

DIRECTED MUTAGENESIS

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INTRODUCTION

Genetic analysis of an organism depends on the isolation of mutants with defined physiological defects. Until recently such mutants could be obtained only by random mutagenesis of a population of organisms, followed by selection or screening for mutants with a characteristic phenotype. The mutational site in mutants isolated in this way could then be localized by complementation or recombinational analysis with other mutants, or by physical mapping procedures. With the advent of methods for specific cleavage and enzymatic manipulation of DNA, cloning of DNA fragments, nucleotide sequence analysis, and rapid chemical synthesis of oligonucleotides of defined sequence, it is now possible to construct mutations at

predetermined sites in a cloned DNA molecule, precisely define the chemical nature of the mutational change, and then test the functional effect of the mutation *in vitro* and/or *in vivo*. This approach to mutational analysis has been termed "reverse genetics," "surrogate genetics," or "genetics by DNA analysis." Widespread application of this approach is already providing new insights into the nature of regulatory elements in DNA and in the future is likely to have a major impact on studies of protein structure and function.

In the sections that follow, methods of *in vitro* mutagenesis which have been used to construct mutations at defined sites will be reviewed. Our objective is not to describe these methods in experimental detail nor to review what has been learned by their application, but rather to bring to the reader's attention recent developments in this widely applicable approach to the analysis of genes and gene products. Other relevant reviews will be found in articles by Smith & Gillam 1981, Maniatis 1980, Morrow 1979, Timmis 1981, Weissmann et al 1979, Shortle et al 1979b, and in the monograph by Kornberg 1980.

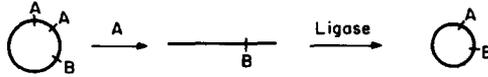
METHODS THAT GENERATE DELETIONS OR INSERTIONS

In the initial stages of a mutational analysis of DNA function it is often desirable to delete segments of DNA from a given region of a cloned gene or gene cluster or to insert a defined sequence in the region of interest. Many procedures have been developed for deleting or inserting DNA at preselected sites (Figure 1). All involve an initial directed incision of a circular DNA duplex, generally in the form of a recombinant bacterial plasmid, enzymatic removal of nucleotides from the ends of the incised molecules or addition of DNA to the ends, followed by ligation and cloning of the constructed mutant genome.

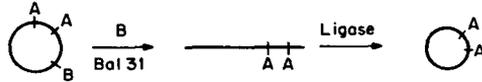
Deletion Mutations

The first *in vitro* construction of deletion mutants involved simple excision of a DNA segment from a circular animal virus genome by partial digestion of the viral DNA with a restriction enzyme that produced cohesive ends. Lai & Nathans (1974) and Mertz et al (1974) cleaved SV40 DNA with restriction enzymes leaving cohesive ends under conditions where digestion did not go to completion, and then transfected monkey cells with electrophoretically purified partial digestion products. Deletion mutants which had undergone precise excision of a restriction fragment ("excisional deletions") resulted, the connecting restriction site having been reformed via intracellular ligation of cohesive ends. In addition, mutants were recovered

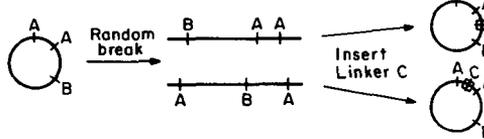
A. EXCISIONAL DELETION



B. EXONUCLEOLYTIC DELETION



C. RANDOM INSERTION OF LINKER



D. DELETION BETWEEN LINKERS

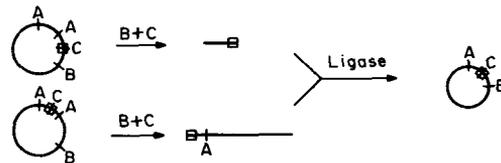


Figure 1 Schemes for construction of deletion and insertion mutants. Letters refer to restriction sites or enzymes.

in which end points of the deletion extended beyond the ends of the linear molecules introduced into cells ("extended deletions"); presumably these arose by limited intracellular exonucleolytic action prior to ligation. Extended deletions also arose after transfection with other shortened, linear DNA molecules, for example those produced by successive cleavage with two different restriction enzymes (Lai & Nathans 1976). Formation of extended deletions can be reduced by *in vitro* cyclization of linear molecules prior to transfection. *In vitro* cyclization also increases the efficiency of SV40 infection of monkey cells (Mertz & Davis 1972) or transformation of *E. coli* by recombinant plasmids (Cohen et al 1973). Except in cases where there is sequence ambiguity in a cleavage site, termini generated by any single restriction endonuclease that produces cohesive ends, and all that produce fully base-paired ends, can be ligated *in vitro* with T4 DNA ligase without further modification. When the termini are incompatible, they can be enzymatically removed by S1 nuclease or (in the case 5' tails) filled in with DNA polymerase prior to blunt end ligation with the T4 ligase.

Several methods have been used to remove a limited number of nucleotides *in vitro* from a predetermined site on a circular DNA molecule. In the initial experiments of this type linear SV40 DNA molecules produced by

digestion with a single-cut restriction endonuclease were treated with λ -exonuclease under conditions where 25–30 nucleotides were removed from each terminus (Carbon et al 1975). The resultant molecules with 3' single-stranded extensions were used to transfect cells, and mutants with deletions at the site of the initial cleavage were readily recovered. In place of λ -exonuclease digestion, subsequent workers have used endonuclease S1 (Backman et al 1979), exonuclease III (Heffron et al 1977), endonuclease BAL-31 (Legerski et al 1978), or a combination of exonuclease III plus S1 (Heffron et al 1977). Transfection with such linear molecules occasionally gives rise to variants which have acquired extraneous nucleotides at the site of the deletion (Wells et al 1979, Magnusson & Berg 1979, Bendig & Folk 1979); this is an additional reason to cyclize molecules by blunt end ligation *in vitro* before introduction into cells.

A useful variation of the above approach allows generation of deletions at random or quasirandom sites in a small circular DNA molecule. Shenk et al (1976) reported that pancreatic DNase in the presence of Mn^{2+} introduces random double-strand breaks in DNA. After partial digestion of SV40 DNA, a circularly permuted set of full-length linear molecules was purified, treated with λ -exonuclease, and used to generate deletion mutants in transfected cells. An alternative procedure for random linearization of a circular duplex involves the use of ethidium bromide to limit pancreatic DNase digestion to a single nick (as described in the section on Bisulfite Reaction at Single-stranded Gaps). The nick can then be extended into a small gap by limited exonucleolytic digestion (Shortle & Nathans 1978) and the circle opened at the gap by S1 nuclease (Pipas et al 1980). A permuted population of linear molecules can often be generated from circular DNA by single cleavage of the duplex with a multicut restriction enzyme in the presence of ethidium bromide (Parker et al 1977). Such molecules can then be the starting point for removal of nucleotides at their ends to generate easily mappable deletions at restriction sites.

Methods have also been described for restriction at a predetermined subset of cleavage sites. For example, distamycin A has this effect on cleavage by *Eco*RI (Nosikov et al 1977). A more general method has been developed by Humayan & Chambers (1979), who annealed a restriction fragment of ϕ X174 DNA to circular single-stranded viral DNA, and digested the resulting partial heteroduplex molecule with *Hinc*II. Cleavage occurred only at the two *Hinc*II sites within the duplex region. When spheroplasts were transfected with the shortened molecules after *in vitro* ligation into circles, 2% of the resulting phage displayed the expected mutant phenotype. Sequence analysis demonstrated the deletion of the segment between the two *Hinc*II cleavage sites as well as an additional nucleotide pair at the same position (presumably resulting from contaminating exonuclease action prior to circularization).

Green & Tibbetts (1980) have reported a method for directed deletion that eliminates the need for a conveniently placed restriction site. In this two-step method a displacement loop (D-loop) is formed between a circular duplex of pBR322 and a single-stranded fragment of DNA that spans the site selected for deletion. Single-stranded fragments for uptake were isolated by gel electrophoresis (Hayward 1972) or small aliquots of a denatured fragment were added periodically during the strand uptake reaction. In both cases, prior to uptake, the fragments were partially degraded to pieces of DNA about 200 nucleotides long and strand uptake was monitored by the conversion of supercoiled to relaxed circles. (Although *recA* protein was not required for D-loop formation in these studies, one would expect considerably higher efficiency in the presence of *recA* protein—see Figure 5.) A D-loop constitutes a target for nuclease S1. Under the conditions described by Green & Tibbetts (1980), S1 linearized the molecule and removed a limited number of nucleotides from the generated ends. Following cleavage by S1, electrophoretically purified linear DNA was circularized with T4 DNA ligase and used to transform bacterial cells. In experiments using purified fragments from the pBR322 gene encoding tetracycline resistance, an average of about 10% of the transformants contained deletions causing tetracycline sensitivity, and all of them mapped to the segment of the gene covered by the fragment originally used for strand uptake. Restriction analysis of these mutants indicated the presence of deletions of less than 15 base pairs in the targeted region.

Once a deletion mutant is obtained it is often desirable to extend the original deletion systematically. Shenk (1977) described a general procedure for accomplishing this by forming a circular mutant/wild type DNA heteroduplex and opening the circle and removing nucleotides at the deletion loop with S1 nuclease. By related procedures a DNA segment between two deletions can be excised (Shenk & Berg 1976) or double deletion mutants can be generated (Konig & Lai 1979). As noted below, insertions of a new restriction site during construction of deletion mutants also provides an entry site for subsequent extension of the deletion *in vitro*.

A class of defined mutants has been constructed by substituting for a wild type segment of DNA a segment already containing the desired sequence alteration. Wallace et al (1980) used a synthetic oligodeoxynucleotide corresponding to the sequence flanking the intron of a yeast tRNA gene. By the steps described for generating base pair substitutions with oligonucleotides (see the section on Mutant Construction with Synthetic Oligonucleotides) a mutant gene was generated lacking the intervening sequence. In a conceptually related approach, SV40 mutants precisely lacking intervening sequences have been generated by using viral mRNA, annealed to viral DNA, as a template for reverse transcriptase (Gruss et al 1979). After removal of the RNA with alkali, the DNA copy was annealed to suitably gapped viral

DNA and the heteroduplex used to transfect cells in the presence of a complementing helper virus. A similar type of polyoma mutant was constructed by replacing a genomic segment with cloned cDNA (Treisman et al 1981).

Insertion Mutations

As already indicated, insertions of DNA can serve not only to interrupt a DNA sequence at a desired position, but also to provide a new restriction target or a new functional element. End-to-end ligation of DNA fragments with complementary or fully base paired termini has been widely used to insert DNA segments into vector molecules as the initial step in molecular cloning (reviewed by Maniatis 1980), to construct novel combinations of genes and regulatory signals (e.g. Backman & Ptashne 1978, Mulligan & Berg 1980, Post et al 1981) and to demonstrate that a mutant phenotype is caused by the presence of a particular DNA segment (e.g. Miller & Fried 1976, Shortle et al 1979a, Katinka et al 1980). A variety of "linker" and "adaptor" molecules containing cleavage sites for one or more restriction endonuclease have been chemically synthesized (Scheller et al 1977, Bahl et al 1977b), and many of these are now commercially available. An early use of such linkers was developed by Heffron et al (1978) to generate a large set of readily mappable insertion mutations in any covalently closed circular DNA molecule. Full length linear DNA molecules of a plasmid containing the bacterial transposon Tn3 were purified following random limited pancreatic DNase I digestion in the presence of Mn²⁺ (Shenk et al 1976). After the termini were made blunt by repair with DNA polymerase I or T4 DNA polymerase, synthetic octamers containing an *EcoRI* recognition site were joined to the ends by using T4 DNA ligase. Excess linkers were removed and the ends rendered cohesive by *EcoRI* digestion. (A pre-existing internal *EcoRI* site was protected by in vitro methylation with *EcoRI* methylase prior to addition of linkers.) Following circularization with *E. coli* DNA ligase, which joins molecules with cohesive ends only, and transformation, more than one half of the transformants had plasmids with a new *EcoRI* cleavage site. These sites were easily mapped by restriction analysis with *EcoRI* in combination with additional enzymes. Nucleotide sequence analysis of the insertion mutants demonstrated the existence of deletions of up to 50 base pairs at the site of some insertions, while other insertions were flanked by short direct repeats, presumably arising from polymerase action at DNase-produced staggered breaks with 5' extensions (Heffron et al 1979). It may be possible to minimize the extent of these extraneous sequence alterations by initially opening the circle by pancreatic DNase nicking in the presence of ethidium bromide followed by gapping and S1 nuclease mediated linearization, as already described. Note that inserted

restriction sites facilitate DNA sequence analysis and the construction of "second generation" mutants. The ability to delete a DNA segment between two newly acquired sites in any pair of insertion mutants is particularly useful (Figure 1).

Boeke (1981) has recently described the construction of one and two amino acid codon insertions in the circular genome of bacteriophage f1. Full length linear molecules were produced by limited digestion of f1 replicative form DNA with *Hinf* I, a restriction endonuclease that leaves a three base 5' extension. Repair with DNA polymerase I and deoxynucleoside triphosphates and recircularization with DNA ligase resulted in a three base duplication of the sequence at the site of initial cleavage. Because *Hinf* I cleaves at multiple sites in f1 DNA, this procedure generated a collection of mutants each with a three base insertion at a different location. Since one codon is added to a protein-coding sequence, no frameshift mutations would be generated; however, simple nonsense mutations were induced as expected from the nucleotide sequence at certain *Hinf*I sites. Similarly, enzymatic repair of linear molecules produced by digestion with *Hpa*II (which leaves a two base extension) and blunt-end insertion of a tetranucleotide resulted in the formation of a set of two codon insertions. A similar approach was used to construct insertion mutations in bacterial plasmids (Backman et al 1979). One can expect increasing use of insertions of DNA segments containing amino acid codons or regulatory elements of various kinds—promoter segments, transcription terminators, processing signals, origins of replication, ribosome binding sites, nonsense codons—either derived from natural sources or synthesized chemically.

More Complex Mutations

A series of deletion/insertion mutants have been constructed that are proving useful for analysing transcriptional control elements or for maximizing expression of cloned genes. By combined use of specific cleavage, exonucleolytic digestion, linker or signal insertions, and recombination of DNA segments, mutants have been constructed with deletions having one fixed end (Sakonju et al 1980, Bogenhagen et al 1980) or with variable distances between regulatory elements (Roberts et al 1979). As illustrated in Figure 2, Bogenhagen et al (1980) constructed a set of mutants in the 3' end of the 5S RNA gene of *Xenopus borealis* cloned into pBR322 by several sequential steps involving incising downstream from the gene, removing nucleotides from the ends of the DNA by exonuclease III and S1 digestion, adding a restriction site linker, and recloning the remaining gene segment. The resulting deletion mutants retained the original 5' part of the gene and were missing variable amounts of the 3' part. In all cases the plasmid vector sequence was identical. A similar set of mutants was constructed on the 5'

side of the gene (Sakonju et al 1980). When the two sets of mutant recombinants were tested for activity as templates for RNA polymerase III transcription *in vitro*, a central segment of DNA was found to be essential for activity (Figure 2). Similar analysis of the VA1 RNA gene of human adenovirus, which is also transcribed by RNA polymerase III, revealed that it too has an internal control region (Fowlkes & Shenk 1980, Guilfoyle & Weinmann 1981). Related approaches have been used to identify promoter elements involved in transcription of genes by RNA polymerase II. After deletion of defined DNA segments, mutant DNA was used as a template for *in vitro* transcription by a HeLa cell extract (Corden et al 1980, Hu & Manley 1981, Tsai et al 1981, Mathis & Chambon 1981), or after transfection of cells in culture (Benoist & Chambon 1980, Osborne et al 1981, Dierks et al 1981) or microinjection of frog oocytes (Grosschedl & Birnstiel 1980a). In contrast to genes transcribed by RNA polymerase III, mutational analysis of RNA polymerase II-dependent genes has revealed transcriptional signals 5' to the gene, including "modulator" elements some distance upstream (Grosschedl & Birnstiel 1980b, Gruss et al 1981, Benoist & Chambon 1981).

Recombinant vectors, such as high expression plasmids (Rao & Rogers 1978, Backman et al 1976, Guarente et al 1980), those combining animal virus regulatory elements and selectable prokaryotic genes with bacterial plasmids (Mulligan & Berg 1980), and those that ensure secretion of a cloned gene product (Villa-Komaroff et al 1978) or translation in the correct reading frame (Charnay et al 1978) might be considered "complex mutants." Construction of such vectors often involves, in addition to recombining DNA segments from diverse sources, positioning of control elements by controlled deletions or insertions of DNA (for review, see Timmis 1981).

METHODS THAT GENERATE BASE SUBSTITUTIONS

After localization of functional elements or protein coding regions of a genome by deletion or insertion of DNA segments, it is often desirable to extend a mutational analysis by constructing base substitution mutations in the region of interest. Methods for directed base substitution are of two general types: those leading to substitutions over a targeted segment of DNA, and those changing specific, predetermined base pairs (Figure 3). In methods of the first type, the targeted segment is either a small fragment of DNA that is mutagenized and then recombined with the rest of the genome, or a larger DNA molecule or even an entire genome that is bio-

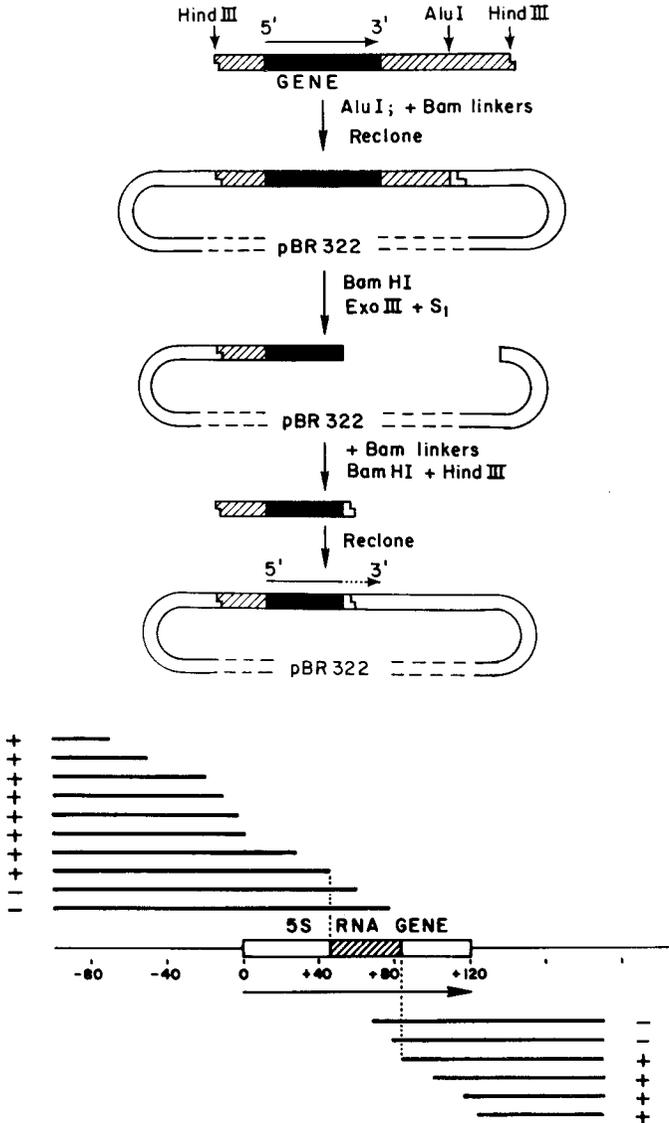


Figure 2 Construction of overlapping deletion mutations with one fixed end, in the 5S RNA gene of *Xenopus* (top), and their effects on in vitro transcription (bottom). The arrow in the figure on the bottom indicates direction of transcription, numbers refer to base pairs from the left end, and bars indicate deleted segments. +, active for initiation of transcription; -, transcriptionally inactive. (From Bogenhagen et al 1980, with permission of *Cell* and MIT Press.)

chemically modified to create a small target for mutagenesis. Methods of the second type involve substituting a synthetic oligonucleotide which contains the desired mutation for the wild type sequence.

Fragment Mutagenesis followed by Recombination

In the conventional approach to isolation of base substitution mutants the entire genome of an organism is exposed to a nucleic acid modifying agent, and mutations are induced throughout the genome. One approach to increase the yield of mutants that fall within a locus of interest is to use a fragment of the genome which carries that locus as a target for the action of a mutagen in vitro. If the mutagenized fragment is returned to its correct position in the intact genome by recombination, then all of the recovered mutant progeny (excluding those that occur spontaneously) should carry mutations only within that part of the genome equivalent to the mutagenized fragment (Figure 3). The first method employing this general strategy was described by Hong & Ames (1971). The targets of their in vitro mutagenesis were fragments of the *Salmonella typhimurium* genome contained in a pool of phage P22 generalized transducing particles. After reaction with the mutagen hydroxylamine, a transducing lysate was used as donor to transduce a recipient *Salmonella* strain carrying a defined auxotrophic marker to prototrophy. Any prototrophic transductants resulting from this cross must have undergone recombination with a fragment of donor DNA that included the locus of the auxotrophic marker. When the donor fragment also contained mutagen-induced lesions in flanking loci, new mutations in these loci could appear in the prototrophic transductants. Since P22 encapsidates approximately 1% of the *Salmonella* chromosome per transducing particle, the new mutations were localized to within less than 1% of the genome length away from the auxotrophic marker. In principle, any gene on the *Salmonella* chromosome, provided it is linked to an easily

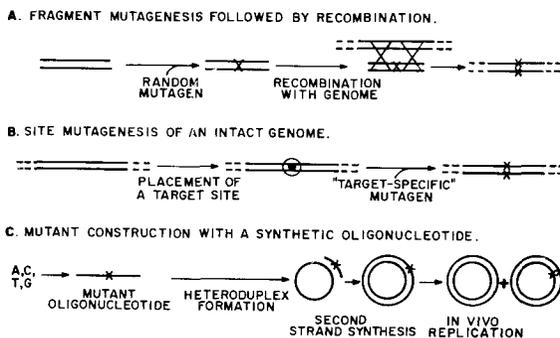


Figure 3 Strategies for constructing base substitution mutants.

transducible marker, can be subjected to mutagenesis by this method (Kleckner et al 1977).

Using a similar approach, Borrias et al (1976) targeted mutagenesis to the *A* gene of ϕ X174 phage by using a restriction fragment that included part of the *A* gene. After reaction with the mutagen methoxyamine and denaturation, the restriction fragment was reannealed to single-stranded virion DNA and the resulting partial heteroduplex molecules transfected into *E. coli*. The partial heteroduplex was formed between virion DNA from an amber mutant in the *A* gene and a mutagenized fragment from wild type which overlaps this amber mutation, and viral progeny that received genetic information from the mutagenized fragment were selected on a suppressor minus strain. These suppressor-independent viruses were screened for a temperature-sensitive plaque phenotype, and nine independent *ts* isolates were identified. Analysis of only one mutant was reported. This mutation was mapped to the mutagenized segment of the *A* gene by demonstrating rescue of the *ts* mutation by the same wild type fragment that had been used for in vitro mutagenesis.

Mutagenized restriction fragments have also been used to induce temperature-sensitive mutations within specific segments of the herpes simplex virus genome (Chu et al 1979). Two large *Eco*RI fragments were treated with hydroxylamine and then introduced, along with intact wild-type viral DNA, into permissive tissue culture cells. Because herpesvirus undergoes very active genetic recombination during its lytic cycle, the mutagenized fragments were incorporated into full length genomes at a significant frequency. By simply screening individual progeny from this coinfection, temperature-sensitive mutants were recovered without using a genetic marker linked to the fragment. Five of these mutants were analyzed by complementation and by marker rescue mapping, and at least four could be assigned a map position within the genomic segments corresponding to the restriction fragments used for mutagenesis. Two of these four mutants were used to identify new complementation groups.

Instead of an isolated restriction fragment, Völker & Showe (1980) mutagenized a small plasmid which carried a cloned *Bgl*II fragment of bacteriophage T4 DNA. After extensive reaction with either hydroxylamine or nitrosoguanidine, the plasmid was transformed into a strain of *E. coli*, and a pool of transformed bacteria served as a permissive host for growth of T4. By infecting with a phage carrying two amber mutations which map at opposite ends of the genomic segment corresponding to the *Bgl*II fragment, recombinants between the homologous sequences on the plasmid and the phage could readily be recovered on suppressor minus strains. With conditions of optimal mutagenesis, approximately 0.5 to 1% of the *am*⁺ phage had acquired a temperature-sensitive mutation. All forty *ts* mutants de-

scribed in this report mapped within the *Bg*III segment. For each mutant isolate, fine structure genetic mapping identified a single mutant site conferring the *ts* phenotype. The sites were distributed throughout the *Bg*III segment, and hot spots for hydroxylamine and nitrosoguanidine mutagenesis which had been identified by mutagenesis of intact phage also registered as hot spots with this procedure.

Using a different strategy in which nucleic acid sequence homology directs a chemical mutagen to a unique sequence on an intact genome, Salganik et al (1980) have specifically mutagenized several segments of the bacteriophage T7 genome. A polyfunctional nitrogen mustard was covalently attached to purified *in vivo* RNA transcripts of specific early viral genes. After forming R-loops with the modified RNA and full length viral DNA molecules, one of the alkylating groups (which was inert during the attachment reaction) was activated chemically, permitting multiple cross-links to form between the RNA and the complementary strand of DNA. When packaged *in vitro* and infected into a permissive *E. coli* strain, these "locally alkylated" viral DNA molecules gave rise to mutant progeny at a frequency of 3 to 12%. In two separate experiments using different early RNAs, all of the recovered mutants (three in one experiment and four in the other) could be assigned by complementation analysis to the viral gene corresponding to the RNA used as substrate for alkylation. No data were reported on the molecular structure of these induced mutations.

Recombination of two or more restriction fragments *in vitro* via DNA ligase-catalyzed joining of sticky ends can, in some instances, be used to regenerate an intact DNA genome (see section on Allele Replacement). In such an instance, genomes carrying mutagen-induced lesions in a defined segment can be generated efficiently *in vitro* by replacing a wild-type restriction fragment in the ligation mixture with a fragment that has been chemically mutagenized. By using a wild-type variant of adenovirus 5 which has only one *Xba*I restriction site in its DNA, Solnick (1981) was able to induce mutations in a small, defined region of the adenovirus genome by reacting the isolated short *Xba*I fragment (4% genome length) with nitrous acid and then ligating it to unmutagenized large *Xba*I fragment (96% genome length). Among the progeny virus recovered on transfection of permissive cells with the *in vitro* recombinants, viral mutants in the early regions 1A and 1B (which map in the short *Xba*I fragment) were detected at a frequency of 20% using a complementation screening procedure.

As can be seen from the results described above, methods employing the strategy of fragment mutagenesis followed by recombination permit site-specific mutagenesis of relatively large genomic segments to be carried out in systems which either (a) exhibit a high level of recombination between the mutagenized fragment and the intact genome, or (b) permit the efficient

recovery of relatively rare recombinants. However, these methods have one intrinsic limitation—the overall efficiency of mutagenesis, as defined by the ratio of mutants to total candidates, is usually quite low when conditions are used that favor the induction of single mutations. A low efficiency of mutagenesis presents no serious problem when the phenotype of the mutants being sought can be easily detected, as is the case for temperature sensitive mutants. However, silent mutations or mutations for which there is no simple assay will not be readily detected among the large predominance of wild-type organisms.

Site Mutagenesis of Intact Genomes

Several methods for site-specific mutagenesis have in common the basic feature that one small, mutagen-sensitive site is generated enzymatically in an intact genome. Through a modification of the local nucleic acid structure, the nucleotide sequence at this target site becomes uniquely susceptible to an *in vitro* mutagenic reaction. Since the intact genome serves as substrate, the necessity of recombining a mutagenized fragment back to its correct genome position is avoided altogether. In addition, the target site can be made sufficiently small that one or only a few tightly clustered mutations are induced by the mutagenic reaction, thereby reducing the likelihood of distant secondary mutations. Perhaps the principal advantage of these methods is their relatively high efficiencies of mutant induction. This is important because, as the fractional yield of mutant progeny increases, the feasibility of applying laborious assays to detect small phenotypic changes, or even nucleic acid sequencing to detect silent mutations, also increases.

INCORPORATION OF NUCLEOTIDE ANALOGUES Mutagenesis by incorporation of nucleotide analogues at precisely defined sites was first applied to the small RNA bacteriophage $Q\beta$ (Flavell et al 1974). The viral or plus strand of RNA can be efficiently replicated *in vitro*, via a minus strand intermediate, in a defined reaction mixture with purified $Q\beta$ replicase plus host factors and the four ribonucleoside triphosphates. By controlling the conditions of reaction, minus strand synthesis can be initiated at any one of three sites on the plus strand; and in the absence of one of the four nucleotides, replicase initiates and then stops at the first position where the missing nucleotide is required. In several consecutive reactions with one or more nucleotides omitted, $Q\beta$ replicase was advanced to a predetermined nucleotide position, which became the target for mutagenesis. The mutagenic reaction directed to this site consisted of a limited synthesis by the replicase with N^4 -hydroxy CTP replacing either UTP or CTP. Because its tautomeric constant (the ratio of enol to keto tautomers) is approximately

one, this nucleotide analogue base pairs equally well with either A or G. During the mutagenic reaction, N⁴-hydroxy CTP is incorporated in the minus strand at one or just a few positions in place of either CTP or UTP, depending on which has been omitted. The mutagenic analogue is then removed and the minus strand completed. On synthesis of the viral plus strand, either ATP or GTP will be incorporated opposite the N⁴-hydroxy C residues. If the analogue was substituted for U (or C) in the minus strand and G (or A) was incorporated during plus strand synthesis, a base substitution is generated in vitro. Using this procedure, four different base substitutions have been introduced into the 3' extracistronic region of Q β , a part of the phage genome in which no mutants had been identified by conventional methods (Weissmann et al 1979). In the construction of one mutant, Q β replicase was advanced to a position on the minus strand opposite +40 on the plus strand, and one N⁴-hydroxy CTP was incorporated in place of UTP. Plus RNA was synthesized after completion of the minus strand and transfected into an *E. coli* host strain. Four of eighteen independent viral isolates recovered from this transfection contained the predicted A \rightarrow G mutation (Domingo et al 1976). A second base substitution, a G \rightarrow A transition, was constructed at position +16 and demonstrated by fingerprint analysis to be present in the in vitro synthesized plus strand RNA (Flavell et al 1974). However, on transfection and screening of 120 viral isolates, this mutant was not recovered (Sabo et al 1975). Therefore the authors concluded that this particular base substitution destroys the viability of Q β phage that carry it. In a second series of experiments, G \rightarrow A substitutions were generated in a segment of the plus RNA that encodes the ribosome binding site for the viral coat gene (Taniguchi & Weissmann 1978). The effect of these sequence changes on ribosome binding were analyzed in vitro, without having to propagate the mutant phage.

Mutagenesis by N⁴-hydroxycytosine incorporation has been extended to duplex DNA by employing the deoxyribonucleotide form of the analogue and using *E. coli* DNA polymerase I in the nick translation mode (Muller et al 1978). The target of mutagenesis in these experiments was a single-strand break (nick) induced by the *EcoRI* restriction endonuclease at its single cleavage site on a small hybrid plasmid. During a limited polymerization reaction initiating at this unique nick (which can reside on either of the two DNA strands), N⁴-hydroxy dCTP was incorporated in place of dTTP at several positions in the nucleotide sequence that forms the *EcoRI* recognition site. After transformation by the mutagenized DNA, it was found that 1.9% of the plasmids isolated from a pool of transformed cells had lost this *EcoRI* site. Seven plasmid isolates from this *EcoRI* resistant fraction were purified and sequenced in the vicinity of the missing *EcoRI* site: three had a single base substitution, three had two substitutions, and one had

three substitutions. In every case the base substitutions were TA→CG transitions at those sites where N⁴-hydroxy dCTP could be substituted for dTTP during DNA synthesis proceeding 5' to 3' from one of the two possible nicks.

BISULFITE REACTION AT SINGLE-STRANDED GAPS The mutagen sodium bisulfite catalyzes the deamination of cytosine to form uracil under mild temperature and pH conditions. While cytosine residues in single-stranded polynucleotides react at nearly the same rate as the free mononucleotide, cytosine residues within the duplex structure of double-stranded DNA are essentially inert to bisulfite attack (for a review of the relevant chemistry, see Hayatsu 1976). Because of this very large differential reactivity of cytosine in single-stranded and duplex DNA, bisulfite can be used for site-specific mutagenesis. The target site is a stretch of single-stranded DNA (a gap) generated enzymatically in an otherwise duplex molecule; cytosine residues exposed in such a gap become susceptible to bisulfite catalyzed deamination.

The first specific sites to be mutagenized using this approach were the two unique restriction sites on SV40 DNA for the *Hpa*II and *Bgl*I restriction enzymes (Shortle & Nathans 1978). To expose the nucleotide sequence recognized by the restriction enzyme, a single nick was induced in supercoiled SV40 DNA at the enzyme's normal cleavage site by inhibiting the cleavage reaction (after the first single-strand break was made) with the intercalating agent ethidium bromide (Parker et al 1977). In a second reaction, this unique nick, which can occur on either strand, was converted into a short, single-stranded gap by the 5' to 3' exonuclease activity of DNA polymerase I from *Micrococcus luteus*. Figure 4 shows that, for the *Bgl*I restriction site, two cytosine residues in each type of gapped molecule are present in a single-stranded conformation, making them accessible to deamination. After viral DNA carrying such short gaps at the *Bgl*I site was incubated with sodium bisulfite in a reaction deaminating approximately 30% of exposed cytosine residues, the gap was filled in by DNA polymerase plus the four deoxyribonucleoside triphosphates. Those molecules which have had one (or more) cytosines deaminated acquire an AU base pair on gap repair, eliminating one of the six GC base pairs comprising the *Bgl*I recognition sequence. This fraction of *Bgl*I cleavage-resistant DNA was recovered and transfected into tissue culture cells permissive for SV40 growth. Of the 23 individual plaques which were analyzed, 19 contained virus which had lost the *Bgl*I site in its DNA. These *Bgl*I-resistant mutants could be grouped into four classes on the basis of plaque size at different temperatures (Shortle & Nathans 1979); the mutant sequence of one member from each class is shown in Figure 4. Three had the base substitution

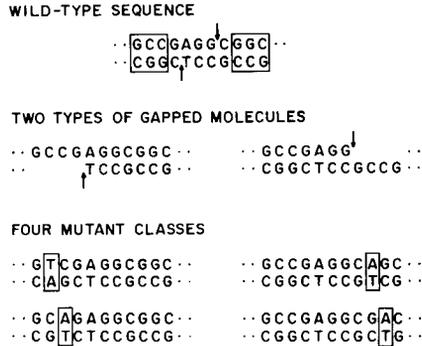


Figure 4 Local mutagenesis at a *Bgl*I site with sodium bisulfite. The boxed sequences at the top indicate the *Bgl*I recognition sequence, and the arrows indicate cleavage sites. See text for details.

expected of bisulfite-induced deamination of cytosine—a CG→TA transition; the fourth mutant had a CG→AT transversion. A second CG→AT transversion appeared in one of two other SV40 mutants induced with bisulfite reaction at a short gap generated by a very limited exonuclease III digestion from the *Bgl*I nick (Shortle & Nathans 1979). The molecular mechanism for these “nonstandard” bisulfite-induced base substitutions is not known.

To apply bisulfite mutagenesis to the nucleotide sequences immediately adjacent to the *Bgl*I restriction site on SV40, a limited synchronous “translation” of *Bgl*I-induced nicks was carried out by using *E. coli* DNA polymerase I under controlled conditions (DiMaio & Nathans 1980). Once the nick had been moved a known distance, it was converted into a short gap and then mutagenized with sodium bisulfite. Sequence analysis of a selected group of SV40 isolates appearing after transfection with this modified DNA revealed that, unlike the mutants within the *Bgl*I recognition sequence, in a majority of mutants more than one CG→TA base substitution had been induced. In one mutant, six base changes were found distributed over a segment of 21 nucleotides. The high frequency of multiple base changes was attributed by the authors to selection of mutants with altered restriction or altered plaque phenotype. It was estimated that about 40% of all viral isolates were mutants.

Restriction enzyme recognition sequences that occur at multiple positions on a circular DNA can also serve as sites for bisulfite mutagenesis, provided that the cleavage reaction of the restriction enzyme can be inhibited with ethidium bromide at the singly-nicked intermediate stage. (Among the many different type II restriction endonucleases, considerable variabil-

ity in the efficiency of this nicking reaction is observed. With some enzymes as much as 90% of the input covalently closed circular DNA can be converted to a nicked circular form, whereas with other enzymes, little or no nicking occurs at any concentration of ethidium bromide.) Although a number of different sites become potential targets for mutagenesis, screening for a mutant at one particular site is simplified by the fact that a mutant site can be readily mapped by restriction analysis with the cognate enzyme.

A collection of mutants within the gene for SV40 T-antigen has been constructed by using bisulfite mutagenesis directed at the *Bst*NI sites on the SV40 genome. Of the 45 isolates derived from the mutagenized DNA and analyzed by restriction, 27 had lost one of 16 different *Bst*NI sites distributed around the SV40 genome, and one isolate had lost two *Bst*NI sites (Peden et al 1980). However, mutations were not induced at the 16 different *Bst*NI sites with the same frequency. One restriction site represented 8 of the 27 single-site mutations, whereas no mutations were recovered at 8 of the *Bst*NI sites. Presumably, this nonrandom distribution reflects differences in the affinity of the restriction enzyme for the various recognition sites in the presence of the intercalating agent ethidium bromide (Nosikov et al 1977). By using the *Pst*I enzyme to introduce specific nicks in SV40 DNA, Rawlins & Muzyczka (1980) constructed a specific amber mutation in the T-antigen gene. One of two *Pst*I sites in SV40 DNA (map position 0.27) includes a CAG glutamine codon. On nicking with *Pst*I plus ethidium bromide and then gapping with the 3'→5' exonuclease activity of T4 DNA polymerase with dTTP present to limit the extent of hydrolysis (Englund et al 1974), the cytosine residue in this codon was specifically exposed to deamination with sodium bisulfite.

The site-specificity of mutagenesis by nucleotide analogue incorporation (see previous section) and by sodium bisulfite reaction at single-stranded gaps resides in the introduction of a single nick at the site to undergo mutagenesis. The number of sites to which these two methods of mutagenesis can be targeted has been increased by the development of a two step procedure that allows placement of a nick in any segment of a DNA molecule for which a corresponding single-stranded fragment can be isolated (Shortle et al 1980). An outline of this procedure is given in Figure 5. In the first application of this segment-specific nicking procedure, two segments of the β -lactamase gene (*bla*) carried on the plasmid pBR322 were mutagenized: between codons 139 and 183 (Segment I), and from a site outside the 5' end of the gene to codon 46 (Segment II). Of the 51 *bla*⁻ mutant plasmids isolated after targeting bisulfite mutagenesis to single-stranded gaps introduced in Segment I, 49 were mapped genetically to a deletion interval that closely approximates this segment of the *bla* gene. Of

the 22 *bla⁻* mutants recovered after Segment II-directed mutagenesis, 14 mapped to a deletion interval contained within Segment II, and the remaining 8 mapped to an interval that partially overlaps Segment II. Nucleotide sequence analysis of a few of these mutants has identified four single CG→TA transitions: glutamine (CAA) at codon 4 mutated to an ochre codon (TAA); proline (CCT) at codon 20 to leucine (CTT); alanine (GCA) at codon 40 to valine (GTA); and arginine (CGA) at codon 41 to an opal codon (TGA) (D. Shortle, P. Grisafi, D. Koshland, and D. Botstein, unpublished results). A majority of the remaining mutants which have been sequenced have, in addition to one or a few tightly clustered CG→TA

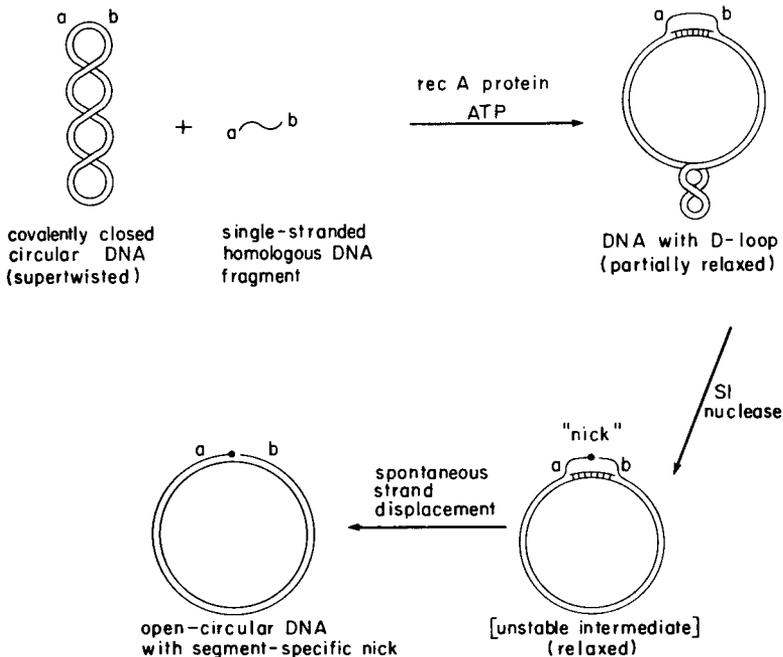


Figure 5 Schematic illustration of the segment-specific nicking procedure. In the first reaction, negatively super-coiled circular DNA and a single-stranded fragment of DNA from the segment of the circle to be nicked are incubated with the *recA* protein of *E. coli* (Shibata et al 1979, McEntee et al 1979). With ATP serving as cofactor, this enzyme catalyzes the annealing of the fragment of the complementary sequence on the DNA circle, forming a stable single-stranded displacement or "D-loop." In the second reaction, the single-strand-specific nuclease S1 breaks a phosphodiester bond in the D-loop, causing rapid breakdown of the D-loop by spontaneous displacement of the single-stranded fragment. The final product is an open circular DNA molecule with a nick located within the segment defined by the single-stranded fragment. (Reprinted from Shortle et al 1980, with permission).

transitions, either a single T inserted at one end of a run of four or more T residues, or a deletion of a single C within a run of three C residues. Preliminary data suggest that these two types of frameshift mutations can be reduced or eliminated by two modifications of the reactions in which the single-stranded gap is generated and repaired (Shortle & Botstein 1981): omission of the single deoxynucleoside triphosphate from the *M. luteus* DNA polymerase I gapping reaction, and the use of DNA ligase to close the nick formed during in vitro gap repair with DNA polymerase.

Finally, when the objective is to induce mutations within a segment of a circular DNA for which there is an easily scoreable phenotype, bisulfite mutagenesis at single-stranded gaps can provide a highly efficient means for localized random mutagenesis (Shortle et al 1979a). In the presence of an optimal concentration of ethidium bromide, pancreatic DNase I induces a single nick essentially at random in covalently closed circular DNA (Greenfield et al 1975). On conversion of these nicks into short gaps, each molecule in the resulting mixture should carry a single bisulfite-sensitive site. When a large number of bisulfite-mutagenized molecules can be screened, it should be possible, in principle at least, to induce mutations at every cytosine residue in the DNA without the limitation of mutational hot spots observed with other methods of random mutagenesis.

NUCLEOTIDE MISINCORPORATION When polymerizing a complementary strand on a primer-template in vitro, purified DNA polymerases are observed to incorporate noncomplementary nucleotides at frequencies orders of magnitude higher than the spontaneous mutation rate measured in vivo (for references, see Kornberg 1980). The frequency of this misincorporation can be substantially increased by one or more manipulations of the reaction conditions: substitution of manganese ion or cobalt ion for magnesium as the divalent ion cofactor; addition of beryllium ion; or creation of large imbalances in the ratio of the four deoxynucleoside triphosphates (for references, see Kunkel and Loeb 1979). Since a short, single-stranded gap in a duplex DNA molecule is a good substrate for many DNA polymerases, nucleotide misincorporation by a DNA polymerase during repair of such a gap in vitro would, in the absence of mismatch repair, generate a base substitution in the newly synthesized strand. If DNA synthesis is terminated by ligation of the nick left when the polymerase has completely repaired the gap, this mutagenic reaction will be confined to those nucleotides within the single-stranded gap; i.e. the short stretch of single-stranded DNA becomes the target for mutagenesis.

In preliminary experiments to examine the feasibility of mutagenesis by nucleotide misincorporation, short gaps have been constructed in pBR322

plasmid DNA at the single cleavage sites for the restriction enzymes *Hind*III and *Cla*I (Shortle & Botstein 1981). When purified circular DNA molecules gapped at the *Hind*III site were incubated with DNA polymerase I from *M. luteus* in the presence of all four deoxyribonucleoside triphosphates plus T4 DNA ligase and ATP, the gap was repaired in greater than 90% of molecules as measured by conversion of the DNA from an open to a covalently-closed circular form. Restriction enzyme analysis of the plasmid progeny appearing after transformation of bacteria with this repaired DNA revealed that all 40 independent isolates screened retained the *Hind*III restriction site. When the first complementary nucleotide required to repair the gap (dATP) was omitted from the reaction mixture and manganese ion was added in addition to magnesium ion, again greater than 90% of the circular DNA molecules were converted to a covalently-closed form. However, 5 out of 36 plasmid isolates recovered after transformation with this DNA were resistant to cleavage by the *Hind*III enzyme. The nucleotide sequence changes found in these five *Hind*III site mutants [A→G (5); A→C (1); A→T (1)] were all consistent with a noncomplementary nucleotide being incorporated in place of the missing nucleotide dATP during gap repair. A higher rate of mutagenesis was observed at the *Cla*I site when the gap misrepair reaction was carried out in the absence of dCTP. Twelve of 36 plasmid isolates had lost this restriction site. Nucleotide sequence analysis of six of the *Cla*I site mutants revealed a pattern of base substitution [C→T (6), C→G (4), C→A (3)] consistent with misincorporation in place of the missing nucleotide dCTP. From these results it would appear that the *in vitro* misrepair of specifically placed single-stranded gaps can be used for site-specific mutagenesis. It is possible that, with the proper ratios of the three added nucleoside triphosphates, the noncomplementary nucleotide which is inserted in lieu of the missing nucleotide can be controlled.

Mutant Construction with Synthetic Oligonucleotides

In the initial stages of genetic analysis of an organism or a particular gene, a wide spectrum of mutants scattered over the region under study is usually the desired product of mutagenesis. As the analysis proceeds and the genomic regions of interest become more sharply defined, the mutants of greatest value also become more clearly defined. The ultimate objective of site-specific mutagenesis would be to enable the investigator to introduce precisely the mutational change desired without a great expenditure of time and effort. Only one of the methods currently in use has the potential to attain this objective—mutant construction with synthetic oligonucleotides.

With the rapid advances taking place in the automated solid-phase synthesis of oligonucleotides, this potential will undoubtedly be realized in the not too distant future. Recent applications of this approach will be briefly reviewed in this section. For a more detailed discussion, see Smith & Gillam (1981).

The basic strategy is to construct a mutant genome *in vitro*—first by synthesizing a defined mutant sequence chemically as an oligonucleotide, and then by enzymatically synthesizing the remainder of the genome onto this oligonucleotide using the wild type genome as template (Figure 3). The most straightforward way this can be done is to synthesize a short oligodeoxyribonucleotide which has a nucleotide sequence complementary to one site on a single-stranded circular DNA, except for the defined mutational change. On annealing to the circular DNA molecule, this mutant oligonucleotide forms a stretch of heteroduplex DNA that can be used as a primer by DNA polymerase for the synthesis of a complete second strand. After closure of the newly synthesized strand by DNA ligase, the product is a covalently-closed duplex circle with one strand carrying the wild-type sequence and the other a specific mutation. On transformation of this hybrid DNA into a cell where it can be replicated, the mutant sequence segregates from wild-type at the first round of replication (assuming there is no mismatch repair). From the resulting mixture of the two types, the mutant genome is recovered by screening for an alteration in phenotype or by one of the physical methods described below.

Although tetra- and pentanucleotides can function as primers for DNA synthesis, three factors necessitate the synthesis of longer mutant oligonucleotides. (a) The oligonucleotide should anneal at a unique site on the circular DNA—the target for mutagenesis. (b) The presence of the mutant nucleotide sequence change creates mismatch(es) which destabilize base-pairing of the oligonucleotide. (c) *E. coli* DNA polymerase I, the enzyme most frequently used to synthesize the second strand, has two exonucleolytic activities that can excise portions of the priming oligonucleotide that do not base pair. A systematic analysis of the requirements for efficient priming by an oligonucleotide containing a single base substitution and for retention of the mutant sequence in the final product has shown that two or three complementary nucleotides to the 3' side and six or seven to the 5' side of the mismatch are usually sufficient for both site-specificity and efficient priming (Gillam & Smith 1979a). When a region of larger non-homology is present in the oligonucleotide-circle heteroduplex, a greater number of base pairs on either side are required.

The first applications of mutant oligonucleotides were in the construction and reversion of amber mutations in gene *E* and gene *G* of the single-stranded DNA bacteriophage ϕ X174 (Hutchison et al 1978, Razin et al

1978, Bhanot et al 1979). In these three reports, the constructed mutants were recovered with efficiencies varying between 1 and 33%, with longer oligonucleotides always giving more efficient mutant recovery. Subsequently, transition, transversion, and small deletion mutations have been constructed in other genes of ϕ X174 (Gillam & Smith 1979a; Gillam et al 1979, 1980). By lowering the temperature of the DNA polymerase reaction to 0°C and by using the large Klenow fragment of *E. coli* DNA polymerase I, the efficiency of mutant construction with shorter primers (8–10 nucleotides) has been greatly increased (Gillam & Smith 1979a). Since the maximum expected yield of mutants on transformation with the completed duplex circle is 50% even with very large primers, methods have been devised to enrich for or detect the mutant nucleotide sequence physically, without reliance on a phenotypic change. By recovering the mixture of mutant and wild-type DNA formed on the first transformation and using this mixture as template for mutant oligonucleotide priming under conditions that selectively stabilize the mutant oligonucleotide-mutant DNA homoduplex, a second round of DNA synthesis enriches for the mutant sequence (Gillam & Smith 1979b). In other words, the mutant oligonucleotide under selective conditions only anneals to mutant viral DNA; therefore, the mutant DNA is primed and replicated, whereas the wild-type DNA is not. After three such cycles of in vitro synthesis followed by transformation, a constructed ϕ X174 mutant containing a single base deletion was enriched from a level of less than 5% after the first transformation to a final level of 100% (Gillam et al 1980). A method which permits direct detection of the constructed mutant among a background of wild-type uses the mutant oligonucleotide as a ³²P-labeled probe for colony hybridization (Wallace et al 1980). If the conditions of hybridization are optimized to destabilize selectively the mutant oligonucleotide-wild-type heteroduplex, the probe will only hybridize to bacterial colonies carrying DNA with the mutant sequence.

In order to apply synthetic oligonucleotides to the construction of mutants of any gene or cloned DNA segment, methods have been developed for placing a given DNA fragment onto a single-stranded circle of DNA which can serve as template for mutant oligonucleotide-primed second strand synthesis. The purified fragment of DNA can be inserted into a single-stranded cloning vector, such as the fd, f1, and M13 bacteriophage derivatives, and single-stranded circular DNA recovered directly from phage particles (Barnes 1980). Using this approach, a mutation in the promoter region of the cloned conalbumin gene has been constructed (Corden et al 1980). Alternatively, a small circular plasmid carrying a cloned DNA segment can be converted to a single-stranded circle by ex-

haustive exonuclease III digestion of singly-nicked plasmid DNA. Using this simple technique, a precise deletion of the 14 base pair intervening sequence in a yeast tRNA^{tyr} gene has been constructed (Wallace et al 1980). As further advances are made in the automated solid-phase synthesis of oligodeoxyribonucleotides, this strategy of mutant construction will undoubtedly become a widely used laboratory technique. Clearly the time is forthcoming when isolation of "the critical mutant" will no longer be a rate limiting step in genetic analysis.

ALLELE REPLACEMENT

Except for the techniques based on fragment mutagenesis followed by recombination, all of the methods of site-specific mutagenesis described in this review have been developed for use on relatively small circular DNAs. Since a particular gene or DNA fragment can usually be inserted into a small circular cloning vector, this general limitation need not be serious. However, when the physiological effects of a constructed mutant allele are to be analysed quantitatively, it is often essential to have one copy of the allele residing at its normal locus in the intact genome. In this section, a few solutions to this important problem of replacing the wild-type allele with a defined mutant allele constructed *in vitro* will be described.

In those few instances when a genome is small enough to be inserted into a circular cloning vector, recovery of constructed mutant forms is straightforward. For example, Peden et al (1980) have cloned the entire SV40 genome into the plasmid pBR322 by cleaving the circular SV40 DNA at its single *Bam*HI site and ligating the full length linear DNA into the *Bam*HI site on the vector. After construction and preliminary analysis of a particular mutant at a defined site within the SV40 segment, the hybrid plasmid was cleaved with *Bam*HI, regenerating a free linear SV40 genome. On transfection into permissive cells, this linear viral DNA was cyclized, restoring the SV40 mutant to a form which can be replicated and then recovered as viral particles for further analysis.

For double-stranded DNA genomes of intermediate size (i.e. viruses and plasmids), a feasible strategy is to clone individual restriction fragments of genomic DNA on circular vectors, construct mutations *in vitro* within the segment of interest, and then reassemble intact genomes carrying this fragment by *in vitro* ligation with the remaining complement of genomic restriction fragments derived from wild-type DNA. Reassembly can be carried out by consecutive ligations (Solnick 1981) or by use of restriction enzymes (such as *Hga*I and *Bgl*II) that leave single-stranded tails at the fragment ends with sequences unique to each cleavage site (Moses & Horiuchi 1979).

In some instances, notably with herpes virus, co-infection of cells with a mutant fragment of viral DNA and wild-type DNA can yield viral mutants by intracellular recombination, as noted earlier (Chu et al 1979, Conley et al 1981, Smiley 1980). To place a small deletion in the VA1-RNA gene constructed in vitro onto the adenovirus 2 genome, Kapoor & Chinnadurai (1981) coinfecting permissive cells with plasmid DNA carrying the mutant gene plus restriction enzyme-cleaved adenovirus DNA. Because the two end fragments of viral DNA contained overlapping sequence homology with the cloned fragment in which the deletion had been induced, intact adenovirus genomes could be assembled by homologous recombination with the plasmid. With this method, mutant virus were recovered at frequencies approaching 100%.

In constructing mutants of cellular genes, a most desirable objective is to return a mutant allele to its proper chromosomal locus. In the case of certain bacteria and yeast the cell's recombinational machinery is able to carry out the requisite homologous recombination, as in classical bacterial transformation. To achieve allele replacement (transplacement) two crossover events between homologous sequences on the chromosome and the exogenous DNA must take place (for an example see Ruvkun & Ausubel 1981). As shown diagrammatically in Figure 6, the crossovers can, in principle, be selected stepwise when the mutant allele resides on a circular DNA molecule which carries one or more selectable genetic markers. If the only pathway to stable inheritance of the genetic marker is via integration into the genome by homologous recombination, then the first crossover is obtained by introducing the circular DNA into a cell and applying selection for the marker. Integration of the DNA molecule by recombination between the wild-type and mutant alleles generates a direct, nontandem repeat of these two gene copies, a structure which is unstable due to homologous recombination between the repeated copies. A second crossover has the effect of excising a complete copy of the circular DNA molecule used for transformation and leaving behind the original arrangement of DNA on the chromosome before transformation. However, when this occurs on the opposite side of the mutation from that of the first crossover (as shown in Figure 6), the mutation originally present on the circular DNA will replace the equivalent wild-type sequence (for a more detailed discussion, see Anderson & Roth 1977, and Oka et al 1971). Using stepwise selection for double recombinants, Scherer & Davis (1979) transplaced the wild-type *his3* gene of *Saccharomyces cerevisiae* with a mutant *his3* allele containing a small deletion within the gene constructed in vitro. The mutant *his3* allele was placed on a vector which carries a *ura3*⁺ gene and can transform yeast with a *ura3* deletion to uracil independence only by integration into the

yeast genome via the sequence homology provided by the inserted *his3* segment. After undergoing transformation of Ura^- to Ura^+ by this composite vector, yeast transformants remained His^+ . However, on growing a pool of transformants in the absence of selection for the Ura^+ phenotype, seven Ura^- segregants (which had excised the transforming DNA segment) were identified; three of these were also His^- . Restriction analysis by Southern transfer of yeast cell DNA from these $\text{Ura}^- \text{His}^-$ segregants confirmed that the wild-type *his3* allele had been replaced by the mutant allele constructed in vitro. As demonstrated by this result, the combination of directed mutagenesis with gene transplacement now makes possible the construction of yeast mutants with defined nucleotide sequence changes.

So far transplacement of eukaryotic cellular genes has been demonstrated only for yeast cells. In the case of cultured mammalian cells transformation with cloned genes or with viral DNA leads to integration of transforming DNA at many different chromosomal loci (Robins et al 1981, Ketner & Kelly 1976, Botchan et al 1976). Nucleotide sequence analysis of cloned SV40-cell DNA joints from transformed cells suggests that integration of viral DNA occurs by nonhomologous recombination between viral and cellular DNA (Sambrook et al 1979). Moreover, when SV40 DNA was used to transform mouse cells already containing integrated SV40 DNA, the newly integrated viral DNA was found by restriction analysis not to have recombined with the resident viral genome (Botchan et al 1979). Comparable experiments have not been reported for transforming mammalian genes, so it is not entirely clear whether sequence homology plays a role in their integration. Why yeast cells appear to differ from mammalian cells in their ease of undergoing the homologous recombinational events required for gene transplacement is not known. The greater abundance of mammalian DNA may be an important factor, as may the presence of active enzymatic systems promoting nonhomologous recombination (Gutai & Nathans 1978,

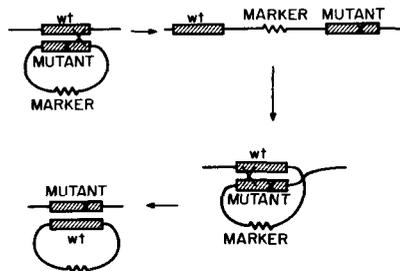


Figure 6 Replacement of a wild-type allele by a mutant allele (present on a recombinant plasmid) by two sequential homologous recombination events.

McCutchan et al 1979). It is also possible that the complexity of DNA mixtures generally used for transformation of mammalian cells obscures any underlying homologous integration (Pellicer et al 1980). This problem deserves increased attention, since directed mutagenesis of cloned DNA combined with gene transplacement in cells of animals and plants would provide a powerful tool for genetic analysis.

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