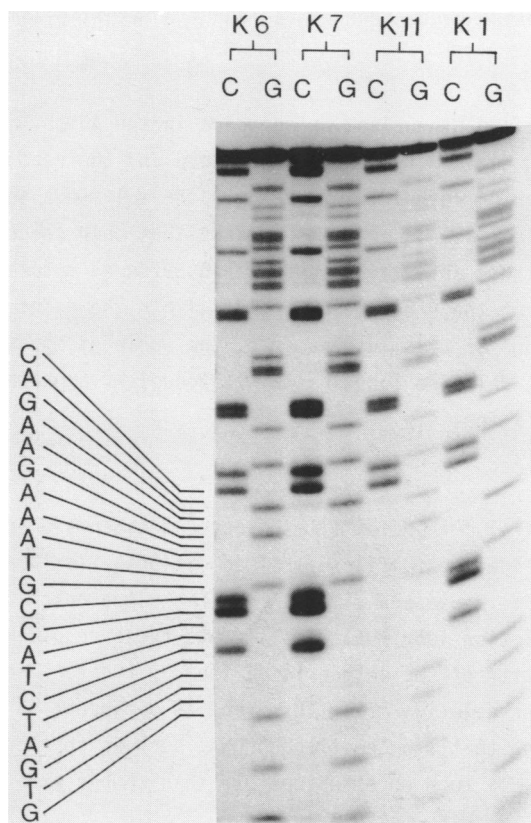


Figure 3. Distribution of G:C base pairs in mutant plasmids Hinf I L-fragments of 109bp length were eluted from polyacrylamide gels, end-labelled with  $\gamma$ - $^{32}\text{P}$ -ATP and strands were separated on an 8% (1:50) polyacrylamide gel. One strand was eluted, subjected to the C- and G-specific chemical sequencing reactions and run on an 8.3M urea 8% (1:20) polyacrylamide gel.

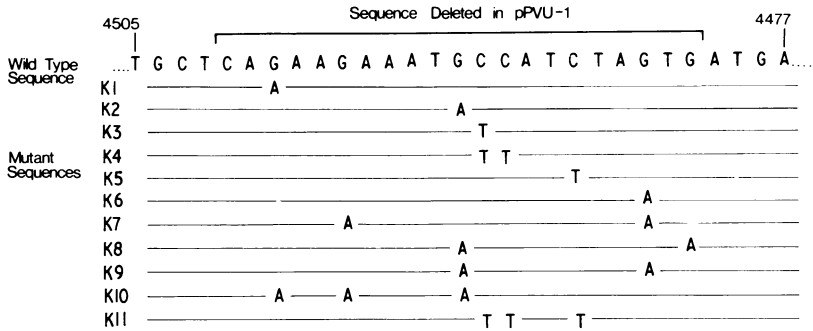


Figure 4. Nucleotide sequence changes found in mutant plasmids.

residues.

The conservation of nucleotide sequence in the 109bp Hinf I fragment outside the region defined by the deletion loop and the retention of the normal distribution of restriction sites for the multi-cut enzymes, Bst NI and Hinf I for all 15 plasmids suggest that only sequences of pPVU-0 corresponding to those absent from pPVU-1 had suffered mutation. Furthermore, as 8 of the 9 possible targets within the deletion loop were affected in the pool of plasmids examined, the whole of the sequence expected to form a deletion loop in the heteroduplex, appears to be susceptible to mutagenesis.

DISCUSSION

Any new general method for site-directed mutagenesis involving cloned DNA should ideally fulfil several conditions. It should be highly specific, efficient, rapid and simple. The technique described here, referred to as deletion loop mutagenesis meets these requirements.

The specificity of the method is defined by the sequences missing from the particular deletion mutant used and is dependent on the well-established finding that single-stranded DNA, such as that present in the deletion loop, is much more susceptible than double-stranded DNA to mutagenesis by sodium bisulphite (21).

The overall yield of mutant plasmids in the application of deletion loop mutagenesis described here was 30%. However, more than 60% of the non-mutant plasmids could be eliminated from further screening by polyacrylamide gel analysis of a single enzyme digest. The extent of mutagenesis found in plasmids of wild-type size depends on the conditions



used for bisulphite treatment. Previous studies (6) have documented the relationship between the extent of bisulphite treatment and the proportion of single-stranded cytosine residues converted to uracil. Clearly, the proportion of single point mutations recovered will be highest for small deletion loops but the method has also been used successfully to generate single point mutations within a deletion loop of 57 nucleotides. Theoretically it could be applied to mutate deletion loops of any size. The only other source of background wild-type plasmid is contributed by the transfection of linear homoduplexes. These form during the hybridisation step but have a very low efficiency of transfection compared with the highly efficient heteroduplexes.

Perhaps the most surprising aspect of the method described here is its simplicity. It was originally anticipated that in vitro DNA synthesis using the mutagenised deletion loop as template would be required to stabilise the C to U conversion by introducing an A residue in the opposite strand. However it was found that transfection of unmutated heteroduplexes without prior repair yielded plasmids of both wild-type and deletion mutant size in roughly equal proportions. Further, in the majority of cases only one size of plasmid was seen in each ampicillin-resistant bacterial colony despite the presence of plasmid DNA at about 20 copies per cell (data not shown). This suggests that one strand of the heteroduplex acts as a template for replication to the exclusion of the other strand. This preference appears to be random and certainly is not related to the strand sense since mutants could be isolated containing C to T conversions in either of the two complementary DNA strands of the wild-type parent plasmid.

Equal segregation of the two strands of the heteroduplex to produce double-stranded plasmids was no longer observed if the heteroduplexes were treated with bisulphite prior to transfection into ED8767. This suggested that the uracil repair system of E. coli (22) was acting on the heteroduplex before replication of the mutagenised strand of the heteroduplex could be accomplished. The use of an E. coli strain deficient in uracil-N-glucosidase, an enzyme thought to play a major role in the recognition and excision of uracil residues from DNA (23, 24), restored to equality the proportion of plasmids containing the wild-type length strand. Indeed, the proportions of single and multiple G:C to A:T conversions observed in these plasmids suggest that K58 fails totally to discriminate against the presence of uracil residues in the wild-type length strands of the heteroduplexes.

No special provision is made in this method for the rapid sequencing

of mutant plasmids but attention is drawn to some recently published procedures that may be applicable (24, 25). Also, as deletion loop mutagenesis can be conducted on plasmids encoding an entire gene or regulatory element, the biological activity of the mutant products may be assayed prior to sequence determination if convenient.

It is anticipated that deletion loop mutagenesis will be used to generate point mutations both de novo and from existing deletion mutants. As there is no requirement for a unique enzyme site spanning the deletion, the method is universally applicable to existing deletion mutants and imposes no constraint on the wide choice of techniques available for constructing deletion mutants as intermediates.

The properties of the mutant SV40 large-T proteins encoded by the plasmids constructed as described above will be reported elsewhere.

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