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Genetics

Rapid and efficient site-specific mutagenesis without phenotypic selection

(M13 cloning vectors/silent mutations/*in vitro* mutagenesis/synthetic oligonucleotides/uracil-containing DNA)

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ABSTRACT **Several single-base substitution mutations have been introduced into the $lacZ_\alpha$ gene in cloning vector M13mp2, at 40–60% efficiency, in a rapid procedure requiring only transfection of the unfractionated products of standard *in vitro* mutagenesis reactions. Two simple additional treatments of the DNA, before transfection, produce a site-specific mutation frequency approaching 100%. The approach is applicable to phenotypically silent mutations in addition to those that can be selected. The high efficiency, ≈10-fold greater than that observed using current methods without enrichment procedures, is obtained by using a DNA template containing several uracil residues in place of thymine. This template has normal coding potential for the *in vitro* reactions typical of site-directed mutagenesis protocols but is not biologically active upon transfection into a wild-type (i.e., ung^+) *Escherichia coli* host cell. Expression of the desired change, present in the newly synthesized non-uracil-containing covalently closed circular complementary strand, is thus strongly favored. The procedure has been applied to mutations introduced via both oligonucleotides and error-prone polymerization. In addition to its utility in changing DNA sequences, this approach can potentially be used to examine the biological consequences of specific lesions placed at defined positions within a gene.**

Procedures for site-specific mutagenesis have become increasingly important for the analysis of gene functions, and many approaches are now available for changing primary DNA sequences (1–3). When sequence changes produce silent, unknown and/or non-selectable phenotypes, it is particularly advantageous to increase the frequency of production of the desired change relative to the unaltered sequence. The low frequencies that are commonly observed apparently result from the sometimes inefficient *in vitro* reactions needed to incorporate the mutation into a biologically active form and from heteroduplex expression phenomena favoring the genotype of the original starting sequence. Several biochemical and genetic approaches have been developed to overcome these limitations (4–11), each having its own advantages but requiring additional, and often time-consuming, steps.

An alternative is presented here that takes advantage of a strong biological selection against the original, unaltered genotype. By using a relatively normal uracil-containing DNA template, prepared by standard procedures (12) after growth on an *Escherichia coli* dut^- ung^- strain (13), several site-specific mutagenesis procedures have been used to produce mutations at high efficiencies without selection and in a few hours. The technique has been applied here to the $lacZ_\alpha$ coding sequence in M13mp2 DNA (14) to create several single base changes required for continuing studies of mutational specificity (15).

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MATERIALS AND METHODS

Phage and Bacterial Strains. The bacterial strains for transfection were *E. coli* NR8051 [Δ(*pro-lac*), thi^-] and NR8052 [Δ(*pro-lac*), thi^-, *trpE9777*, *ung*-1]. *E. coli* strain BW313 (*dut*, *ung*, *thi*-1, *rel*A, *spo*T1/F'*lys*A) was obtained from D. Sagher and B. Strauss (University of Chicago). *E. coli* CSH50 [Δ(*pro-lac*), *ara*, *thi*/F'*tra*D36, *pro*AB, *lac*I^qZΔM15] and bacteriophage M13mp2 were from J. E. LeClerc (University of Rochester). The M13mp2 mutant containing a G-C → C-G transversion at position +82 was from the collection of mutants described previously (15).

Enzymes and Chemicals. Homogeneous *E. coli* uracil glycosylase and dUTPase were gifts from B. Duncan (Institute for Cancer Research, Philadelphia, PA) and L. Bertsch and A. Kornberg (Stanford University), respectively. *E. coli* DNA polymerase I, large fragment, was from Bethesda Research Laboratories. T4 DNA polymerase and T4 polynucleotide kinase were from P-L Biochemicals. T4 DNA ligase and restriction endonuclease *Pvu* II were from New England Biolabs. Avian myeloblastosis virus (AMV) reverse transcriptase was from Life Sciences (St. Petersburg, FL). Deoxynucleoside triphosphates (HPLC grade) were from P-L Biochemicals. All other chemicals were of reagent grade.

Preparation of Uracil-Containing Template. M13mp2 phage containing uracil in the DNA were prepared as described (12). BW313 cells were grown at 37°C with vigorous shaking in YT medium (yeast extract, 5 g/liter; tryptone, 8 g/liter; NaCl, 5 g/liter) supplemented with 20 μg of thymidine per ml and 100 μg of deoxyadenosine per ml. At a cell density of 4×10^8 per ml, the cells were centrifuged (15 min at 2000 × *g*), washed with YT medium, resuspended in fresh YT medium prewarmed to 37°C and containing 0.25 μg of uridine per ml, and shaken vigorously for 5 min. M13mp2 phage were then added at a multiplicity of infection of 5. Incubation at 37°C was continued overnight. After a single cycle of growth on BW313, relative phage survival was decreased to 0.1% when compared on an ung^- (BW313) versus an ung^+ (CSH50) host. Phage obtained from this multiplication were used for a second cycle of growth, identical to the first, but producing phage exhibiting the relative survival values shown in Table 1 (experiment 1). The culture was centrifuged at 5000 × *g* and the phage (≈10^{11} per ml) were precipitated from the supernatant with 3% polyethylene glycol (PEG 8000)/0.5 M NaCl. After centrifugation at 5000 × *g* for 15 min, the phage pellet was resuspended in 50 mM Tris·HCl, pH 8.0/1 mM EDTA/100 mM NaCl, then phenol extracted twice, chloroform/isoamyl alcohol (24:1) extracted twice, ethanol precipitated, and resuspended in 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA.

Uracil Glycosylase Treatment. Treatment of either normal or uracil-containing M13mp2 DNA was performed in a 25-μl reaction mixture containing 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, 4 μg of M13mp2 single-stranded DNA (10^{12} mole-

Abbreviation: AMV, avian myeloblastosis virus.

cules), and either buffer only or 100 ng (2.5×10^{12} molecules) of highly purified *E. coli* uracil glycosylase (16). Incubation was at 37°C for 30 min. Reaction mixtures were then placed on ice and 1 μg each of the control and glycosylase-treated DNAs were used for transfection of competent cells, prepared as described (15), made from *E. coli* NR8051 (ung^+) or NR8052 (ung^-).

dUTPase Treatment of Substrates. Deoxynucleoside triphosphates were treated with highly purified dUTPase (17) in a 50-μl reaction mixture containing 5 mM (each) dATP, dTTP, dCTP, and dGTP, 30 mM Hepes (pH 7.8), and 800 units of dUTPase. Incubation was for 20 min at 22°C, after which the dNTP substrates were used directly for the *in vitro* DNA synthesis reaction described in the legend to Table 2.

DNA polymerase reactions were performed as described in the legends to the tables; transfections, plating, and chain-terminator DNA sequence analyses were performed as described (15).

RESULTS

The Approach. Single-stranded viral DNA is prepared from phage grown in an *E. coli* dut^- ung^- strain. This strain is deficient in the enzyme dUTPase, the product of the *dut* gene, resulting in an increased intracellular pool of dUTP, which competes with TTP for incorporation into DNA (18). Uracil thus incorporated is not removed due to the deficiency in the product of the *ung* gene, uracil glycosylase (19). The M13mp2 template DNA purified from the phage thus contains 20–30 uracil residues per genome (12). This DNA is biologically active in a strain incapable of removing the uracil (Table 1, ung^- host). Furthermore, this DNA has nearly normal template coding potential, having only a 2-fold higher mutation frequency than templates prepared on a wild-type *E. coli* strain, when compared for loss of α-complementation (14, 15) on an ung^- host (legend to Table 1). This is consistent with the expectation that uracil both replaces and codes like thymine. Such a template can, however, be biologically inactivated through the action of uracil glycosylase (16), which removes uracil bases, creating abasic sites. Such sites are lethal in single-stranded DNA (15, 20, 21). This is detected as decreased survival, by 5 orders of magnitude, when comparing biological activity of uracil-containing phage on an ung^- versus ung^+ host (Table 1, experiment 1). The same phenomenon is observed when DNA extracted from the virions is used to transfect competent cells (Table 1, experiment 2). That this is indeed due to uracil in the template is shown by the decreased survival of uracil-containing DNA, even in an ung^- host, if this DNA is treated before transfection with the highly specific purified uracil glycosylase (16) to remove the uracil residues (Table 1, experiment 2). Thus, when a uracil-containing template is applied to a standard site-specific mutagenesis protocol, the DNA newly synthesized *in vitro*, containing the desired mutation but no uracil, should have a strong selective advantage over the template containing the unaltered genotype.

Table 1. Survival of uracil-containing M13mp2 phage and DNA in ung^- and wild-type *E. coli* cells

DNA content	Titer: ung^- host	Titer: ung^+ host	Survival, %
Experiment 1: Intact phage infection			
Wild type	1.7×10^{11}	1.3×10^{11}	76.0
Uracil-containing	1.8×10^{12}	1.1×10^{7}	0.0006
Experiment 2: DNA transfection			
Wild type	3.0×10^{4}	2.7×10^{4}	90.0
Uracil-containing	2.0×10^{4}	0	<0.005
Wild type + glycosylase	2.8×10^{4}	2.6×10^{4}	93.0
Uracil-containing + glycosylase	0	0	<0.005*

*This relative survival is calculated by using the titer obtained with untreated uracil-containing DNA in the ung^- host (2.0×10^4). The mutation frequency (for loss of α-complementation) was 6.5×10^{-4} for wild-type M13mp2 DNA and 12.0×10^{-4} for uracil-containing M13mp2 DNA, when transfected into competent ung^- cells and plated as described (15).

Production of a Missense Mutation. The experimental details have been established first by using a selectable missense mutation in the coding sequence for the β-galactosidase α-peptide present in M13mp2 DNA.

The results obtained by using a uracil-containing template to change a G (viral strand) to a C at position +82 are shown in Table 2. In this instance, codon 14 is changed from arginine to proline, resulting in an α-peptide with decreased ability to complement β-galactosidase activity (14, 15). Plaques produced from transfection by DNA having the desired change are therefore lighter blue than wild-type M13mp2 plaques when plated on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)-containing plates. A 268-base *Pvu* II restriction endonuclease fragment prepared from the mutant (light blue) replicative form DNA (non-uracil-containing) was hybridized to the wild-type (i.e., blue) uracil-containing template. *In vitro* DNA synthesis and ligation was performed to incorporate this fragment into a covalently closed circle. Analysis of the product of this reaction on an agarose gel (not shown) indicated that ≈20% of the input DNA was converted to covalently closed double-stranded circles. The remainder was either not ligated, only partially copied, or remained uncopied. Transfection with this DNA mixture without selection against the template (i.e., in ung^- host cells) produced a mutant phenotype frequency of 6.7%. This value is 50-fold above the background frequency and is typical of current site-specific mutagenesis protocols before enrichment for only copied molecules.

In contrast, transfection of an aliquot of this same mixture of DNA molecules into competent ung^+ cells gave a 51.0% mutation frequency, a 7.6-fold increase. As expected, in the absence of DNA synthesis, no survivors were observed in the ung^+ transfection. In addition, the low yield of total survivors and mutants produced from *in vitro* reactions containing no ligase demonstrates that both arose primarily from covalently closed complementary strand circles.

Several variations of this experiment have been performed to further increase the mutation frequency. Although transfection of the double-stranded heteroduplex clearly selects against the phenotype of the uracil-containing strand, it is possible that this methylated template strand could act to instruct mismatch correction (9, 22) within the complementary strand but in favor of the template phenotype, before the viral template strand is destroyed. Alternatively, the newly made complementary strand could be used *in vivo* as a template to repair the many abasic sites produced in the viral strand upon removal of uracil. Therefore, the product of the *in vitro* reaction was treated, before transfection, with either uracil glycosylase or uracil glycosylase followed by alkali, to hydrolyze the apyrimidinic sites and disrupt hydrogen bonding. This latter strategy should effectively produce, as the sole source of biological activity, covalently closed complementary (i.e., mutant) single-stranded circles. The results of transfection of DNA treated in this way are shown in the last two lines of Table 2. As expected, glycosylase treatment before transfection yielded a mutant frequency of >50% even in the ung^- host, consistent with the concept that uracil removal, whether *in vitro* with the purified enzyme or *in vivo*, is responsible for the increase in frequency. More importantly, glycosylase followed by alkali treatment gave a mutation frequency of 81% (ung^-) to 89% (ung^+). Five light blue mutants were examined by DNA sequence analysis, and all five had the expected G → C change at position +82.

Table 2. Efficiency of mutagenesis with uracil-containing M13mp2 DNA

Experimental condition	*ung*⁻ transfection			*ung*⁺ transfection		
	Total pfu	Mutants	%	Total pfu	Mutants	%
Sham-primed	1492	2	0.13	1	0	—
Primed, uncopied	2180	42	1.90	0	0	—
Copied						
No ligase	2715	24	0.88	234	2	0.85
+ Ligase	3695	248	6.70	1157	589	51.0
+ Ligase + glycosylase	256	136	53.0	277	145	52.0
+ Ligase + glycosylase + alkali*	26	21	81.0	38	34	89.0

For each variable, 1 μg of uracil-containing M13mp2 DNA (wild type for *lac*) was used. To this was hybridized (except for the sham-primed control) 4 μg equivalents of a 268-base-pair *Pvu* II restriction endonuclease fragment that spans positions −123 to +145 of the $lacZ_\alpha$ gene in M13mp2 and is derived from a (light blue) mutant (15) having a single G → C base change at position +82. The resulting heteroduplex thus contains a G(+)·G(−) mismatch at position +82. DNA synthesis reactions were performed in a 50-μl volume containing the primer-template, 20 mM Hepes (pH 7.8), 5 mM dithiothreitol, 10 mM $MgCl_2$, 200 μM rATP, 100 μM dATP, dTTP, dCTP, and dGTP, 16 units of dUTPase, 1 unit of *E. coli* DNA polymerase I (large fragment), and, for the + ligase condition, 10 units of T4 DNA ligase. Incubation was for 30 min at 37°C and reactions were terminated by adding EDTA to 15 mM. For each condition, 200 ng was used for transfection into competent *ung*⁻ or *ung*⁺ *E. coli* cells as described (15). Wild-type and light blue mutant M13mp2 plaques were scored as described (15), by plating on minimal plates supplemented with isopropylthio-β-D-galactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). The copied, ligated DNA (200 ng) was treated with uracil glycocylase, and then 100 ng of this DNA was adjusted to 0.2 M NaOH (pH 12.8), incubated at 37°C for 5 min, and neutralized; 25 ng was used for transfections. pfu, Plaque-forming units.

*The decrease in biological activity associated with this treatment could result from several factors, including decreased efficiency of transfection of complementary strands or single-stranded versus double-stranded DNA or damage due to alkali treatment.

This set of experiments was performed by using deoxynucleoside triphosphates that had been preincubated with dUTPase (17) to hydrolyze any dUTP contaminants in the commercial preparations of substrates. Any such dUTP could be incorporated into the complementary strand, destroying its selective advantage (21). However, in order to establish the general applicability of this technique and because dUTPase is not commercially available, the effect of excluding the dUTPase treatment was examined. Parallel reactions were performed with and without dUTPase and the products were transfected, without further treatment, into *ung*⁺ cells. Survival values were similar and the mutant frequencies were both 51%. This suggests that, using highly purified but commercially available substrates, dUTPase treatment is not necessary. In subsequent experiments (Table 3) such treatment was omitted.

In a further attempt to improve the ease with which this approach can be applied, a parallel reaction was performed with a uracil-containing template prepared from phage produced in only one cycle of growth on the *E. coli dut*⁻ *ung*⁻ host (see *Materials and Methods*). The mutant frequency was 41%, in contrast to the 51% shown for the experiment in Table 2, and consistent with the observation that relative survival (*ung*⁺/*ung*⁻ host) of the (non-mutant) template is 100-fold greater with only one growth cycle. Since a 41% site-specific mutation frequency is more than adequate for many applications, a single growth cycle may often suffice.

Oligonucleotide-Directed Missense Mutagenesis. In many instances site-specific mutations are introduced via oligonucleotides (1–3). The results of such an approach with a uracil-containing template are shown in Table 3 (protocol 1). In this instance a proline codon is changed to an arginine codon through a C → G change at position +82. A 15-base oligonucleotide containing the desired change was incorporated into a covalently closed complementary strand circle, using a uracil-containing M13mp2 template. The reaction was performed with *E. coli* DNA polymerase I, large fragment, at low temperature in an attempt to maximize utilization of the oligonucleotide and minimize strand displacement. Analysis of the products of the reaction by agarose gel electrophoresis (not shown) demonstrated very little production of covalently closed circular double-stranded DNA. This was reflected in low survival upon transfection into an *ung*⁺ host. Despite this limitation, the mutation frequency was 40%, clearly demonstrating the strong selection against the uracil-containing phenotype. Of four (blue) mutants examined, all four had the desired sequence change.

Site-Specific Misincorporation Mutagenesis to Produce a Nonsense Codon. Uracil-containing templates can be effectively used to create specific mutations via misincorporation using an error-prone DNA polymerase and a single dNTP substrate (7). This approach has been used (Table 3, protocol 2) to change codon 6 of the $lacZ_\alpha$ gene to an ochre codon through a C → A change at position +58. Using a 17-base oligonucleotide (complementary to positions +59 to +75) hybridized to a wild-type (i.e., blue) uracil-containing template, AMV polymerase was used to incorporate a single dTMP residue opposite a template C at position +58. This single-base mutation was then "fixed" by addition of the remaining three dNTP substrates to allow further chain elongation. T4 DNA polymerase and T4 DNA ligase were then added, to produce covalently closed double-stranded molecules. Using 500 μM dNTPs, this reaction is highly efficient, typically converting 20–50% of the input template to the double-stranded form. Transfection of the products of this reaction using *ung*⁺ host cells yielded 53% colorless plaques. Sequence analysis of five colorless mutants confirmed the presence of the C → A change at the intended position in each case.

Site-Specific Mutagenesis Without Phenotypic Selection. In parallel with the experiment just described, a similar experiment was performed to incorporate dAMP opposite a template C at position +58 (Table 3, protocol 3). This produces a C → T missense mutation coding for leucine rather than serine at amino acid 6 in the $lacZ_\alpha$ peptide. Amino acid changes in this region are expected to be silent, so this experiment was performed without color selection on the plates. Ten plaques, produced from transfection of the products of the *in vitro* reaction into *ung*⁺ cells, were subjected to DNA sequence analysis. Six had the desired change.

The ability to place a phenotypically silent change at a desired position in a gene has many uses. For example, under appropriate conditions to be reported elsewhere, the run of the four thymine residues at positions +70 through +73 in

Table 3. Efficiency of site-specific mutagenesis using different protocols

Mutation	Complementary strand		Viral strand phenotype	Mutant selection	% mutants	Sequence analysis
	Bases	Phenotype				
Protocol 1: Oligonucleotide-directed mutagenesis with selection						
C → G	15	Blue	Light blue	Blue	40.0	4/4
Protocol 2: Misincorporation mutagenesis with selection						
C → A	17	Blue	Blue	Colorless	53.4	5/5
Protocol 3: Misincorporation mutagenesis without selection						
C → T	17	Blue	Blue	None	60.0	6/10
T → C	15	Blue	Blue	None	50.0	5/10

Protocol 1: The mutation was introduced via the 15-base oligonucleotide (P-L Biochemicals) complementary to nucleotides +76 to +90 of the $lacZ_\alpha$ gene in M13mp2. The 5'-OH termini were phosphorylated, and the 15-mer was hybridized at a 2:1 primer-to-template ratio to a (light blue) mutant M13mp2 template containing a C at position +82, thus creating a C·C heteroduplex. Synthesis was performed as described in the legend to Table 1, but at 0°C for 15 min followed by 16°C for 120 min. Protocol 2: Wild-type (blue) uracil-containing M13mp2 template was primed with a 17-mer (P-L Biochemicals) complementary to nucleotides +59 to +75 of the $lacZ_\alpha$ coding region. The desired site-specific change, a C → A change at position +58, was created by misincorporating a T opposite a template C [a C(+)·T(−) mismatch] using the error-prone AMV polymerase and dTTP only. Reactions were performed as follows. To a 25-μl volume containing the oligonucleotide-primed DNA (0.5 μg), 20 mM Hepes (pH 7.8), 10 mM $MgCl_2$, and 500 μM dTTP, were added 4 units of AMV DNA polymerase (Life Sciences). After 10 min at 37°C, the remaining three dNTPs were added to 500 μM and incubation at 37°C was continued for 5 min. The final additions were dithiothreitol to 2 mM, rATP to 200 μM, 5 units of T4 DNA ligase, and 0.5 unit of T4 DNA polymerase (P-L Biochemicals). Incubation was at 37°C for 60 min, and the reactions were terminated by adding EDTA to 15 mM. Protocol 3: The C → T change was produced as described in protocol 2, except that misincorporation of A opposite C at position +58 was produced by using AMV polymerase and dATP only. Although this experiment was performed without visual selection for plaque color, and later re-examination of the plates containing the mutant plaques showed that this change unexpectedly resulted in a very slight decrease in blue color intensity. The T → C change at position +72 was introduced by using a 15-base oligonucleotide (Bethesda Research Laboratories) complementary to positions +73 to +87, AMV polymerase, and dGTP only. As expected, this mutation was silent. In all cases, the unfractionated products of the reactions were used to transfect competent ung^+ host cells, which were then plated as described (15).

the $lacZ_\alpha$ coding sequence represents a mutational hot spot for frameshift mutations. To facilitate future studies with this DNA target it was desirable to alter the DNA sequence without altering the phenotype (dark blue color). This has been done by changing a T to a C at positions +72 (Table 3, protocol 3). This silent change, at the first position of codon 11, was introduced by misincorporation of dGMP opposite T at position +72, using as a primer a 15-base oligonucleotide complementary to positions +73 through +87. Of 10 plaques chosen at random and having identical wild-type blue color, the DNA of 5 had the desired silent base change.

DISCUSSION

These results illustrate the utility of uracil-containing templates for obtaining altered DNA sequences without phenotypic selection. The critical new step is the preparation of template DNA from phage grown in an *E. coli* dut^- ung^- host rather than in the usual wild-type host. Standard protocols already in widespread use can then be directly applied to obtain the results in a few hours. At the frequency of mutant production obtained here, it is only necessary to sequence two clones to obtain one with the desired change. The recent construction of an *E. coli* dut^- ung^- strain containing an amber suppressor (unpublished data) allows production of uracil-containing templates for DNA sequences cloned in any of the single-stranded M13 cloning vectors containing amber codons, making the technique more generally useful. In principle, this approach can also be applied to double-stranded uracil-containing templates, using strand-displacement reactions or strand-separation techniques.

Though single-base substitution mutations have been produced here, this approach may be even more useful for production of other sequence alterations, such as insertions or frameshifts. Attempts to produce these types of changes may produce even lower mutant frequencies. These frequencies may be enhanced many-fold using uracil-containing templates, particularly by using the additional *in vitro* treatments with uracil glycosylase and alkali. In theory this would allow transfection of the desired change in the form of a single-stranded complementary strand, rather than a double-stranded form in which the desired alteration is looped-out and subject to elimination by repair processes *in vivo*.

The idea to exploit the normal thymine-like coding potential of uracil in combination with the ability to convert these residues to lethal lesions was developed specifically for several uses in this laboratory. For example, the method has been used to introduce specific, and sometimes silent, changes in the primary DNA sequence of a defined mutational target (15). This is most clearly illustrated by the introduction of a TAA ochre codon, for use in reversion studies, and a silent T → C change, to reduce a mutational hot spot, in the coding region of the $lacZ_\alpha$ gene. Both changes, produced with a minimal effort, have been used to critically evaluate hypotheses concerning mechanisms of mutagenesis in a defined *in vitro* system (unpublished data). In addition, studies on control of transcription of the *lac* operon are now feasible, using mutants constructed to contain two (symmetric) base changes in the binding site for the cyclic AMP receptor protein.

In addition to the obvious utility of this approach for highly efficient site-specific mutagenesis, uracil-containing templates may be used to introduce specific premutagenic lesions at defined positions within a gene. This can be done either by using oligonucleotides containing the premutagenic lesion of interest or by specifically incorporating modified precursors at defined sites.

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