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Improved oligonucleotide site-directed mutagenesis using M13 vectors

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ABSTRACT

An improved method is described for the construction of mutations in M13 vectors using synthetic oligonucleotides. The DNA is first cloned into a novel M13 vector (based upon M13mp18 or M13mp19), which carries a genetic marker that can be selected against, such as an EcoK or EcoB site, or an amber mutation in an essential phage gene. In this "coupled priming" technique, one primer is used to construct the silent mutation of interest, and a second primer is used to eliminate the selectable marker on the minus strand. After primer extension and ligation, the heteroduplex DNA is transfected into a strain of E.coli which is repair deficient and selects against the plus strand marker. Over 50 mutants have been constructed with this approach, and the yields can be excellent (up to 70%). For the stepwise construction of mutations using separate rounds of mutagenesis, the EcoK and EcoB markers offer a particular advantage over the amber marker. They permit selection in each round, as it is possible to cycle between the two markers. However for construction of multiple mutations over a short region, long synthetic oligonucleotides with multiple mismatches to the template can offer an alternative strategy.

INTRODUCTION

Oligonucleotide site-directed mutagenesis is now widely used to construct point mutations, insertions and deletions in DNA fragments cloned into M13 vectors. At its simplest, "single priming", the oligonucleotide is hybridised to the single stranded template and is extended with Klenow. The partial extension product is then transfected into competent E.coli, where it gives rise to mutant and wild type progeny phage. Since the 5' end of the primer is exposed, the mismatch may be edited out by 5' -> 3' exonucleases in vivo, and in the "all-the-way-round" (1) "double priming" (2,3) or "gapped duplex" (4) techniques the 5' end is therefore protected by ligation. However the frequency of mutant phage is often low, and is affected by at least two other factors in vivo. Firstly repair of the mismatch may occur, correcting the unmethylated minus strand in favour of the plus strand template (4). Secondly progeny phage are derived from both the plus and minus strands of M13, although there is a 2:1 bias in favour of the minus

strand as the template (5,6). Thus transfection of the heteroduplex into repair deficient (repair-) strains of E.coli and selecting for phage progeny derived from replication of only the minus strand, should enhance the yields of mutant. Recently this strategy was employed using the "gapped duplex" technique and an amber marker, (7), although it has also proved possible to eliminate the plus strand more directly (8). We now present a technique based upon a "double priming" method using the restriction markers EcoK and EcoB.

The vectors M13mp18 and M13mp19 have been engineered by inserting either an EcoK site (to give M13K18 and M13K19) or an EcoB site (to give M13B18 and M13B19) in the polylinker sequence. An amber mutation in gene IV has also been constructed to give the vectors M13mp18amIV and M13mp19amIV. These vectors thus contain markers which can be selected against in non-supressor (su2-), K strains (rk+mk+) or B strains (rb+mb+) as appropriate. By priming on plus strand template with both the mutagenic primer and a "selection primer" to remove the selectable marker from the minus strand, and transfecting into cells which are repair- and select against the marker, a selection for progeny phage derived from replication of the minus strand is obtained (Fig. 1). However after one round of mutagenesis, the selectable amber marker is lost, but EcoK or EcoB markers can be retained. For example, starting with the template containing the EcoK site, a "selection primer" is used which removes the EcoK site by converting it into an EcoB site. For a second round of mutagenesis, a second "selection primer" is used which removes the EcoB site by converting it back into an EcoK site (Fig. 2). Thus by reciprocating between EcoK and EcoB selection, it is possible to undertake multiple rounds of mutagenesis utilising strand selection in each round.

MATERIALS AND METHODS

Bacterial Strains

The following E.coli strains were used:

TG1=K12,Δ(lac-pro),supE,thi,hsdD5/F'traD36,proA+B+,lacI^q,lacZΔM15 (9);

AC2522=B/r,Hfr,sul-1 (10);

HB2151=K12,ara,Δ(lac-pro),thi/F'proA+B+,lacI^q,lacZΔM15;

HB2154=HB2151,mutL::Tn10 and HB2155=AC2522,mutL::Tn10.

HB2151 was constructed by crossing P90C (11) and BMH 71-18 (12). HB2154 and HB2155 were constructed by P1 transduction of the mutL::Tn10 insertion from the strain BMH 71-18mutL (6) into HB2151 and AC2522 respectively.

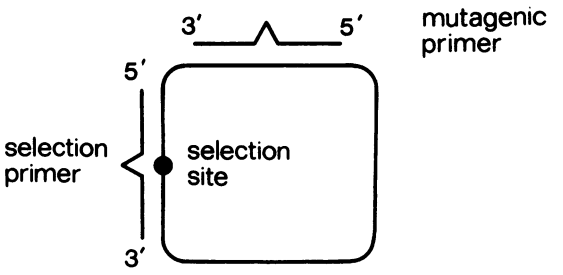

Figure 1. "Coupled Priming" Oligonucleotide-directed Mutagenesis

Phage Strains

The novel M13 vectors M13K18 and M13K19 were constructed by first inserting a blunt ended 30 bp synthetic "selection cassette" into the HincII site of M13mp18 and M13mp19 (13) respectively (Fig. 3). The cassette has an EcoK site which overlaps part of an EcoB site to allow interconversion of the two markers by site-directed mutagenesis, and additional restriction site for StuI,NruI and XhoI. The reading frame of the lacZ α-peptide is maintained in the "selection cassette" (with no stop codons) to allow blue/white screening for recombinant phage (14). The EcoB site in gene II of M13 was then removed by site-directed mutagenesis, transfecting into HB2155 (rb+mb+, repair-) to select against the original site. Sequencing confirmed that the EcoB site had been removed and the vectors were designated M13K18 or M13K19.

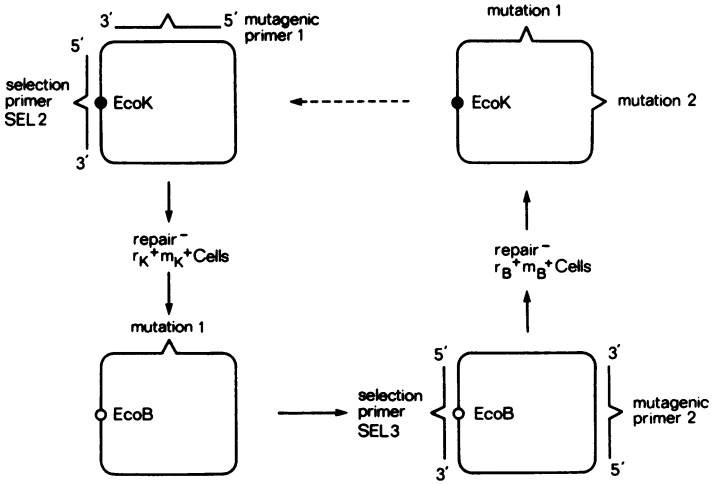

Figure 2. Scheme for "Cyclic Selection" Mutagenesis

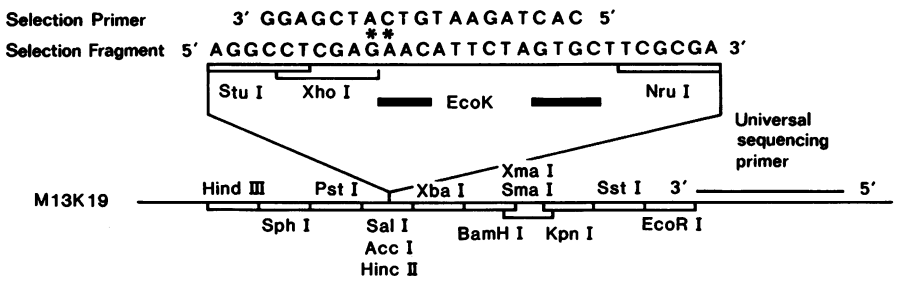

Figure 3. Selection Mutagenesis Vector M13K19

Mutations were subsequently constructed in the tyrosyl-tRNA synthetase (TyrTS) gene from Bacillus stearothermophilus, which was recloned from its original background in M13mp93 (15) into the SmaI site of M13K18 and M13K19 vectors. However the EcoB site in the TyrTS gene was first removed by site-directed mutagenesis, transfecting into the host strain HB2155 (rb+mb+,repair-) to select against the original site. This yields the vectors M13K18TyrTS and M13K19TyrTS.

For "coupled priming" mutagenesis utilising an amber marker, an amber mutation was constructed at position 5327 in M13 by site-directed mutagenesis of the original M13mp93TyrTS vector. This yields the vector M13mp93amIVTyrTS. For more general use, M13mp18 and M13mp19 vectors were also converted to amIV versions.

Oligonucleotides

Oligonucleotides were synthesised either manually (G35,G48,G51,amIV and SEL1) or by Biosearch DNA synthesizer (SEL2,SEL3,wtII,B1 and B2) using phosphotriester chemistry or by Applied Biosystems DNA synthesizer (SC1,SC2,CO and MET) using phosphoramidite chemistry. All oligonucleotides were purified by preparative gel electrophoresis using pre-electrophoresed thin 20% native polyacrylamide gels containing 2 μg/ml ethidium bromide, and were visualized by long wave ultra violet light. The amIV primer (5'AAGAGTCTATCCATCAC 3') was used to construct an amber mutation at position 5327 within M13 gene IV, which may be removed using the selection primer SEL1 (5' AAGAGTCTGTCCATCAC 3'). The two complementary oligonucleotides SC1 (5' AGGCCTCGAGAACATTCTAGTGCTTCGCGA 3') and SC2 (5' TCGCGAAGCACTAGAATGTTCTCGAGGCCT 3') were used to construct the selection site in M13K18 and M13K19. The selection primer SEL2 (5' CACTAGAATGTCATCGAGG 3') was used to convert the EcoK selection site into an EcoB site, and SEL3 (5'CACTAGAATGTTATCGAGG 3') to convert the EcoB selection site back into an EcoK site. The primer B1

(5'GCAAAATTCAATAATAAAGC 3') was designed to remove the EcoB site from gene II of M13 without changing the amino acid sequence: the mutation was verified by dideoxy sequencing using the wtII primer (5'GCTGAATCTGGTGCTGT 3'). The primer B2 (5'ATGTAGTTAACGCTGAA 3') was designed to remove the EcoB site from the TyrTS gene without changing the amino acid sequence. The mutagenic primers were designed to construct point mutations in the TyrTS gene (position of mismatch shown by asterisk after corresponding nucleotide): G35 (5'CAAACCCGCC*GTAGAG 3') for converting Cys35 to Gly (16), G48 (5' GCCAAGC*C*GCCGATAT 3') for converting His48 to Gly (17) and G51 (5' CAAAATGC*C*GGCCAAG 3') for converting Thr51 to Gly (18). Multiple mutations (Fig. 4) were constructed in the TyrTS gene using the 38mer oligonucleotide CO (5' CTGCTGGAAGCGTTTCAGGCACAAAAGCGGGACCAAGT 3') to direct 6 amino acid changes Ala50 to Val, Thr51 to Pro, Ile52 to Leu, Thr54 to Cys, Met55 to Leu and Arg56 to Lys; and the 44mer oligonucleotide MET (5' ATATGCAAACTGCCATTCGCA TACGGCAACGCGCAGTAGAGCGT 3') to direct 7 amino acid changes Gly36 to Ala, Phe37 to Leu, Asp38 to Pro, Pro39 to Tyr, Thr40 to Ala, Ala41 to Asn, and Asp42 to Gly. Oligonucleotides were kinased for mutagenesis and for probes as previously described (17).

Construction of Mutants

Single stranded DNA for the template for mutagenesis was prepared as for dideoxy sequencing (19), except that small RNA primers were eliminated by a RNase digest of the resuspended PEG pellet, before the phenol extraction.

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   GlyHisLeuValProLeuLeuCysLeuLysArgPheGlnGlnAla      Mutant Enzyme
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     3'TGAACCAGGGCGAAAACACGGACTTTGCGAAGGTCGTC 5'      Mutagenic Primer (CO)
           * * **     ****  ***
5'GGCCACTTGGCCACCATTTTGACGATGCGCCGCTTCCAGCAGGCG3'    Template

   GlyHisLeuAlaThrIleLeuThrMetArgArgPheGlnGlnAla      Wild Type Enzyme


   ThrLeuTyrCysAlaLeuProTyrAlaAsnGlySerLeuHisIle      Mutant Enzyme
   ------------------------

3'TGCGAGATGACGCGCAACGGCATACGCTTACCGTCAAACGTATA 5'    Mutagenic Primer (MET)
                 *   ********  *** *
5'ACGCTCTACTGCGGGTTTGACCCGACGGCGGACAGTTTGCATATC 3'   Template

   ThrLeuTryCysGlyPheAspProThrAlaAspSerLeuHisIle      Wild Type Enzyme
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Figure 4. Construction of multiple oligonucleotide-directed mutations in the tyrosyl-tRNA synthetase of Bacillus Stearothermophilus

The "self-priming" activity of the templates used for mutagenesis represented 1-2% of the primed activity as assayed by incorporation of α-[32P]-dATP.

Single stranded M13 template (0.67pmol) was mixed with phosphorylated primers (10pmol of mutagenic primer and 10pmol of "selection primer") in 10μl 10mM Tris-HCl (pH 8.0), 10mM $MgCl_2$. The primers were annealed by cooling from 80°C to 25°C over about 30 minutes and extended with ∿1U DNA polymerase I Klenow fragment (gift from A.R.Fersht) in the presence of 5U T4 DNA ligase (Anglian Biotechnology) as previously described (17). After 6 to 20 hours at 12°C, small aliquots were then used to transfect $CaCl_2$ treated E.coli cells (20). After transfection of repair- strains (HB2154 or HB2155), lawn cells were provided by repair+ strains (HB2151 or AC2522 respectively) in order to minimize the exposure of the phage to the mutator phenotype of the repair- strains.

In each case 200 plaques resulting from the transfected DNA were toothpicked on to L-plates and grown up as colonies of infected bacteria for 8 to 20 hours. A nitrocellulose blot was prepared and probed with the mutagenic oligonucleotide as previously described (17) except that the filters were baked for only 15-30 minutes at 80°C in vacuo and prehybridized for only 5-10 minutes at 67°C. The percentages of colonies that hybridised strongly to the mutagenic oligonucleotides after a stringent wash were scored, and are listed as "mutant frequency" in Tables 1,2 and 3.

Putative mutant phage were plaque purified by toothpicking from the colony blot plate into 1ml of L-broth. After heating at 70°C for 5-20 minutes to kill the bacteria, 1-2μl of this phage suspension was then streaked out on to a fresh L-plate using a sterile wire loop and a lawn provided by overlaying with 3ml H-top agar containing 200μl of an overnight culture of TG1 diluted 10-fold in L-broth. Mutations were verified by dideoxy sequencing (21) using a family of sequencing primers located at intervals throughout the TyrTS gene (22).

Test for EcoK/EcoB selective marker

For multiple rounds of mutagenesis using the EcoK/EcoB reciprocating markers, it is easy to check that the mutant phage carries the new marker at the stage of plaque purification. Potential mutant phage are normally plaque purified as they may also contain wild type phage. By plaque purifying the phage on TG1 (rk-mk-,rb-mb-) any phage which escaped restriction by virtue of being modified will now be unmodified. Subsequent streaking of the phage on two different strains (as in plaque purification described above) will identify the selective marker. Thus the difference in plaque yield of phage

Table 1: The use of Amber Selection for Mutagenesis

TyrTS cloned in:	Extension Ligation /hours	Sucrose Gradient	Mutant Frequency in Host Strains: TG1 repair+ su2+	HB2151 repair+ su2-	BMH71-18 mutL repair- su2+	HB2154 repair- su2-
M13mp93	4.5	-	0.5	1.5	12.5	15
M13mp93	19	+	3.5	5	29	37
M13mp93amIV	4.5	-	2	14	16	32
M13mp93amIV	19	+	4.5	37.5	32	70

The mutagenic primer G48 and the selection primer SEL1 were annealed to a M13 template (M13mp93TyrTS or M13mp93amIVTyrTS) extended and ligated. The heteroduplex DNA was then used to transfect various E.coli hosts (TG1,HB2151,BMH71-18mutL, and HB2154).

Table 2: Comparison of Amber, EcoK and EcoB Selection for Mutagenesis

TyrTS cloned in:	Selection Primer	Mutagenic Primer	Mutant Frequency in Host Strains: TG1 repair+ rk-mk- su2+	HB2154 repair- rk+mk+ su2-	HB2155 repair- rb+mb+ su2-
M13K19	SEL2	G48	7.5	58.5	-
M13B19	SEL2	G48	4.5	25.5	-
M13K19	SEL3	G48	5	-	42.5
M13B19	SEL3	G48	4.5	-	72
M13mp93amIV	SEL1	G48	3.5	61.5	-
M13mp93	SEL1	G48	3	30.5	-
M13K19	SEL2	G51	1.5	8	-
M13B19	SEL2	G51	1.5	2.5	-
M13K19	SEL2	G35	3.5	37	-
M13B19	SEL2	G35	6	18.5	-

A mutagenic primer (G35,G48 or G51) and a selection primer (SEL1,SEL2 or SEL3) were annealed to a M13 template (M13K19TyrTS, M13B19TyrTS, M13mp93TyrTS or M13mp93amIVTyrTS), extended and ligated for 17 hours, and used directly to transfect an E.coli host (TG1,HB2154 or HB2155).

Table 3: Mutiple Mutations

Template	Mutagenic Primer	Mutant Frequency in Host Strains: TG1 repair+ rk-mk-	HB2154 repair- rk+mk+
M13K19TyrTS	CO	1.0	5.5
M13B19TyrTS	CO	1.5	2.5
M13K19TyrTS	MET	4.5	33
M13B19TyrTS	MET	4.5	12

A mutagenic primer (CO or MET) and a selection primer (SEL 2) were annealed to a M13 template (M13K19TyrTS or M13B19TyrTS) extended and ligated for 17 hours and used directly to transfect an E.coli host (TG1 or HB2154).

containing a single unmodified EcoK site on TG1 (rk-mk-) compared with HB2151 (rk+mk+) is 30-100 fold. Similarly the difference in plaque yield of phage containing a single unmodified EcoB site on TG1 (rb-mb-) compared with AC2522 (rb+mb+) is also 30-100 fold. The difference in plaque yield is readily detected without accurate serial dilution, by streaking on a lawn of TG1 (rk-mk-,rb-mb-), and either HB2151 (rk+mk+) or AC2522 (rb+mb+) as appropriate.

RESULTS AND DISCUSSION

Coupled priming using an amber marker

Using the "coupled priming" approach with an amber marker in M13 gene IV, we have attempted to evaluate the individual effects of 5' editing of the oligonucleotide in vivo, of mismatch repair and of strand selection (Table 1). The selection primer (SEL1) lies to the 3' side of the mutagenic primer (G48) of the TyrTS gene. The G48 oligonucleotide has two mismatches with template: C/C, normally repaired with low efficiency and C/A, normally repaired with high efficiency whilst the selection primer has a single G/T mismatch with the template, normally repaired with high efficiency (6). Under the conditions of limited extension/ligation (4.5 hr) very little closed circular DNA is formed (checked on an alkaline sucrose gradient). Therefore the 5' end of the mutagenic oligonucleotide should be exposed in the transfected heteroduplex to any 5' editing in vivo. However in the longer extension/ligation (19hr), which was purified on an alkaline sucrose gradient, the 5' end should be ligated and protected against such editing.

The closed circular DNA gave a two fold improvement in mutant frequencies in several strains of E.coli (Table 1), suggesting that in this case, 5' editing of the mutagenic oligonucleotide may reduce mutant frequencies by a factor of two.

Eliminating point mismatch repair appears to increase mutant frequencies by 6-8 fold (comparing the repair- su2+ and repair+ su2+ strains in Table 1). The further effect of strand selection in the M13mp93amIVTyrTS vector is about 2 fold (comparing repair- su2+ and repair- su2- strains in Table 1). This agrees with Kramer et al. (6), but in this case we also find a strong effect of strand selection in the presence of mismatch repair (comparing the repair+ su2+ and repair+ su2- strains in Table 1). This may indicate that in this vector the mutagenic primer and the selection primer are on the same "repair tract", and are therefore repaired together or not at all.

Placing the mutagenic primer 5' to the selection primer exposes the 5' end to in vivo editing. Could this be avoided by placing the mutagenic primer to the 3' side of the selection primer? Perhaps surprisingly, constructing an amber mutation in M13 gene II, and utilising a selection primer 5' to the TyrTS gene, gave poor yields of mutants (not shown). Similar results were obtained with an EcoK or EcoB marker located to the 5' side of the mutagenic primer. This may be due to strand displacement of the mutagenic oligonucleotide by the in vitro extension from the selection primer.

The "coupled priming" technique using an amber marker has been used to construct 32 single and double mutations in the TyrTS gene using 16mer to 18mer oligonucleotides (HB, unpublished results). The frequency of mutations obtained was in the range 2% to 68% with 16% of mutations obtained at frequencies of >50% and 38% of mutations obtained at frequencies of 20% to 50%. Nine mutations involved the same double mismatch (T/G and C/C) and were obtained with frequencies in the range 2% to 39%. This variability in frequency may reflect the quality of the synthetic mutagenic primers or the specificity of priming at the target site (1,3), but not mismatch repair, as repair- strains were used and the primers have the same mismatches.

Coupled priming using an EcoK or EcoB marker

A drawback of using an amber marker is that after one round of mutagenesis the selectable marker is removed. This problem may be overcome by generating a second selectable marker at the same time as removing the first. In E.coli there is a type one restriction-modification system in both K strains (rk+mk+) and B strains (rb+mb+) (23,24). A number of features of

the EcoB and EcoK systems suggested that they might be exploited as a pair of reciprocating genetic markers for site-directed mutagenesis. If double stranded DNA containing an EcoK site is introduced into a K strain (rk+mk+), then the DNA will be modified if one strand is already modified, or it will be restricted if neither strand is modified (similarly for an EcoB site in a B strain). However hybrid sites containing the EcoK or EcoB site recognition sequence on one strand, but a mutationally altered sequence on the other strand are not recognized by the corresponding restriction or modification enzymes. A hybrid site may be devised allowing the recognition sequence of the EcoK (5'AAC N6 GTGC3') and EcoB (5'TGA N8 TGCT3') systems to be interconverted by a single base change (Fig.3). Thus site-directed mutagenesis, using an oligonucleotide with a single mismatch forms the basis for reciprocating between EcoK and EcoB sites.

First the EcoK and EcoB selection technique was compared with amber selection using the G48 primer to the 5' side of the selection primer (SEL1,SEL2, or SEL3) (Table 2). As in Table 1, there is a 6-8 fold improvement in using repair- cells, and a further 2-3 fold improvement with strand selection. Using the primers G35 (a 16mer with a single mismatch to the mutagenic template C/T) and G51 (a 16mer with two mismatches to the mutagenic template C/C and C/A) gave similar results (Table 2). The frequency of G35 and G51 mutations was increased 2-3 fold by using a repair- strain and a further 2-3 fold by using EcoK selection against the template strand. Thus strand selection, using either an amber mutation, or an EcoK or EcoB site gives a useful improvement in mutant frequency.

EcoK/EcoB reciprocating markers

For construction of mutants using EcoK/EcoB reciprocating markers, it is critical that the required silent mutant is tightly coupled to the new selection marker. However, phage may escape restriction not only by mutation of the selection marker but by methylation. In order to assess the contribution of methylation, colony blots were prepared in duplicate for the experiment in Table 2 and probed with either the mutagenic primer or the selection primer SEL2 or SEL3 as appropriate. Only 2-5% of the phage which hybridised strongly to the mutagenic primer (G48) did not hybridise to the selection primer. This shows that methylation is not a serious problem and that the selection marker and required mutant are 95% coupled. Practically it is easy to check whether any one mutant has the new marker by plating the phage on different host strains (see Materials and Methods).

As a test for cyclic selection, up to three rounds of mutagenesis have

been used to construct all possible single, double and triple mutations using the mutagenic primers G35,G48 and G51. After the first round of mutagenesis (Table 2), several clones which hybridised strongly to the mutagenic primers (G35,G48 and G51) were plaque purified. All the clones sequenced were demonstrated to have the required mutation at the target site by dideoxy sequencing (21) using one of a family of sequencing primers located at intervals throughout the TyrTS gene (22). Mutation of the EcoK selection site to an EcoB site was verified for the mutant phage by the reduced plaque yield on AC2522 (rb+mb+) compared with TG1 (rb-mb-).

The Gly35 and Gly51 single mutants were then used in a second round of mutagenesis to construct the double mutants Gly35Gly48, Gly35Gly51 and Gly48Gly51 using EcoB selection. Several hybridisation positive clones were then sequenced to verify the mutations. Mutation of the EcoB selection site to an EcoK site was verified for the mutant phage by the reduced phage yield on HB2151 (rk+mk+) compared with TG1 (rk-mk-). Finally a third round of mutagenesis was invoked to construct the triple mutant Gly35Gly48Gly51 from the double mutant Gly35Gly51 using EcoK selection. Clearly by reciprocating between the two selectable markers it is possible to construct multiple mutations, imposing a strand selection in each round.

Alternative strategies for making multiple mutations

Instead of constructing mutations stepwise in multiple rounds of mutagenesis, it would be ideal if several mutants could be made in a single round by priming with several mutagenic oligonucleotides at the same time. In preliminary experiments, we have found that the frequency of a double mutant is no higher than the product of the frequencies of the two single mutants. However, for constructing multiple mutations within a limited region, long synthetic oligonucleotides can be used successfully. The oligonucleotides CO (38mer) and MET (44mer) have multiple mismatches (11 and 13 respectively) to the mutagenic template to direct many simultaneous amino acid changes (6 and 7 respectively) in the tyrosyl-tRNA synthetase enzyme (Fig. 4).

The frequency of phage hybridising strongly to the two mutagenic oligonucleotides was low (<5%) using a repair+ host. This frequency was improved 2-3 fold by using a repair- host strain without selection and a further 2-3 fold by using a repair- host strain and EcoK selection against the template strand (Table 3).

Several clones which hybridised strongly to the mutagenic oligonucleotides (MET and CO) were plaque purified and then sequenced. 5 out

of 6 clones had the required MET mutation and 2 out of 6 clones had the CO mutation. The other clones included a number of deletions. The kinetic properties of these mutant tyrosyl-tRNA synthetase enzymes prepared from these mutant phage will be presented elsewhere.

CONCLUSION

This paper presents a reliable and exceedingly simple method for the construction of oligonucleotide directed mutations at frequencies of up to 70%. This "coupled priming" technique relies upon linking the required "silent" mutation to a selectable marker using host strains deficient in point mismatch repair. Several rounds of mutagenesis can be undertaken by cycling between EcoK and EcoB markers. It should be possible to extend the EcoK and EcoB cyclic selection system to the "gapped duplex" method (7) or to enable facile plasmid mutagenesis (25).

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