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A rapid and efficient method for region- and strand-specific mutagenesis of cloned DNA

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The single-stranded viral DNA of an M13 phage recombinant containing the early promoter region of SV40 was hybridized with linear, double-stranded replicative form DNA of a related M13 phage containing a short deletion in the cloned SV40 sequence. The heteroduplexes formed between these DNA molecules contained a short, defined single-stranded region in an otherwise duplex molecule. These heteroduplexes were treated with sodium bisulphite to deaminate exposed unpaired cytosines to uracil residues. The single-stranded region was filled in with DNA polymerase I, which incorporates adenine opposite the mutated uracils, and the DNA then transfected into the M13 host JM103. Viral DNA from the resultant plaques was used for the rapid dideoxy-DNA sequencing procedure; all of the plaques studied contained point mutations within the desired area. This method allows the very rapid and efficient generation of region-directed point mutants which can be quickly sequenced.

Key words: DNA sequencing/*in vitro* mutagenesis/M13 phage/sodium bisulphite

Introduction

The study of the molecular mechanisms of gene expression in both prokaryotic and eukaryotic systems often requires the *in vitro* generation of site-directed point mutations. Many such methods have been published (for example, Borrias *et al.*, 1976; Hutchinson *et al.*, 1978; Shortle and Nathans, 1978, 1979; Weber *et al.*, 1981; Giza *et al.*, 1981; Shortle *et al.*, 1980; reviewed by Shortle *et al.*, 1981). However, these methods suffer from one or more disadvantages in that the mutated region may be large or not easily controlled, specific synthesized oligonucleotides may be necessary which may not be routinely available, the background of unmutagenized DNA may be potentially high, the mutants formed may not be amenable to immediate sequence analysis, or complicated preparation of isolated DNA strands or fragments may be required. This paper describes a rapid method of directing point mutations into a defined small region of the SV40 early promoter, which had been cloned into M13. The method requires no special materials, uses easily isolatable DNA molecules, and is very rapid. The background of unmutagenized DNA was zero and each mutant could be immediately sequenced.

Results

The scheme of the method is shown in Figures 1 and 2. In this particular series of experiments the desired single-stranded region was generated by cutting the double-stranded replicative form DNA (RF-DNA) of M13.SV.23 with *Bam*HI (the molecule contains an unique *Bam*HI site at the deletion in pMKD52; Figure 1) followed by denaturation of this linear double-stranded molecule and hybridization with the single-stranded DNA (SS-DNA) from the "wild type" undeleted clone, M13.SV.8. However, a specific single-stranded region could be formed by other methods as discussed below. The predicted products of this hybridization are heteroduplexes between the viral SS-DNA of M13.SV.8 and the complementary strand of M13.SV.23 RF-DNA, linear homoduplexes of M13.SV.23 and unhybridized single strands (Figure 2). The heteroduplexes contain a short, defined single-stranded region between nucleotides 69 and 112 which exposes 24 unpaired cytosine residues in the "wild-type" viral strand (Figure 4). Sodium bisulphite specifically deaminates cytosine bases in single-stranded DNA (Shapiro *et al.*, 1973; Kai *et al.*, 1974). After bisulphite mutagenesis (see Materials and methods) a number of these cytosine residues will have become uracil residues and therefore, when the region is filled in with DNA polymerase I, deoxyadenine will be incorporated at these positions in the non-viral strand. These mutated molecules were recovered by transfection of the mixture into JM103. The plaques that appear are derived exclusively from the mutated heteroduplexes since the M13.SV.8 SS-DNA will have been mutated throughout the genome and therefore in essential genes, and the linear forms of M13.SV.23 have a very poor ability to give rise to plaques compared with the circular heteroduplexes. During replication of the heteroduplexes, the non-viral strand will produce viral strands with thymine incorporated at the positions of the mutated cytosines. The original "wild-type" viral strand contains uracil at these positions, which will be excised by the host enzyme uracil-N-glycosylase (Tye *et al.*, 1978). If these gaps are not repaired before the first round of replication of the mutant heteroduplex, then the original viral strand may not be replicated, and therefore all plaques isolated would be derived from the non-viral strand of the heteroduplex. In fact, this appears to be the case in the experiments described here. M13.SV.23 contains a *Hinc*II site at position 32 in the SV40 sequence, while M13.SV.8 does not (Figure 1); therefore, it is possible to differentiate between phage derived from the viral or non-viral strands of the mutated heteroduplex. All the plaques screened in these experiments, even after replating of the original isolates, contained the *Hinc*II site (data not shown). Therefore, it appears that after the degree of bisulphite mutagenesis used here, the viral strand containing uracil was not replicated. However, the sequence of the mutated region in the phages obtained would be the same whether they were derived from the viral or non-viral strand of the heteroduplex.

The plaques obtained were picked and inoculated into 2 ml cultures with *Escherichia coli* JM103. The phage were propagated overnight and SS-DNA prepared. Six of these phages were initially screened by sequencing with the M13 12-base primer, and running only the dideoxyguanosine terminated products on a sequencing gel with the corresponding "wild-

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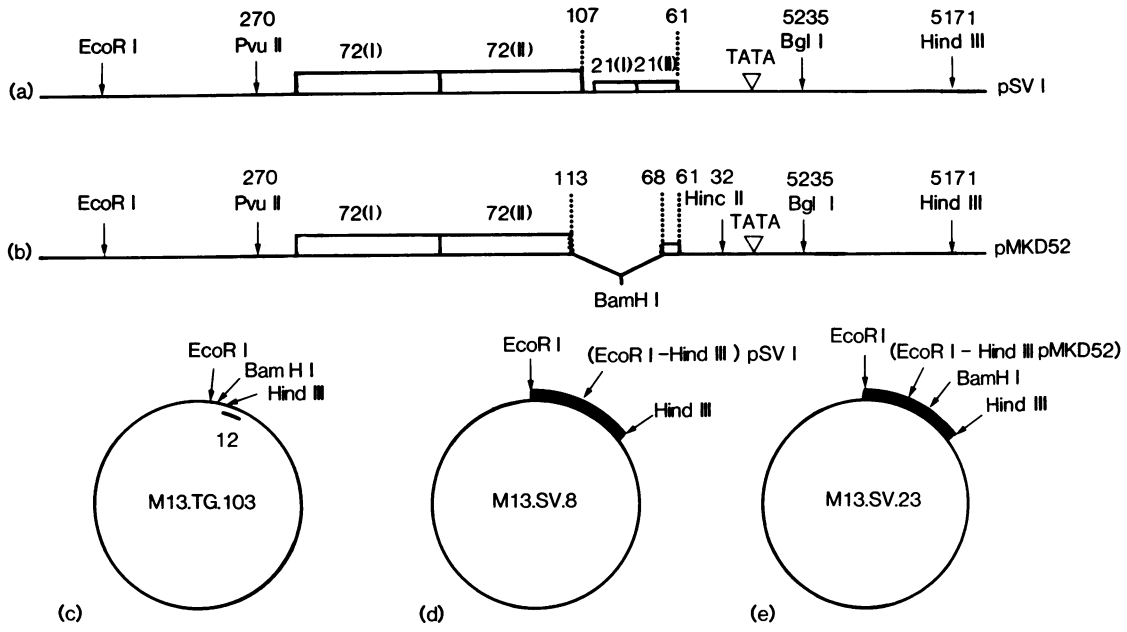

Fig. 1. The clones used in this work are as follows: **(a)** the early promoter region of SV40 as cloned into pSV1 (Benoist and Chambon, 1980); some relevant restriction sites are shown. TATA refers to the TATA box upstream of the early RNA major cap sites. 72 (I) and (II) are the 72-bp direct repeats, and 21 (I) and (II) are the 21-bp direct repeats. The numbers refer to the nucleotides as numbered in the BBB system (Tooze, 1980). **(b)** The equivalent region in pMKD52. Nucleotides 69 – 112 have been deleted and a *Bam*HI linker inserted at this position. The *Hinc*II site is derived from the SV40 variant CS1096 (Di Maio and Nathans, 1980). **(c)** M13.TG.103. The cloning sites and the position of the 12-base universal primer are shown: **(d)** the *Eco*RI/*Hind*III fragment from pSV1 cloned into M13.TG.103 to give M13.SV.8; **(e)** the *Eco*RI/*Hind*III fragment from pMKD52 cloned into M13.TG.103 to give M13.SV.23.

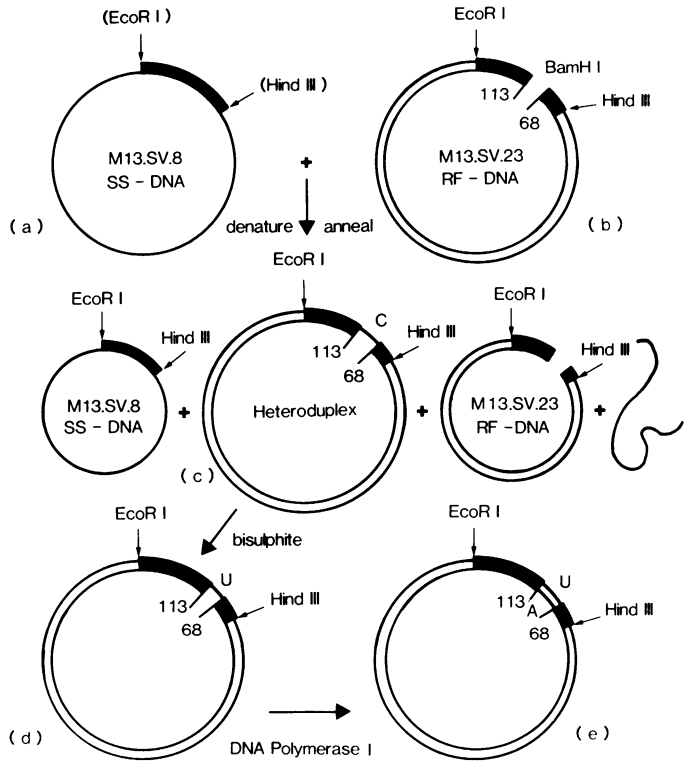

Fig. 2. A diagram of the method. SS-DNA of M13.SV.8 **(a)** was hybridized with *Bam*HI cut M13.SV.23 RF-DNA **(b)** to give the predicted hybridization products of SS-DNA, circular heteroduplexes with an exposed single-stranded region between nucleotides 69 and 112 **(c)**, and double-stranded linear homoduplexes of M13.SV.23 RF-DNA. Unpaired cytosine residues exposed to bisulphite mutagenesis are represented by a C in **(c)**. The mutated heteroduplexes (now with a number of uracil residues [U in **(d)**] were treated with DNA polymerse I and all four deoxynucleotide triphosphates to give the mutant double-stranded circular molecule in **(e)**, where some C-G pairs have been mutated to U-A.

type" control. It was clear that all of these viruses contained a number of point mutations, since all had lost some bands in the G track compared with wild-type, indicating the presence of thymine instead of cytosine at these positions in the mutants. All the mutants were different (results not shown). To check that the mutations were in fact C→T transitions, two of the mutants were studied further. Using a 36-bp primer prepared from a *Hinc*II/*Bgl*I digest of pRE4 (Moreau *et al.*, 1981) by the DE-81 paper elution method (Dretzen *et al.*, 1981), both dideoxyguanosine and dideoxyadenosine terminated products of mutant M13.SV.B11 and M13.SV.B12 non-viral DNA strands were sequenced next to the control M13.SV.8 (Figure 3). The use of the prepared primer merely brings the mutated region in this experiment into a lower mol. wt. portion of the gel.

The results in Figures 3 and 4 clearly show that both mutants have gained an A at each position where a G has been lost, indicating a C→T transition in the viral strand. Furthermore, it is clear that the base changes have occurred only within the deletion area of pMKD52 and not outside it, showing that these mutants have arisen from the predicted heteroduplexes with the expected single-stranded region. The relative mobilities of the oligonucleotide bands in the mutants have become slightly different from the corresponding bands in the wild-type, presumably because of the sequence changes in the mutants. Under these conditions of bisulphite treatment B11 suffered 16, and B12 17, mutations out of a possible 24. Obviously, a lower mutagenesis rate could be obtained, if required, by reducing the time of incubation with bisulphite. The mutated regions are now available for recloning back in pSV1 in order to study their effects on SV40 early region transcription. These results will be described elsewhere.

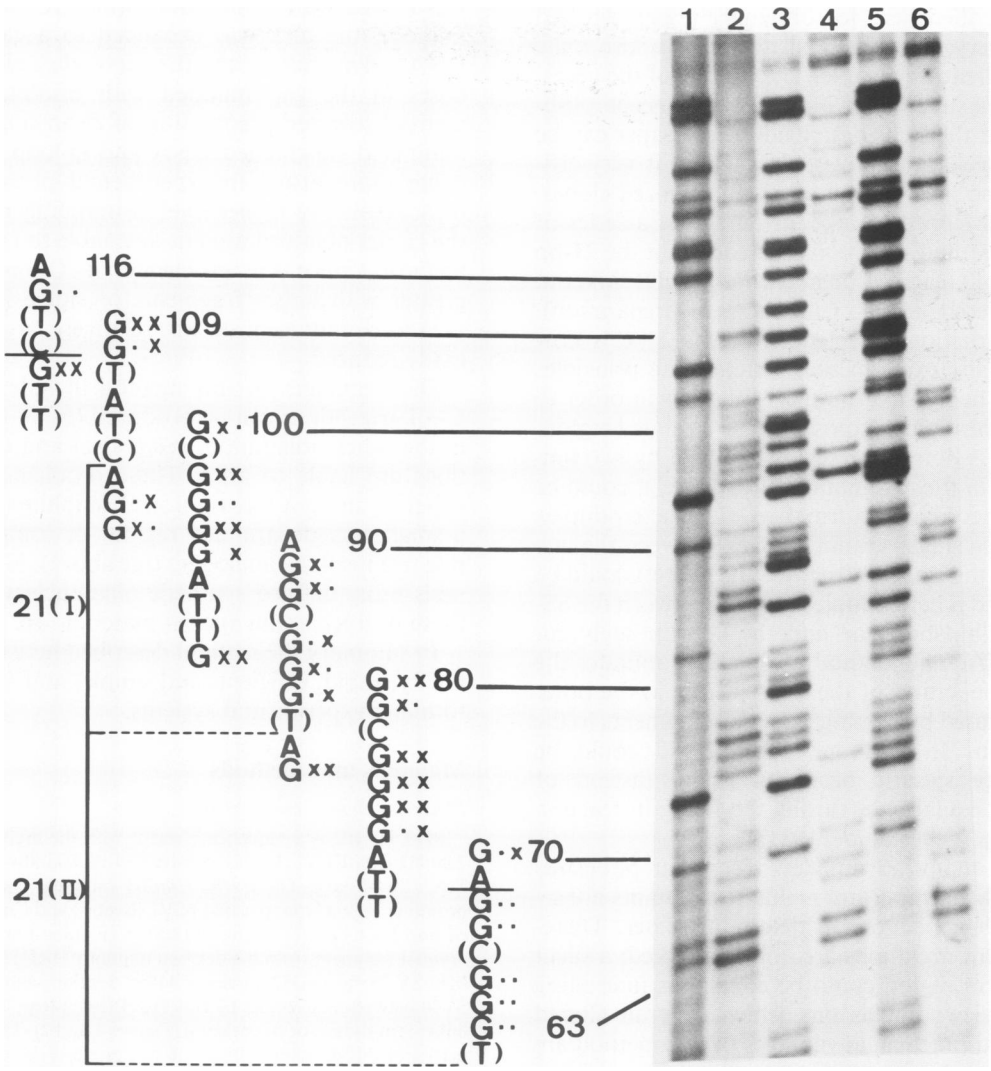

Fig. 3. A sequencing gel of the non-viral strands of "wild-type" M13.SV.8 (tracks **1** and **2**), the mutants M13.SV.B11 (tracks **3** and **4**), and M13.SV.B12 (tracks **5** and **6**). Tracks **1, 3,** and **5** show dideoxyA terminations, tracks **2, 4,** and **6** show dideoxyG terminations. The sequence of this region is shown on the left. The numbers refer to the nucleotide position in the SV40 sequence (see Figure 4); the two horizontal bars within the sequence show the limits of the deletion in pMKD52, and the 21-bp repeats are indicated. To the right of the sequence are two columns of dots and crosses. The left-hand column refers to M13.SV.B11 and the right to M13.SV.B12. Dots indicate that the G in the "wild-type" remains a G in the mutant, while crosses show those G bases that have been changed to A residues. The strand sequenced here is that complementary to that of M13.SV.8 SS-DNA.

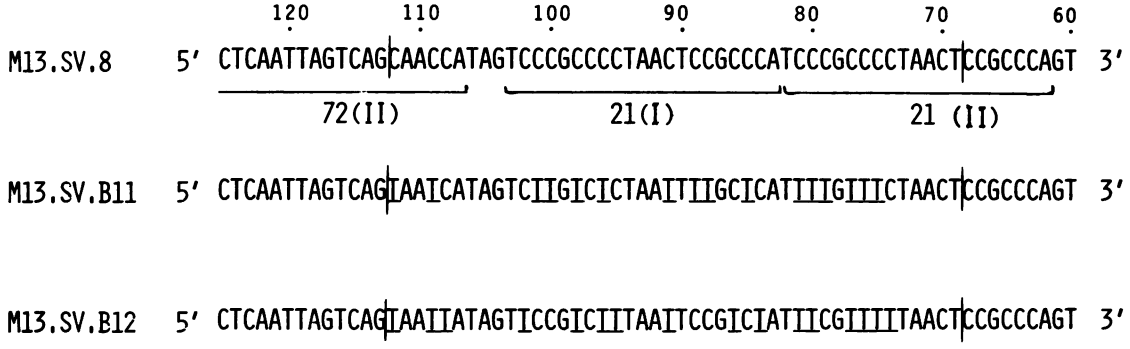

Fig. 4. The sequence (SV40 early gene non-coding strand) of the wild-type 21-bp repeat region in the SS-DNAs of M13.SV.8 and the mutants M13.SV.B11 and M13.SV.B12. The sequence shown is of the strand complementary to that sequenced in Figure 3. The 21-bp repeats are indicated, and the start of the 72-bp repeat (II) shown. Vertical lines indicate the extent of the deletion in pMKD52 and M13.SV.23 (all bases between the lines have been deleted). The sequences of the mutants were different from that of the wild-type only between these lines as determined between nucleotides 30 and ~210 (except for the *Hin*cII site at nucleotide 36, which had been inherited from pMKD52 in all cases, see text). The underlined T bases in the mutants are those changed from C in the "wild-type".

Discussion

The method described here for obtaining region-directed point mutations is very rapid, especially when an appropriate deletion mutant is already available. The cloning of the necessary fragments into a suitable M13 vector can be accomplished in <1 week, and the steps described in this paper can be completed in <2 weeks. Moreover, the method is very efficient since there is no background of unmutagenized clones. The method was developed to take advantage of a series of deletion mutants that had been constructed within the 21-bp repeat region of SV40, each of them with a *Bam*HI linker at the deletion (R. Everett and P. Chambon, in preparation). Although the construction of deletions of this type is now commonplace in many experimental systems, the principles of this method can be applied to other situations in order to obtain the required bisulphite-exposed single-stranded region. For example, if the region of interest is bounded by two unique restriction sites in the recombinant RF-DNA, it could be excised prior to heteroduplex formation. The heteroduplex formed would have a single-stranded region between the two sites. Alternatively, the isolated fragment of interest could be cloned into M13, and a heteroduplex formed between the SS-DNA of the recombinant clone and the appropriately cut M13 vector RF-DNA. The method as described mutates the cytosines in only one strand of the DNA; those in the other strand could be mutated by cloning the same fragments in the opposite orientation. In addition, the method could be modified to mutate specific bases by using, instead of bisulphite, nucleotide analogues during the filling-in reaction (Muller *et al.*, 1978; Weber *et al.*, 1981).

The method has many advantages over other published methods. Perhaps most important is that the mutants are sequenced first and their phenotypes determined later. Therefore, potentially silent mutations are not overlooked; a silent mutation in a control region could be equally as interesting from a biological viewpoint as one which gave an altered phenotype. Principal practical advantages of this method are its rapidity, efficiency, ease of control of the region mutagenized, and its use of easily prepared materials. In addition, purification of the mutated heteroduplexes from the other DNA forms in the mixture is not necessary (see above).

While this work was in progress a similar method using plasmid molecules was published (Giza *et al.*, 1981). This method requires the use of isolated separated plasmid strands to obtain a gapped molecule; the identity of the separated strands would have to be determined for each different experiment before heteroduplexes could be made. The mutated molecules in this method (also obtained by bisulphite treatment) are not amenable to rapid sequencing. In contrast, by using the M13 method many mutants can be sequenced very rapidly without scaling up beyond a 2 ml overnight culture. Other approaches to bisulphite mutagenesis have been described (Shortle and Nathans, 1979; Shortle *et al.*, 1980). In the first, gapped DNA is produced by treatment of plasmid molecules with a restriction enzyme in the presence of ethidium bromide to produce a unique nick, followed by limited digestion with *Micrococcus luteus* DNA polymerase. This is applicable only if the site of interest is at a restriction site. The nick can be translated with *E. coli* DNA polymerase I before gap creation, but this is not easily controlled (Di Maio and Nathans, 1980). In the second method, single strands from isolated fragments were used to create D-loops with closed circular plasmid molecules in a reaction catalysed by *E. coli* recA protein. The exposed single-stranded region was nicked with S1 nuclease (which results in the loss of the D-loop), the nick was expanded to a gap using *M. luteus* DNA polymerase I, and the exposed single-stranded cytosine bases in the gap mutated with bisulphite. However, the method required preparation of clean single strands, uncontaminated supercoiled DNA and D-loop structures. Besides being more prone to experimental problems, this method must result in a higher level of unmutagenized DNA. Again, the clones obtained are not amenable to rapid sequencing. In addition, since the efficiency of D-loop production was greater with larger fragments of single-stranded DNA, the area to be mutagenized was relatively large and could not be easily reduced.

Disadvantages of this method include the fact that only C→T transitions can be produced (or G→A using the clones in the other orientation, see above) and that multiple mutations are liable to arise. These problems could be avoided either by using a less vigorous bisulphite treatment, exposing a smaller single-stranded region, or using deoxyribonucleotide triphosphate analogues (see above). Site-directed mutagenesis using defined synthetic oligonucleotides does not have these problems, but special materials are involved.

In summary the method described here is of general applicability, rapid, efficient, and simple, and should be of benefit to many experimental systems.

Materials and methods

Plasmids, phages, and bacteria

M13.TG.103 is a sequencing vector with the multi-cloning site 5′-*Eco*RI-*Bam*HI-*Hind*III-3′. It was constructed and kindly supplied by Transgene S.A., Strasbourg. The *Eco*RI-*Hind*III fragment containing the SV40 early promoter region from plasmid pSV1 (Benoist and Chambon, 1980) was cloned into M13.TG.103 to give M13.SV.8 (Figures 1 and 2). The same *Eco*RI-*Hind*III fragment from the deletion mutant pMKD52 was also cloned to give M13.SV.23; pMKD52 carries a 44-bp deletion within the 21-bp repeats of SV40 between nucleotides 69 and 112 (BBB system; Tooze, 1980). A *Bam*HI linker is at the site of the deletion (Figures 1 and 2). The construction and properties of this deletion mutant will be described elsewhere (R. Everett and P. Chambon, in preparation). The host strain for M13 was *E. coli* JM103 (Messing *et al.*, 1981).

Purification of viral SS-DNA and RF-DNA

M13 phage were isolated from the supernatant of infected cultures after pelleting the bacteria. The phage were concentrated by precipitation with polyethylene glycol and purified by CsCl step-gradient centrifugation using the methods described by Wasylyk *et al.* (1980) for fd phage. RF-DNA was isolated by CsCl ethidium bromide gradient purification of supercoiled DNA from cleared lysates of infected cultures (Clewell and Helinski, 1970).

Heteroduplex formation

0.7 μg of SS-DNA of M13.SV.8 was mixed with 1 μg of *Bam*HI cut RF-DNA of M13.SV.23 in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 M NaCl in a final volume of 10 μl. The mixture was taken up into a glass capillary that was flame-sealed at the ends before incubating in a boiling water bath for 3 min. The capillary was then incubated at 65°C for 6 h and allowed to cool slowly to room temperature before ethanol precipitation of the DNA.

Bisulphite mutagenesis

The precipitated heteroduplexes were redissolved in 0.1 x SSC, treated with 3 M sodium bisulphite at 37°C for 4 h, and the reaction terminated by dialysis exactly as described by Shortle and Nathans (1978). After dialysis, the mutated DNA was ethanol precipitated.

Filling-in of gapped, mutated heteroduplexes

1 μg of mutated DNA was treated for 3 h at 18°C with 2 units DNA polymerase I in the presence of all four deoxynucleotide triphosphates at a concentration of 50 μM each. The holoenzyme was used so that the associated exonuclease activities could remove the unpaired bases at the extremities of the single-stranded region derived from the *Bam*HI linker in M13.SV.23.

Transfection of JM103 with mutated DNA

Competent JM103 bacteria were prepared by the method of Dagert and

Ehrlich (1979) and stored at −90°C. 0.5 μg of the treated DNA was transfected into the bacteria, which were either plated out directly onto M9 plates for plaque formation, or inoculated into L-broth liquid medium and grown overnight before the progeny phage in the supernatant were diluted and plated out for single plaques.

DNA sequencing

The insert DNA in phage from isolated plaques was sequenced after growth of the phage in 2 ml cultures and isolation of the viral DNA template as described by Sanger *et al.* (1980). The primer used was either the commercially available 12-base universal primer (Collaborative Research), or the isolated *Hin*cII-*Bgl*I fragment (nucleotides 5242−34) from pRE4 (Moreau *et al.*, 1981). Sequencing reactions and gels were as described by Smith (1980).

Enzymes

Restriction enzymes, DNA polymerases I holoenzyme, and Klenow fragment were obtained from commercial sources and used according to the suppliers' specifications.

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