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Chromogenic identification of oligonucleotide-directed mutants

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ABSTRACT

We describe a simple plaque color assay for identifying oligonucleotide-directed mutations in cloned DNA fragments. The basis of the method is to: 1) fuse the sequence of interest in-frame to the *E.coli lacZ* gene to produce a blue plaque phage, 2) mutate the site of interest to a stop codon to generate a white plaque phage, and 3) revert the stop codon and surrounding nucleotides to give a blue plaque phage containing one or more desired amino acid changes. The advantages of this cyclic method are that it produces truncated as well as amino acid substituted protein molecules, it can be repeated to introduce additional mutations, and it eliminates the need for labor intensive screening. Essentially any piece of DNA can be mutated using this method if the fragment has one open reading frame. If there is an open reading frame between the site and the *lacZ* gene, ATG codons can be inserted at the target site. We have used this method to produce termination and amino acid substitution mutants in the yeast *CUP1* gene.

INTRODUCTION

Oligonucleotide site-directed mutagenesis is a powerful technique for studying structure-function relationships in DNA , RNA and protein molecules. It requires a synthetic oligonucleotide exactly complementary to the sequence of interest except for a mismatch that directs the change. The oligonucleotide is extended with DNA polymerase and sealed with DNA ligase *in vitro*. The duplex molecules are transformed into an *E.coli* host and the mutant identified. A limitation of the technique is the low frequency of production of DNA molecules with the desired base change relative to the background of molecules containing the unaltered sequence. This is presumably due to the inefficiency of the extension reaction *in vitro* and to mismatch repair processes in the host bacterium (1). Many techniques have been used to enrich for mutant molecules and thus mimimize laborious screening. These include the isolation of closed circular duplex DNA on alkaline sucrose gradients (2), insertion of a genetic marker on the template strand so that it is selected against (3,4), and incorporation of uracil into the template so that it may be eliminated by treatment with uracil glycosylase (5).

Here we present a convenient plate assay that does not enrich for mutant molecules but instead allows easy identification of clones containing the desired basepair changes. The technique requires making a fusion protein to the *lacZ* gene on an M13 vector; this results in β-galactosidase activity, producing a blue plaque phenotype when the DNA is transfected into an *E.coli* host and

plated on media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). An oligonucleotide, designed to introduce a termination codon into the cloned fragment at the target site, is then annealed to the DNA, elongated *in vitro* by DNA polymerase I, sealed with DNA ligase, and transfected into competent cells. Phage containing this termination codon will not produce the α-complementing amino domain of β-galactosidase and therefore be visible as white plaques against a background of parental blue plaques. Next, the M13 DNA containing the termination codon is used as a template for an oligonucleotide which has been designed to convert the termination codon to a codon with the desired base change, again producing a functional α-complementing domain of β-galactosidase. After DNA elongation, ligation and transfection, these phage will produce plaques which are blue against the background of parental white plaques. Therefore, in two cycles of mutagenesis reactions and plating, the desired clone is produced, eliminating the need to enrich for mutagenized molecules or to do extensive screening of plaques to find those containing the desired base pair changes. This blue/white cycling can be repeated indefinitely to introduce any number of desired base changes. Another use of the method is that truncated protein molecules are generated when a protein coding sequence is cloned in-frame with the *lacZ* gene and a stop codon is introduced.

We have chosen to apply this plaque phenotype assay to mutagenesis of the *CUP1* gene of the yeast, *Saccharomyces cerevisiae*. The *CUP1* gene product is a small protein, belonging to the metallothionein family, that binds eight copper atoms through its twelve cysteine residues (6). As a means of determining the function of the individual cysteine residues in metal binding we produced truncated proteins and proteins in which neighboring cysteine residues, corresponding to positions 17 and 19, 32 and 34, 44 and 46, and 57 and 58, were converted to serine residues in pairwise combinations. We developed the plaque phenotype assay as a means of rapidly screening for both truncations and amino acid substitutions of the *CUP1* gene product.

MATERIALS AND METHODS

Strains and Vectors

E.coli strain JM101 (7) was used as the recipient in all transfection experiments. The *CUP1* coding and flanking sequences were originally cloned as an approximately 1.1 kb Bam H1 fragment into M13mp8. The insert was subjected to sequence analysis and was identical to that of Karin et al. (8). To create a smaller fragment containing the *CUP1* gene, we created Sst II restriction sites flanking the gene. This was done by annealing oligonucleotides 8 and 9 (Table 1) simultaneously to the *CUP1* -mp8 template and performing extension and ligation reactions as described below. The closed circular DNA was isolated on an alkaline sucrose gradient as described by Winter et al. (9), then neutralized with an equal volume of 1 M Tris-HCl, pH 7.5, and dialyzed against 10 mM Tris-HCl, pH 8, 1 mM EDTA overnight. Clones resulting from transfection were screened by restriction analysis of duplex phage DNA for the Sst II site introduced by each of the oligonucleotides. Of 16 clones examined, 2 had the desired Sst II

fragment. The Sst II fragment from one of these clones was then cloned into M13mp10sstII. M13mp10sstII was constructed in three steps: 1. A deletion between the NarI-EcoRI sites of M13mp10 was made to create mp10delta. 2. The lac203 EcoR1 fragment containing the Plac L8UV5 promoter and first eight codons of *lacZ* (10) was inserted into the EcoRI site of mp10delta to make mp10uv1. 3. The synthetic oligonucleotide 5' GCCGCGGC 3' was inserted into the SmaI site of mp10uv1 to make M13mp10sstII. The oligonucleotide restores the *lacZ* translation phasing across the polylinker and introduces a unique SstII cloning site. The orientation of the *CUP1* SstII insert was determined by sequence analysis. The *CUP1* coding region contained within this Sst II fragment lies in-frame with the portion of the *lacZ* gene 3' to the polylinker region (Figure 1). DNA from this clone was used as the template for all subsequent steps of the mutagenesis procedure.

The larger Bam H1 fragment from one of the *CUP1* -mp8 clones containing the double Sst II sites was subcloned into YEp13, a yeast expression vector containing the yeast *LEU2* gene and 2 μ

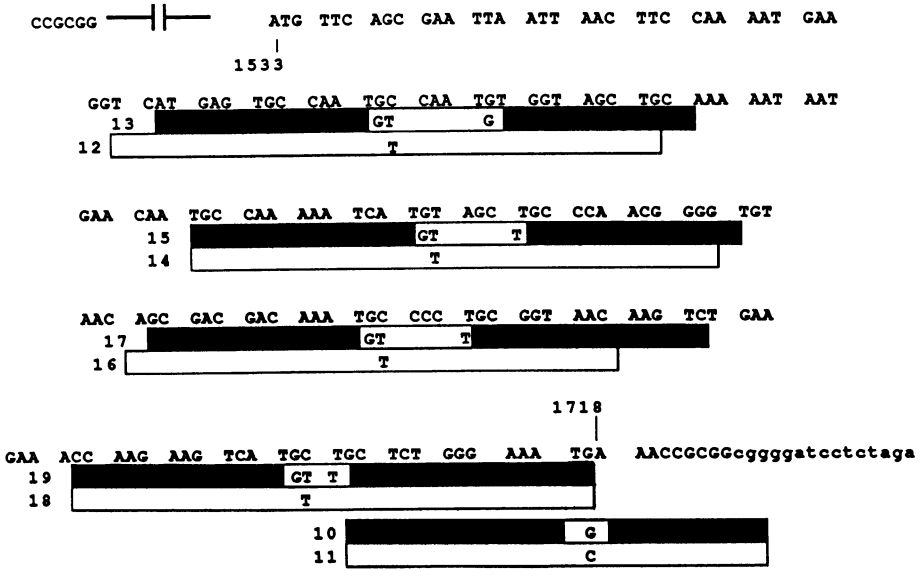

Figure 1. Shown above is the coding sequence for the CUP1 gene, numbering of the coding sequence is as determined by Karin et al. (8). Upper case letters designate bases which code for CUP1 or bases in the Sst II linker. Lower case letters designate bases in the M13mp10 polylinker region. Translation proceeds across the polylinker into the lacZ gene. The interrupted line represents the 5' noncoding region of CUP1 which is cloned into the M13 vector. Black boxes represent oligonucleotides which create readthrough codons, white boxes represent oligonucleotides which create stop codons. The bases inside the boxes represent the bases of the oligonucleotide which mispair with the wildtype CUP1 sequence. The numerical designation for each oligonucleotide appears to the left of the boxed region.

origin of replication (11). The resulting plasmid was designated YEp3362xSst. In order to create a cassette into which the Sst II fragments from the various mutants could be inserted, YEp 3362xSst was digested with Sst II, ligated, and transformed into *E. coli* strain MC1061 (12). DNA from the transformants was screened by restriction analysis for those which had lost the Sst II fragment. The resulting plasmid was designated YEp336ΔSst.

Oligonucleotides

Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method on an Applied Biosystems model 380, ABI, Foster City, Ca. Crude oligonucleotides were purified by electrophoresis of approximately 300 μg on a denaturing polyacrylamide gel, excision of the gel slice containing the longest extension product (visualized with uv shadowing), and elution of the DNA by dialyzing the gel slice against water overnight. The sequence of all oligonucleotides used is shown in Table 1. Oligonucleotides were phosphorylated before use in the annealing reactions as follows: 100 pmol of oligonucleotide was incubated with 7.5 U of polynucleotide kinase in 30 μl of 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 500 mM ATP at 37°C for 50 minutes and then heated to 70°C for 10 minutes.

Annealing, Extension and Ligation Reactions

The annealing of the oligonucleotide to the template and the extension and ligation reaction were performed essentially as described by Winter et al. (9) except that the annealing reaction was done in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ at 70°C for five minutes before cooling to room temperature. The extension reactions were usually incubated for 16 hours at 16°C, however, incubation for 3 hours was sufficient to obtain mutant clones. The plating efficiency with a 3 hour incubation is 1-10% that with a 16 hour incubation. The extension products were then diluted into TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA); typically 1 μl of a 1:100 and a 1:1000 dilution were transfected into JM101. In order to assure that the DNA isolated from a plaque was homogeneous, plaques from these platings were picked and the phage grown as below. These phage were either used directly to re-infect JM101 or single-stranded DNA was made from the phage and used to transfect JM101. Plaques from these subsequent platings were assumed to be homogeneous.

Transfections and Phage Growth

Transfections were performed with $CaCl_2$ treated cells (7). Cells were plated by adding 10 μl 100 mM IPTG (isopropyl-β-D-thiogalactoside), 3 ml Luria (LB) top agar, 50 μl 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethyl formamide, and 150 μl log phase JM101 to a tube containing the DNA and competent cells and then pouring onto a fresh LB plate preincubated at 37 °C. Phage were grown by stabbing plaques with a toothpick, then incubating the toothpick with 1.5 ml of log phase JM101 in LB at 37° C for 5 hours with vigorous shaking. After incubation, cultures were centrifuged at 12,000 x g in 1.5 ml centrifuge tubes for 10 minutes. Single-stranded phage DNA was prepared from the supernatant fraction (13) and double-stranded DNA prepared from the cell pellet by the quick alkaline method as described by

Maniatis et al. (14). To be used as a template, the single-stranded DNA was concentrated ten-fold by ethanol precipitation to a final concentration of about 400 μg/ml.

DNA Sequencing

The sequence of DNA was determined by the dideoxynucleotide chain termination method of Sanger et al. (15) as modified by Biggin et al (16).

Reagents

Restriction enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, Inc., and International Biotechnologies, Inc.; T4 DNA ligase was from either New England Biolabs, Inc. or Bethesda Research Laboratories; the Klenow fragment of DNA polymerase I was from Bethesda Research Laboratories; polynucleotide kinase was from PL Biochemicals. Deoxy- and dideoxynucleoside triphosphates were purchased from PL Biochemicals. ^{35}S-dATP (>600 Ci/mmol) was purchased from Amersham.

RESULTS

CUP1-lacZ Fusion

The first step in mutagenizing the *CUP1* gene was to construct a *CUP1-lacZ* fusion on an M13 vector such that translation of the *CUP1* gene was in phase with the *lacZ* gene. This was done by cloning a Sst II DNA restriction fragment containing the *CUP1* gene (386 bp; 198 bp upstream of the ATG and 3 bp after the TGA termination codon) into M13mp10sstII. Several recombinant phage were isolated and the *CUP1* insert determined to be in the same orientation as the *lacZ* gene by sequence analysis using an M13 universal sequencing primer. Because this clone contains part of the 5' nontranscribed sequence of the *CUP1* gene, which contains translational stop codons in all three reading frames, expression of the *lacZ* gene in this construction depends on translational reinitiation at the *CUP1* ATG. A *CUP1-lacZ* fusion protein capable of promoting β-galactosidase α-complementation in *E.coli* was produced by changing the *CUP1* TGA stop codon to a readthrough TCA (serine) codon. This was done by annealing and extending oligonucleotide 10 (Table 1) as described in the methods section, transfecting dilutions of the reaction mixture into *E.coli* host JM101 and plating on LB agar plates with X-gal indicator. After overnight incubation at 37 °C, the two plaque phenotypes observed were white and deep blue. Deep blue plaques were observed at a frequency of less than 0.1% of the total number of plaques and were observed at a similar frequency in control annealing-extension reactions that contained no oligonucleotide. Overnight incubation of the plates at 4°C revealed a third plaque phenotype, pale blue, which was present at approximately 10% of the total. Deep blue and pale blue plaques were isolated and sequenced. All pale blue plaques contained the oligonucleotide 10 directed mutation while the deep blue plaques contained rearrangements of the *CUP1-lacZ* template. From this result and the DNA sequence data, it is clear that one can isolate deep blue plaques at very low frequencies that are rearrangements of the M13 template and not the desired products of the oligonucleotide-directed experiments. The pale blue plaque phenotype presumably results from the

TABLE 1. Sequence of oligonucleotides used. The number in parentheses refers to the length of the oligonucleotide. Bases which are underlined create mismatches with the template.

OLIGONUCLEOTIDE	SEQUENCE
8 (32)	5' GAATATATTAAAGACCCGCGGTTTCATTTCCC 3'
9 (30)	5' TGCTGGAACGGTTCCGCGGAAAAGACGCAT 3'
10 (26)	5' CCCGCCGCGGTTTGATTTCCCAGAGC 3'
11 (26)	5' CCCGCCGCGGTTTCATTTCCCAGAGC 3'
12 (30)	5' CAGCTACCACATTGTCATTGGCACTCATGA 3'
13 (30)	5' TGCAGCTACCAGATTGTGATTGGCACTCAT 3'
14 (30)	5' CCCCGTTGGGCAGCTTCATGATTTTTGGCA 3'
15 (31)	5' ACCCCGTTGGGCTGCTTGATGATTTTTGGCA 3'
16 (27)	5' GTTACCGCAGGGTCATTTGTCGTCGCT 3'
17 (30)	5' ACTTGTTACCGCTGGGTGATTTGTCGTCGC 3'
18 (28)	5' GATTTCCCAGAGCATCATGACTTCTTGG 3'
19 (28)	5' GATTTCCCAGAGCTTGATGACTTCTTGG 3'

inefficiency of the fusion gene in providing β-galactosidase α-complementation (see discussion). The reason for the slow production of pale blue color in this experiment is not known, but this problem did not re-occur. In this regard, it has been found that plating the reaction mixtures on minimal plates or on plates lacking glucose (7) can produce plaques with a deeper blue color.

Introduction of Stop Codons in the *CUP1* Gene

The next step of the mutagenesis strategy was to introduce termination codons into the *CUP1* gene at various positions. This produced the templates used in the subsequent steps of the mutagenesis as well as a set of carboxy-terminal truncated *CUP1* proteins. In four separate reactions oligonucleotides 12, 14, 16 and 18 (Table 1) were annealed to the *CUP1-lacZ* template and extension and ligation reactions carried out. The mismatches created by these oligonucleotides create TGA stop codons at positions 17, 32, 44 and 57 of the *CUP1* protein, respectively. White plaques were obtained after transfection of the reaction mixtures at a frequency ranging from 0.6% to 11.6% (Table 2) and were easily distinguishable from the pale blue plaques produced by the template. To determine the frequency of spontaneous white plaques, the template alone was diluted and plated. No white plaques were observed on any of the plates; however, on one plate, with approximately 2000 pale blue plaques, two deep blue plaques were visible, presumably rearrangements as described above. From the oligonucleotide primed plates,

TABLE 2. Primer-directed frequency of mutagenesis.

template:oligo[2]	mismatch(es) template:oligo	plaque phenotype[1] blue/white	percentage mutant plaques
wildtype: 12[2]	C:T	38/5	11.6
wildtype: 14	T:T	1600/10	0.6
wildtype: 16	C:T	1360/21	1.5
wildtype: 18	C:T	192/11	5.4
CUP1-12: 13	G:G/G:G	6/200	3.0
CUP1-14: 15	G:G/T:T	10/300	3.3
CUP1-16: 17	G:G/T:T	13/200	6.5
CUP1-18: 19	G:G/T:T	16/250	6.4
CUP1-13: 11	C:C	1200/200	14.3
CUP1-15: 11	C:C	1440/344	19.3
CUP1-17: 11	C:C	800/112	12.2
CUP1-19: 11	C:C	696/88	11.2

[1]Numbers resulting from plating appropriate dilutions of extension/ligation reactions.
[2]Sequences of oligonucleotides appear in Table 1.

white plaques were picked, single-stranded DNA isolated, and the entire *CUP1* gene from each clone was sequenced. For oligonucleotide 12 primed template, 5 white plaques were picked for sequencing. Four had the correct stop codon substitution and one had the correct substitution as well as a base insertion and deletion in the sequence complementary to oligonucleotide 12. For oligonucleotide 14 primed template, DNA from 2 white plaques was sequenced with one having the correct stop codon substitution and the other a rearrangement. For oligonucleotides 16 and 18 primed templates, DNA from 2 white plaques from each was sequenced and all had the correct stop codon substitutions. A clone with the correct sequence from each was picked, plaque purified, and designated *CUP1*-12, *CUP1*-14, *CUP1*-16, *CUP1*-18, respectively.

In order to determine the effect of these truncations on the ability of the *CUP1* protein to detoxify copper, the Sst II fragments containing the *CUP1* coding sequence from each of the above clones were sub-cloned into YEp336ΔSst, a yeast expression vector containing pBR322 sequences, the yeast *LEU2* gene, and the yeast 2 μ circle origin of replication (see Materials and Methods). The resulting plasmids, designated 336/12, 336/14, 336/16, and 336/18, were transformed independently into 55.6B, a *leu2* yeast strain which lacks the *CUP1* gene and is

TABLE 3. Growth of yeast strains transformed with plasmids carrying various truncations of the CUP1 gene on media containing copper. Symbol designations are as follows: + indicates good growth; +/- indicates moderate growth; - indicates no growth.

Strain	0	0.025	0.075	0.1	0.2	0.5	2.5	4	mM$CuSO_4$
55.6B	+	-	-	-	-	-	-	-	
55.6B(336/12)[1]	+	+	-	-	-	-	-	-	
55.6B(336/14)	+	+	-	-	-	-	-	-	
55.6B(336/16)	+	+	+	+	+	+/-	-	-	
55.6B(336/18)	+	+	+	+	+	+	+/-	+/-	
55.6B(YEp336 2xSst)	+	+	+	+	+	+	+/-	+/-	

[1]Names in parentheses indicate the plasmid with which the recipient strain, 55.6B, has been transformed.

extremely copper sensitive (17). Two transformants from each truncation were then streaked onto a series of plates (17) containing increasing amounts of copper. These results, shown in Table 3, clearly demonstrate the correlation between the length of the *CUP1* protein and the copper resistance of the yeast transformants. It is interesting to note that the shortest truncation, which contains only a single cysteine residue, is still capable of conferring some copper resistance, at least contained on this high copy number plasmid.

Serine Substitutions and Neighbor Conversion

DNA from the phage which contained termination codons at different positions in the *CUP1* gene was used as the template for the next step of the mutagenesis. Since our goal was to introduce codon substitutions which would convert neighboring cysteine to serine codons, we designed oligonucleotides which would simultaneously convert the termination codon and a neighboring cysteine codon into serine codons. To this end, DNA from each of the stop mutants *CUP1* (12-18) was annealed to oligonucleotides 13, 15, 17 and 19, respectively, the oligonucleotides were extended and ligated and the DNA used for transformation. The results are shown in Table 2, this time monitoring for the appearance of blue plaques. The percentage of white to pale blue plaque phenotypes observed in these double base changes varied from 3 to 6 depending on the oligonucleotide used. DNA from 5 pale blue plaques from *CUP1*-12/oligonucleotide 13 were subjected to sequence analysis and all had the expected double change. Likewise, DNA from 6 pale blue plaques of *CUP1*-14/oligonucleotide 15 was analyzed and again all had the expected double change. Clones of *CUP1*-16/oligonucleotide 17 and *CUP1*-18/oligonucleotide 19 were not sequenced at this point. All of these double codon substitution mutants were plaque purified and designated *CUP1*-13, *CUP1*-15, *CUP1*-17, and *CUP1*-19. These sequence results illustrate the

usefulness of the pale blue vs. deep blue phenotype in identifying plaques containing the mutation of interest. Of eleven white plaques analyzed, two had undergone rearrangements, however, all of the pale blue plaques which were analyzed contained only the desired basepair changes.

Restoration of the *CUP1* TGA Codon

Before transferring each of the double codon substitution mutants into a yeast shuttle vector, it was neccesary to restore the natural *CUP1* TGA stop codon. *CUP1* (13-19) templates were annealed to oligonucleotide 11 to restore the *CUP1* TGA stop codon which changes the plaque phenotype again from blue to white. The frequency of this converison is shown in Table 2. One white plaque from each reaction was picked, purified, and the entire insert subjected to sequence analysis. All contained the desired cysteine to serine changes and, in addition, the original termination codon of the *CUP1* gene with no additional mutations.

DISCUSSION

Oligonucleotide-directed mutagenesis is a revolutionary tool to study the structure and function of DNA, RNA, and proteins. One can precisely insert, delete, or substitute any number of bases in any DNA sequence. All that is required is: 1.) the availability of the DNA of interest, 2.) a synthetic oligonucleotide with the desired change, and 3.) a method to identify the mutated DNA molecule.

In this paper we demonstrate the feasibility of using plaque phenotype to identify oligonucleotide directed substitutions as an alternative to other methods. We have used this system to mutagenize the yeast *CUP1* gene, which codes for a cysteine-rich protein that confers heavy metal resistance to yeast. The technique is a two-step process generating carboxy-terminal deletion mutants as intermediates to making codon substitutions. The efficiency of this technique results from the fact that mutants are identified by plaque color, thereby eliminating nearly all labor intensive procedures normally required for enriching and detecting oligonucleo tide-directed mutations.

There are several real and potential difficulties with the system as we have described it. Perhaps the most apparent problem is the tendancy of M13 to spontaneously rearrange generating plaque phenotypes similar to those generated by the mutagenic oligonucleotide. However, because of the low frequency of this event (<0.1%) relative to the average oligonucleotide-dependent plaque phenotype change (1-15%), and also because the pale blue plaque phenotype observed with many *lacZ* fusions is distinct from dark blue plaques of rearranged molecules, this becomes a trivial problem. We have observed the pale blue phenotype in a number of different clones. One example is a simple insertion into M13mp8 of a 189 bp fragment from phage lambda that contains a transcription termination site with an open reading frame through the insert. This suggests that the *lacZ* α-complementation activity is decreased resulting in the pale blue phenotype. Another example is a substitution of the *lacZ* ribosome binding site and first 5 codons with the ribosome binding site and start codon of the E.coli *galK* gene fused to codon 6 of *lacZ*. This suggests that the *galK* ribosome binding site is a poor site or that the first 5 codons of *lacZ* are important for the

deep blue phenotype. Either possibility is consistant with the *CUP1-lacZ* fusion activity and we have no data to support one or the other. From our experience we suggest that many gene fusions in M13 vectors will produce a pale blue phenotype if the fusion substitutes for the *lacZ* start and first 5 codons or if the fusion introduces a large number of codons (greater than 30) into *lacZ* at the polylinker. Another potential shortcoming of the method might arise if the *lacZ* gene fusion had internal ATG codons in a sequence context that *E.coli* ribosomes recognize to initiate translation. Since the *CUP1* gene has no internal ATG codons, we have not addressed this problem. Perhaps one could use DNA fragments coding for smaller regions of a gene and elimate many if not all internal ATG codons.

Another potential problem, common to all methods for oligonucleotide-directed mutagenesis, is the introduction of sequence changes other than the desired change. The possibility for this increases if one uses *E.coli* hosts that have mutator genes (3,5). It is essential that all DNA subjected to mutagenesis be sequenced completely before conclusions are drawn regarding the effect of any particular mutant. Our solution to this has been to mutagenize relatively small DNA fragments (<1 Kbp), to sequence the DNA completely, and then to transfer the fragment to another vector that has not been exposed to mutagenesis.

Development of a suitable fusion protein may require a bit of manipulation to position the target DNA with an open reading frame in the same phase as the *lacZ* gene. To eliminate some of these difficulties we have constructed two M13 vectors that in conjuction with currently available M13 vectors have *lacZ* translation crossing the M13 polylinker site in all three reading frames. One of these vectors, mp10uv1 is described in materials and methods. The other vector, mp8uv1 was constructed in the same way except that it used M13mp8 as the starting vector. M13mp10 has translation crossing the SmaI site of the polylinker [CCC][GGG], while mp10uv1 has translation crossing the site CC[CGG]G. mp8uv1 has translation crossing the site C[CCG]GG. In conjunction with other M13 vectors one can mix sequences upstream and downstream of the polylinker to make a large set of restriction sites with the desired translation phasing.

The technique described here requires that a stop codon be substituted at or adjacent to the target codon. Since several codon changes can be accomplished with one oligonucleotide, the stop codon need not be the same as the target codon. One can use an oligonucleotide to change a stop codon back to the original codon and convert a neighboring codon in one step. In this regard, we suggest that the system does not require two oligonucleotides to change a specific codon. It is possible to design oligonucleotides that have a two base degeneracy at one positon, creating both a stop and the desired codon. In cases where this is not possible (eg. creating GTG) one can use neighboring codons to create stops and restore the normal codon using the same oligonucleotide, while changing the target codon to the desired sequence.

In summary, we have shown that cycling between stop and readthrough codons in oligonucleotide-directed mutagenesis produces altered plaque phenotypes which greatly facilitate the identification of molecules carrying the mutation of interest.

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