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REVIEW ARTICLE

Site-directed mutagenesis

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Introduction

The nucleotide sequence of a cloned DNA fragment may be changed at will by site-directed mutagenesis using synthetic oligonucleotides (reviewed by Itakura *et al.*, 1984; Craik, 1985; Bostein & Shortle, 1985; Smith, 1985). The most commonly used approach is to use an oligonucleotide complementary to part of a single-stranded DNA template but containing an internal mismatch to direct the mutation. In addition to single point mutations, this approach may also be used to construct multiple mutations, insertions and deletions. An alternative strategy is to replace the region to be mutated by a fragment obtained by ligation of a number of synthetic oligonucleotides. In this review, recent advances in these alternative strategies to the construction of site-directed mutations using synthetic oligonucleotides will be considered.

The last few years has seen rapid advances in the solid phase synthesis of oligonucleotides (reviewed by Itakura *et al.*, 1984; Gait, 1984; Caruthers, 1985) which combined with the development of automated synthetic systems (reviewed by Caruthers, 1985; Kaplan, 1985) has led to the routine use of short synthetic oligonucleotides (10mers–20mers) in molecular biology. Oligonucleotides may be purified either by h.p.l.c. (see McLaughlin & Piel, 1984) or by polyacrylamide gel electrophoresis (see Wu *et al.*, 1984). However the efficiency of chemical synthesis is becoming so high that unpurified oligonucleotides may be used directly in some cases (Sanchez-Pescador & Urdea, 1984).

Mutagenesis using mismatched oligonucleotides

The underlying strategy behind many mutagenesis methods is to anneal a mutagenic primer to a single-stranded template (M13 or plasmid converted to a single-stranded form). The primer is then extended with the Klenow fragment of *Escherichia coli* DNA polymerase I using deoxynucleoside triphosphates in the presence of T4 DNA ligase to ligate the ends of the new strand. After transfecting competent *E. coli*, the heteroduplex DNA gives rise to the mutant and wild type progeny DNA (Fig. 1).

If the frequency of mutants is routinely greater than 50% then mutants may be identified directly by sequencing a few clones. However, where the frequency of mutants is more variable, hybridization screening with the 5′-^{32}P-labelled mutagenic oligonucleotide provides a convenient way of identifying mutants (Wallace *et al.*, 1980). Hybridization screening may be done under reasonably stringent conditions (elevated temperature) so that the probe selectively hybridizes to the mutant DNA to which it is perfectly matched. Alternatively, the hybridization may be done under non-stringent conditions (room temperature) and the probe then selectively dissociated from wild type DNA by a series of washes at higher temperatures (Fig. 2; Suggs *et al.*, 1981).

Clones which remain hybridized to the mutagenic primer after a stringent wash are then sequenced to check the mutation. In addition to sequencing the region of interest, it is necessary to sequence the rest of the DNA fragment to check that no additional mutations have occurred (Villafranca *et al.*, 1983; Wilkinson *et al.*, 1984). This is most conveniently done by running the T, C, G

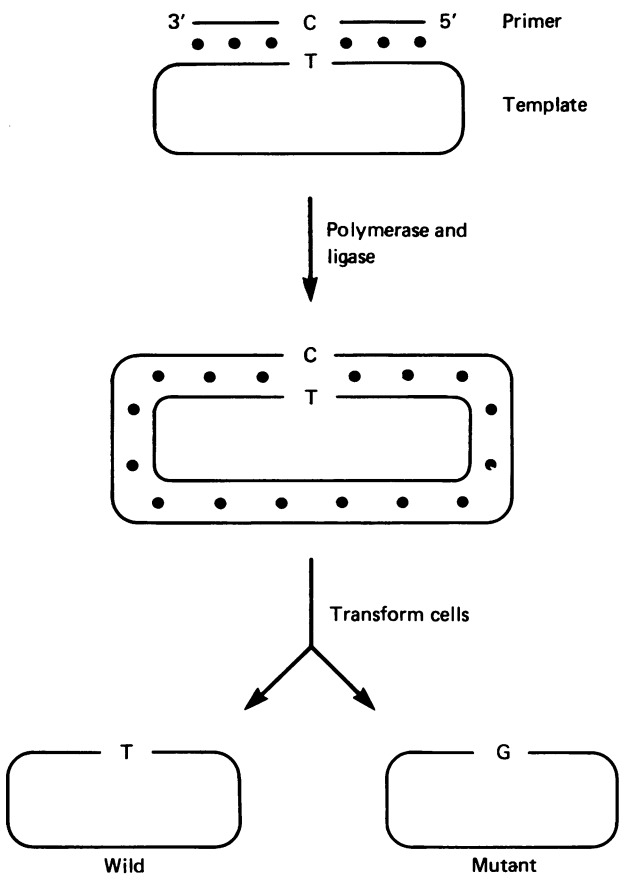

Fig. 1. Mutagenesis using mismatched oligonucleotide

A mismatched oligonucleotide (C to a T) is annealed to a single-stranded DNA template (M13 in this case), extended with the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. After transformation of *E. coli*, mutant and wild type progeny molecules result (from Winter & Fersht, 1984).

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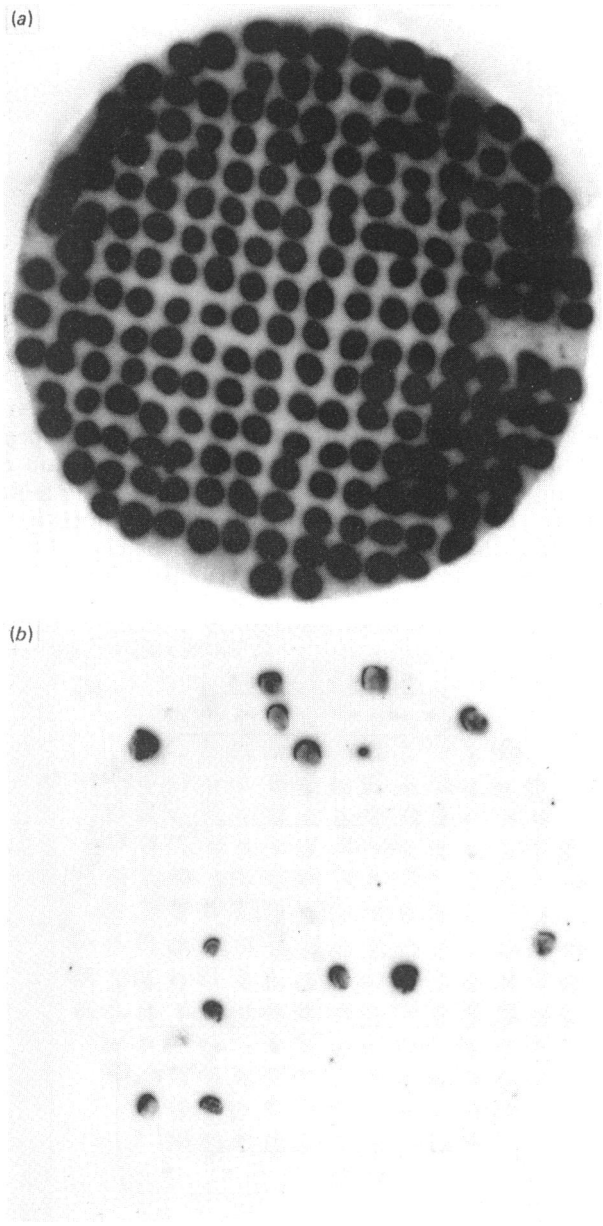

Fig. 2. Colony blot hybridization screening for mutants

A mutagenic primer, G48, (see Table 1) was 5′-^{32}P-labelled by using T_4 polynucleotide kinase and [γ-^{32}P]ATP and then hybridized to M13-infected colonies of *E. coli* which had been blotted on to a nitrocellulose filter. After a non-stringent wash (room temperature), the probe remained hybridized both to mutant DNA and also to wild type DNA, to which it shows two mismatches (*a*). After rewashing the filter under stringent conditions (high temperature), the probe was selectively dissociated from wild type DNA leaving it hybridized to the mutant DNA to which it is perfectly matched (*b*) (from Carter, 1985).

and A sequencing reactions from mutant and wild type clones on adjacent tracks on a sequencing gel (Wilkinson *et al.*, 1984). In constructing some mutations a restriction endonuclease site is created or destroyed, which may be exploited in screening for mutants (Corden *et al.*, 1980; Charles *et al.*, 1982; Wang *et al.*, 1984; Gillam *et al.*, 1984, 1985) or for enriching for mutants (Corden *et al.*, 1980).

In order to obtain high yields of mutants, several problems must be overcome in constructing the heteroduplex DNA and after transfecting it into *E. coli* (Smith, 1985; Carter, 1986). The most significant problem *in vitro* is probably displacement of the mutagenic oligonucleotide by the Klenow fragment after extending completely round the template leading to loss of the mutation. Ligation may be promoted over the competing displacement reaction by titration of the ratio of the Klenow fragment and ligase used, so improving the mutant yield (Hutchison *et al.*, 1978; Gillam & Smith, 1979). The Klenow fragment is often obtained by proteolytic digest of DNA polymerase I. However, any contaminating DNA polymerase I activity in the Klenow fragment may remove the mismatch by nick translation after extending completely round the template (Baas *et al.*, 1981). This problem has decreased with improvements in the quality of commercially available preparations of Klenow fragment, but may be overcome by using enzyme from a genetically engineered clone in which a truncated DNA polymerase I gene corresponding to the Klenow fragment is expressed directly at a high level. The mutagenic primer may sometimes spuriously prime at sites other than the target site as shown by assays *in vitro* (Zoller & Smith, 1982, 1983, 1984). By designing the mutagenic primer to minimize complementarity to other sites this problem may be reduced. Small RNA or DNA molecules present in the template preparation may give rise to spurious 'self-priming' events. Suitably purified template may be prepared by caesium chloride gradient centrifugation, although RNAase treatment is a convenient and acceptable alternative (Carter, 1985; Carter *et al.*, 1985*a*,*b*).

The *in vitro* synthesized (mutagenized) strand of heteroduplex DNA may contain trace amounts of deoxyuridine (dU) residues as a result of deamination of dCTP to dUTP. After transfection these deoxyuridine residues trigger a repair mechanism which may result in loss of the mutation by nick translation (Kunkel, 1985). The use of dUTPase-treated dNTPs or h.p.l.c.-purified dNTPs should overcome this problem. Klenow fragment may be arrested in the synthesis of DNA by extensive secondary structure in the template. A single-stranded DNA binding protein (T4 gene 32 protein) has been used to alleviate this problem (Craik *et al.*, 1985).

Methylation at GATC sites by the *dam* methylase (Marinus & Morris, 1975; Hattman *et al.*, 1978) directs a mismatch repair system in *E. coli*. This may reduce mutant yield by directing mismatch repair towards the methylated template strand (Radman *et al.*, 1980; Glickman, 1982; Kramer *et al.*, 1984*a*). The mutant yield may also be reduced by progeny DNA derived from the template strand of the transfected heteroduplex. However for M13 there is a 2:1 bias in favour of progeny derived from the minus (mutagenized) strand, because of the asymmetric way in which the DNA is replicated (Enea *et al.*, 1975; Kramer *et al.*, 1984*a*).

Mutagenesis using single-stranded vectors

Single-stranded vectors derived from the filamentous phage M13 and fd provide a direct source of single-stranded DNA for mutagenesis and for dideoxy sequencing of mutants constructed. Occasionally it is found that a DNA sequence is not stably maintained in M13, but this problem may also be encountered with other types of vector. Expression of the mutant gene will generally require recloning of the mutant into an

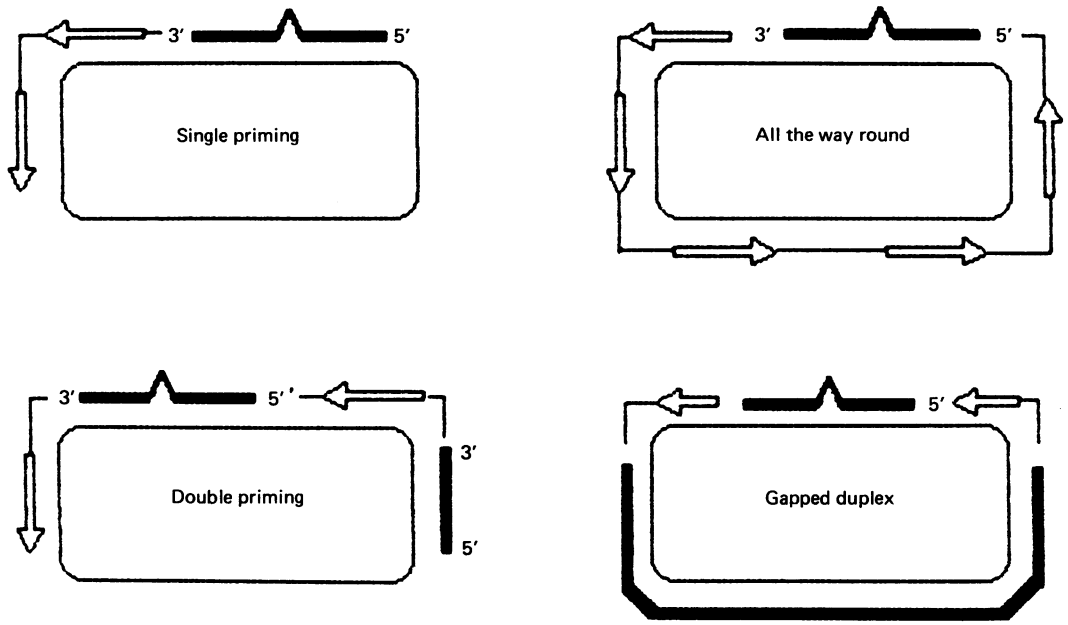

Fig. 3. Simple strategies for M13 mutagenesis

The simplest approach to M13 mutagenesis is 'single priming', where a mutagenic primer is annealed to the single-stranded template, extended briefly with Klenow fragment and used to transfect an *E. coli* host. The mismatch may be removed *in vivo* by the action of 5′→3′ exonucleases but this may be overcome in several different ways. After extending 'all-the-way-around' the template the ends of the new strand may be ligated. In the 'double priming' technique a second primer 5′ to the mutagenic primer is used to protect the mismatch after extension and ligation. In the 'gapped duplex' technique, mutagenesis is carried out in a single-stranded region formed by annealing the template with a restriction fragment from the vector. Again the 5′ end of the oligonucleotide is protected after extension and ligation (from Carter *et al.*, 1985*a*).

expression vector, although in some cases high levels of gene expression may be obtained from the M13 clone directly (Winter *et al.*, 1982; Bloxham *et al.*, 1983). DNA fragments are readily cloned into M13 for mutagenesis by using Messing's M13mp vectors (reviewed by Messing, 1983). These M13mp vectors have a multiple cloning site containing unique or paired restriction endonuclease sites for a number of enzymes and there is a simple colour screen for recombinant phage (Messing *et al.*, 1977).

Several strategies have been used to protect the 5′ end of the mutagenic oligonucleotide (by ligation) from removal of the mismatch by 5′→3′ exonucleases *in vivo* (Fig. 3). After extending completely round the template ('all-the-way-around') and ligating, closed circular DNA may be isolated by agarose gel electrophoresis in the presence of ethidium bromide (Simons *et al.*, 1982) or by alkaline sucrose gradient centrifugation (Kudo *et al.*, 1981; Zoller & Smith, 1982). In the 'gapped duplex' technique, mutagenesis is conducted in a short single-stranded region left after annealing the M13 template with a restriction fragment from the M13 vector (Kramer *et al.*, 1982). Hence the 5′ end of the oligonucleotide is protected after extension and ligation. In the 'double priming' technique, a partial extension is made from a mutagenic oligonucleotide and a second oligonucleotide 5′ to the mutagenic oligonucleotide to protect the mutation (Norris *et al.*, 1983; Zoller & Smith, 1984).

Reduction in the yield of mutants by mismatch repair of the M13 heteroduplex DNA has been avoided by constructing hemi-methylated 'gapped duplex' DNA, using unmethylated template and a methylated restriction fragment of the vector. Mismatch repair should then correct the mismatch in favour of the mutagenized strand (Kramer *et al.*, 1982; Marmenout *et al.*, 1984). A more powerful method uses *E. coli* host strains deficient in mismatch repair which also select genetically against progeny phage derived from the plus strand of M13 (Kramer *et al.*, 1984*b*). The 'gapped duplex' is constructed (Fig. 4) using template from one of Messing's widely used M13mp vectors containing amber mutations in two essential phage genes and a restriction fragment from a wild type vector (Kramer *et al.*, 1984*b*). After transfecting into a non-suppressor host strain which is also deficient in point mismatch repair, only phage which have incorporated the amber-to-wild-type mutation will be viable. A variant procedure using amber selection and a hemi-methylated 'gapped duplex' to direct mismatch repair towards the mutagenized strand has also been reported (Bauer *et al.*, 1985).

Mutagenesis using repair deficient host strains and amber selection has also been developed with a 'coupled priming' technique (Carter *et al.*, 1985*b*). One primer is used to generate the 'silent' mutation of interest and a second primer is used to remove an amber mutation previously introduced into gene IV of M13 (Fig. 4). After extension and ligation from these two primers, the heteroduplex DNA is used to transfect a non-suppressor repair deficient *E. coli* host strain. For a model point mutation, eliminating point mismatch repair was found to give a 6–8-fold increase in the mutant frequency (comparing the Repair$^-$ Su2$^+$ and Repair$^+$ Su2$^+$ strains in Table 1). Amber selection against the plus strand of M13 was found to give an additional 2-fold increase in the frequency (comparing the Repair$^-$ Su2$^-$ and Repair$^-$ Su2$^+$ strains in Table 1).

A disadvantage of these amber selection procedures

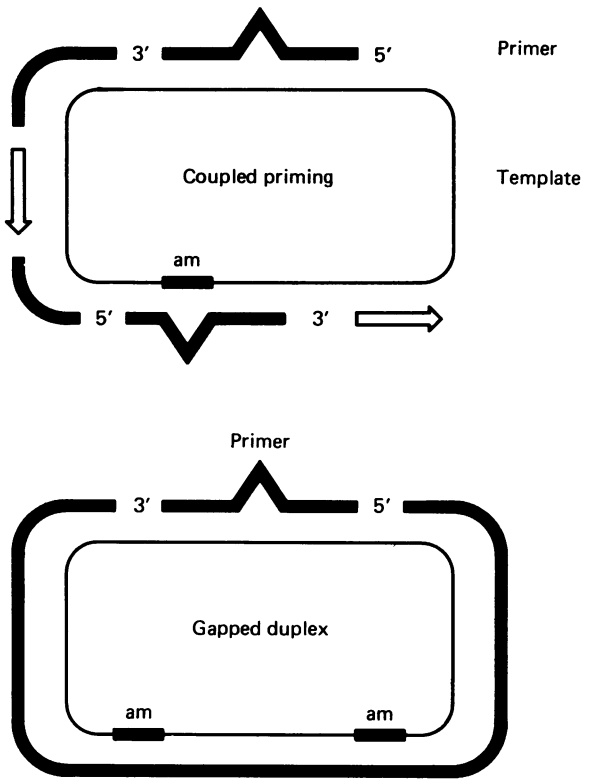

Fig. 4. Improved M13 mutagenesis using strand selection

Enhanced mutant yields are obtained by using repair-deficient *E. coli* host strains and amber selection against progeny phage derived from the template strand of M13. In the 'coupled priming' technique, a primed extension is made from a mutagenic oligonucleotide and a selection oligonucleotide to remove an amber mutation in the template. After transfection into a non-suppressor (repair-deficient) host strain, only wild type phage are viable. Amber selection using the 'gapped duplex' techniques uses an amber M13 template with a restriction fragment from a wild type vector (from Carter *et al.*, 1985*a*).

(Kramer *et al.*, 1984*b*; Carter *et al.*, 1985*b*; Bauer *et al.*, 1985) is that the selectable marker is removed in the first round of mutagenesis so further mutations cannot be constructed using selection. This problem has been overcome by generating a second selectable marker at the same time as the first one is removed, allowing a series of mutations to be constructed by cycling between the two markers (Carter *et al.*, 1985*b*; Carter, 1986). [The markers used are the restriction modification systems found in K strains (*Eco*K) and B strains (*Eco*B) of *E. coli*.] In this approach one oligonucleotide is used to construct a 'silent' mutation of interest and another oligonucleotide used to remove one selectable marker (*Eco*K or *Eco*B site) and at the same time generate a second selectable marker (*Eco*B or *Eco*K site).

Progeny phage derived from the plus (template) strand of M13 have been eliminated directly by preparing the template in a *dut ung* host strain of *E. coli* which results in few deoxyuridine residues being incorporated into the template in place of thymidine (Kunkel, 1985). After extension completely round the template from a mutagenic primer and ligation, the plus strand may be destroyed *in vitro* by treatment with uracil glycosylase and alkali. After transfection very high mutant frequencies (~ 90%) have been obtained (Kunkel, 1985). The heteroduplex DNA may be transfected directly after the extension and ligation steps, since a very strong biological selection is obtained against the template strand. The frequency of a model point mutation was compared using this simplified variant of the Kunkel procedure and the 'coupled priming' technique using amber or *Eco*K selection (Table 2). The frequency of mutants was similar in each case (~ 70%) and no further improvement was obtained by combining the two approaches. This mutant frequency is very similar to that reported (76%) for the same model mutation in *lacZ′* using the 'gapped-duplex' method with amber selection (Kramer *et al.*, 1984*b*).

An alternative strategy for enriching for mutants in M13 relies upon the observation that double-stranded DNA with an α-phosphorothioate analogue of one of the deoxynucleoside triphosphates incorporated into one strand can be selectively nicked on the unmodified strand by using a suitable restriction endonuclease (Vosberg & Eckstein, 1982; Potter & Eckstein, 1984; Taylor *et al.*, 1985*a*). For mutagenesis, covalent circular DNA is obtained by extension from a mutagenic primer on a M13 template in the presence of three dNTPs and one α-phosphorothioate dNTP analogue. The plus strand of M13 is then selectively nicked with a restriction endonuclease. After exonuclease III treatment, repair DNA synthesis and transfection, mutant progeny phage were obtained in yields of 40–66% (Taylor *et al.*, 1985*b*).

Table 1. Effect of strand selection and repair-deficient host strains upon frequency of mutants constructed in M13

A mutagenic primer, G48 (5′ GCCAAGCCGCCGATAT 3′, mismatches underlined), and a selection primer, SEL1 (5′ AAGAGTCTGTCCATCAC 3′, mismatch underlined), to remove an amber mutation from gene IV of M13 were annealed to a M13TyrTS template, extended and ligated for 17 h at 12 °C. Closed circular heteroduplex DNA was then isolated after alkaline sucrose gradient centrifugation and used to transfect various *E. coli* host strains ($Su2^+$ = suppressor, $Su2^-$ = non-suppressor; $Repair^-$ = repair deficient, $Repair^+$ = repair proficient). The percentage of G48 mutants identified by oligonucleotide hybridization screening is shown (from Carter *et al.*, 1985*b*).

	G48 mutants (%) in host strains			
Vector background	TG1 $Repair^+$ $Su2^+$	HB2151 $Repair^+$ $Su2^-$	BMH71-18 mutL $Repair^-$ $Su2^+$	HB2154 $Repair^-$ $Su2^-$
M13mp93	3.5	5	29	37
M13mp93amIV	4.5	37.5	32	70

Table 2. Comparison of 'coupled priming' technique and deoxyuridine-containing template for mutagenesis in M13

The mutagenic primer for a blue→white model mutation, B/W (5′ GGTTTTCCTAGTCACGA 3′, mismatch underlined) and a selection primer SEL1 (see Table 1) or SEL2 (to mutate the *Eco*K selection site into an *Eco*B site) were annealed to a M13 template [M13mp19amIV prepared in TG1 or the *dut ung* strain RZ1032 (Kunkel, 1985) and M13K19], extended and ligated for 4 h at 12 °C. The heteroduplex DNA was then used directly to transfect various *E. coli* host strains (TG1, HB2151, BMH 71-18 mutL and HB2154) in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside and isopropylthiogalactoside. The percentage of white plaques obtained is shown. The data were obtained by participants on the EMBO Site-Directed Mutagenesis course held at the EMBL, Heidelberg, Germany, in November 1985, organized by Professor R. Cortese and Dr. G. Winter (see Carter *et al.*, 1985a,b; Carter, 1986).

Template	Primers	White plaques (%) in host strains: TG1 Repair$^+$ Su2$^+$ $r_K^-m_K^-$	HB2151 Repair$^+$ Su2$^-$ $r_K^+m_K^+$	BMH71-18 mutL Repair$^-$ Su2$^+$ $r_K^+m_K^+$	HB2154 Repair$^-$ Su2$^-$ $r_K^+m_K^+$
M13mp19amIV	B/W, SEL1	9.3	47.8	47.5	68.0
M13K19	B/W, SEL2	6.5	34.2	62.1	66.0
M13mp19amIV (*dut ung*)	B/W, SEL1	74.8	58.5	54.0	68.4

In constructing many mutations over a limited region, the synthesis of long oligonucleotides with multiple mismatches to the template provides an alternative strategy to the sequential construction of mutations. A 44mer with 13 mismatches to the template was used to direct seven simultaneous amino acid changes in the tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* (Carter *et al.*, 1985*b*). Deletions may be made using an oligonucleotide whose two ends are complementary to different regions of the template, so that in the annealing reaction the intervening region of template is looped out. In constructing large deletions the frequency of clones which hybridize strongly to the mutagenic oligonucleotide is often low, and only a fraction of these clones carry the required mutation (Osinga *et al.*, 1983; Chan & Smith, 1984). The frequency of a correctly generated kilobase deletion was improved 100-fold by using *Eco*K selection (Waye *et al.*, 1985). This was achieved by inserting four *Eco*K sites within the region to be deleted to provide a very strong selection against the starting template. Insertions may be made using an oligonucleotide complementary to part of the template but containing an extra bit of sequence which is looped out in the annealing reaction. An insertion of four nucleotides was made into a M13 vector using a 21mer oligonucleotide to generate additional restriction sites (Norrander *et al.*, 1983). The size of the insert is limited by the length of the oligonucleotide. Thus for large insertions a more convenient approach is to clone a synthetic fragment into unique restriction sites created by site-directed mutagenesis.

Mutagenesis using double-stranded vectors

The attraction of mutagenesis using double-stranded vectors is that most cloning experiments are done with such vectors and they allow expression of cloned genes. Denatured double-stranded DNA can be sequenced directly by the dideoxy method using oligonucleotide primers (Smith *et al.*, 1979; Hong, 1982) or by the chemical method (Maxam & Gilbert, 1977).

In constructing oligonucleotide-directed mutations in plasmids, the plasmid is normally converted to a single-stranded or partially single-stranded form. This may be achieved by first nicking the supercoiled plasmid in the presence of ethidium bromide with a restriction endonuclease (Greenfield *et al.*, 1975) or DNAase I (Parker *et al.*, 1977) and then digesting to completion with exonuclease III (Wallace *et al.*, 1980). After purification by caesium chloride gradient centrifugation, the isolated mixture of plus and minus strands (only one of which is a target for the mutagenic oligonucleotide) is used for mutagenesis in a manner analogous to M13 single-stranded DNA. The main problems with this approach is that the frequency of mutants obtained is generally very low ($< 0.5\%$) and isolation of single-stranded DNA is relatively time-consuming.

The use of a 'double priming' technique for plasmid mutagenesis removes the need to isolate single-stranded DNA (Schold *et al.*, 1984). Covalent double-stranded DNA is denatured by boiling and then annealed with a mutagenic primer and a second primer (perfectly complementary) located on the 5′ side of the mutagenic primer. After a brief extension reaction with Klenow fragment, the mixture is used directly to transform *E. coli*. The simplicity of this approach should encourage its further use, particularly if the low frequency ($< 0.5\%$) of mutants can be improved, for example by using repair deficient host strains and strand selection as used with M13. A similar 'double priming' strategy using linearized plasmid DNA has been reported in which the mutagenized region is excised and then recloned into the parent vector (Hollenberg *et al.*, 1984).

Several approaches have been used to construct 'gapped-duplex' plasmid DNA for oligonucleotide-directed mutagenesis. The simplest approach is to nick the plasmid at a restriction site near the target sequence followed by limited digestion with exonuclease III to produce the 'gapped duplex' (Dalbadie-McFarland *et al.*, 1982). An alternative way of constructing the 'gapped duplex' is to anneal denatured linear recombinant DNA (cut far away from the insert) with denatured linear vector DNA (cut at the site of insertion) (Oostra *et al.*, 1983). This technique has been modified to enhance the yield of mutants (Moriniga *et al.*, 1984; Straus *et al.*, 1985; Inouye & Inouye, 1986). Another method is to linearize the plasmid near the target site and expose the target by

limited digestion using exonuclease III. After extension from a mutagenic oligonucleotide and ligation, the resulting double-stranded DNA is used to transform an *E. coli* host (Gutteridge *et al.*, 1982; Lewis *et al.*, 1983; Efimov *et al.*, 1985).

Single-stranded plasmids ('phasmids') have been developed, which contain a selectable marker, a multiple cloning site and origins of DNA replication for plasmids and a filamentous phage (Dente *et al.*, 1983; Levinson *et al.*, 1984; Zagursky & Berman, 1984). When a male (F') *E. coli* host strain carrying such a plasmid is infected with a 'helper phage' then single-stranded DNA from the plasmid (and also the 'helper') is packaged and secreted. After isolation of the mixture of single-stranded DNA from the phage, mutagenesis against a target site in the plasmid is carried out directly (see Valenzuela *et al.*, 1985). After mutagenesis single-stranded DNA may be isolated directly, allowing characterization of the mutation by dideoxy sequencing.

Mutagenesis by total chemical synthesis

The main attraction of constructing mutants by total chemical synthesis is that the frequency of mutants obtained may approach 100%. Improvements in oligonucleotide chemistry and the simultaneous synthesis of large numbers of oligonucleotides by using segmented solid supports (Frank *et al.*, 1983; Matthes *et al.*, 1984; Ott & Eckstein, 1984) has made the synthetic approach to mutagenesis much more appealing, particularly for very extensive mutagenesis projects.

The total chemical synthesis strategy for constructing mutants involves removing a fragment from a clone to be mutated using unique restriction sites and replacing it with a synthetic mutant homoduplex (Lo *et al.*, 1984; Wharton *et al.*, 1984; Eisenbeis *et al.*, 1985). Suitable restriction endonuclease sites may be present in the starting clone (Lo *et al.*, 1984; Hui *et al.*, 1984; Eisenbeis *et al.*, 1985) or introduced by mismatched oligonucleotide mutagenesis (Matteucci & Heyneker, 1983; Kadonaga *et al.*, 1984; Wharton *et al.*, 1984) or introduced in the construction of larger synthetic fragments (Nambiar *et al.*, 1984; Roberts *et al.*, 1985). In constructing a series of mutants over an extended region, the duplex is constructed several times using a modified pool of oligonucleotides to generate each mutant. Thus the oligonucleotide pool is very similar in each case except that a pair of oligonucleotides for the wild type duplex is replaced by a pair which introduces the required mutation. The full length mutant homoduplex is then purified by gel electrophoresis prior to cloning (Lo *et al.*, 1984; Eisenbeis *et al.*, 1985) or cloned directly — 'shotgun ligation' (Grundström *et al.*, 1985). A number of mutations in a limited region have been obtained by 'cassette mutagenesis', where a small restriction fragment is replaced by a single pair of complementary oligonucleotides which may contain a mixed sequence at one or more positions (Murphy & Baralle, 1983; Matteucci & Heyneker, 1983; Hui *et al.*, 1984; Wells *et al.*, 1985). A 'cassette mutagenesis' method has been used to replace one amino acid with the 19 other alternatives using pools of mixed oligonucleotides (to limit the amount of required oligonucleotide synthesis) to construct the 'mutagenic cassette' (Wells *et al.*, 1985).

Choice of strategy for mutagenesis

Over the last 3 or 4 years there has been an avalanche of alternative methods for constructing oligonucleotide-directed mutations. Currently the method of choice in constructing mutants is using mismatched oligonucleotides with M13. A small fragment to be mutated should be subcloned into M13. Mutants are very simply and reliably constructed by a limited extension from a mismatched oligonucleotide primer and transfection of the heteroduplex DNA into a repair-deficient *E. coli* host strain (see Carter, 1986). An improved frequency of mutants may be obtained by using a number of more elaborate procedures (see Kramer *et al.*, 1984*b*; Carter *et al.*, 1985*a*,*b*; Kunkel, 1985; Taylor *et al.*, 1985*b*). It is essential to determine the complete nucleotide sequence of the mutagenized fragment, which is very readily achieved in M13. The mutagenized fragment is then reintroduced into the parent clone for further characterization and expression of the mutant.

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