

Mutational Analysis Using *in vivo* Suppression of Amber Stop Codons: The INTERCHANGE(TM) *in vivo* Amber Suppression Mutagenesis System

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Amber suppressor tRNAs have been isolated which allow insertion of any one of 12 amino acids at an amber (TAG) stop codon. Using a set of strains containing these various suppressor tRNAs, it is possible to determine the effect of substituting each of these 12 amino acids at a particular site by making a single amber mutation.

Introduction

The ability to specifically substitute amino acids is a key technology for studying protein structure: function. A wide variety of techniques have been employed to make these substitutions, including site-directed mutagenesis, random mutagenesis and library approaches. Each technique has its own advantages and disadvantages and is best suited for answering different types of questions. As superior protein structure and homology models are developed, site-directed mutagenesis will play an increasing role in probing the molecular determinants of protein structure, enzyme activity and molecular recognition. When amino acids or regions in proteins are identified as playing a role in protein function, researchers invariably want to make substitutions at these sites to study their effects on protein activity. This process can be tedious and time-consuming. For this reason, the number of substitutions analyzed is often limited. Library approaches are often used to increase the number of sites examined. All possible substitutions are made at predetermined locations using degenerate oligonucleotides and site-directed mutagenesis. The number of sites examined often must be limited due to constraints on library size. Since the mutants are generated randomly, there is no direct way to assess the effect of a particular substitution on the activity of the target protein. Screens are required to identify those mutants having a particular activity, and the interesting mutants are sequenced to determine the amino acid substitution. Often, hundreds of mutants must be analyzed to identify key substitutions.

Amino acid substitution by amber stop codon suppression provides an alternative to both library and standard site-directed mutagenesis procedures for studying the effect of amino acid replacement on protein structure: function. Miller and coworkers (1,2) have developed a set of 12 amber suppressor tRNAs which insert different amino acids at the site of the stop codon. *E. coli* strains containing these amber suppressors are now available from Promega as part of the INTERCHANGE(TM) *in vivo* Amber Suppression Mutagenesis System. By placing an amber stop codon at the position of interest, the amber suppressor strains can be used to insert various amino acids at that site. The effects of 12 different amino acid substitutions at that site can be easily determined. This tremendous savings in time and expense allows a systematic series of substitutions throughout the protein to identify those sites which are important for protein structure and function. This approach has been used to extensively analyze the *lac*

repressor (3) and T4 lysozyme (4). In the case of T4 lysozyme, 163 positions were mutated, and the results of 2,015 amino acid substitutions obtained.

Suppressor strains

Table 1 lists the amber suppressor strains included in the INTERCHANGE System and the amino acids which they substitute. Some of the suppressors are chromosomal mutants and the remainder are synthetic constructs (2) on ColE1 plasmids. The collection allows 12 of the 20 potential amino acid substitutions at a single site. The strains also contain a chromosomal amber mutation in the arginine biosynthetic pathway, which selects for suppressor activity when plated on minimal media. This provides a control to assure the functionality of the suppressor when assaying amber mutants of the protein of interest.

Table 1. Amber Suppressors and Amino Acids.

Strain	Amino Acid	Suppressor Source
Amber-Cys	Cysteine	plasmid
Amber-Glu	Glutamic Acid	plasmid
Amber-Phe	Phenylalanine	plasmid
Amber-Gly	Glycine	plasmid
Amber-His	Histidine	plasmid
Amber-Pro	Proline	plasmid
Amber-Arg	Arginine	plasmid
Amber-Lys	Lysine	chromosomal (Su5, <i>supG</i>)
Amber-Leu	Leucine	chromosomal (Su6, <i>supP</i>)
Amber-Gln	Glutamine	chromosomal (Su2-89, <i>supE</i>)
Amber-Ser	Serine	chromosomal (Su1, <i>supD</i>)
Amber-Tyr	Tyrosine	chromosomal (Su3, <i>supF</i>)

The strains are provided as low efficiency competent cells., and vectors carrying the amber mutant of interest can be easily transformed into these strains. Propagating the transformed strains on minimal media maintains selection for the amber suppressor and ensures adequate levels of full-length protein. Individual isolates of each of the suppressor transformants can be conveniently maintained as freezer stocks in covered microtiter dishes at -70°C.

Many common strains such as JM109 and HB101 contain amber suppressors (*supE44*). Amber

mutations placed in these strains will be suppressed and produce full-length protein. A nonsuppressing strain is also included with the suppressor set. Expression of the amber mutant in this strain provides a reference point for basal activity of the amber fragment when measuring the effect of the amino acid substitution. The nonmutated gene of interest should also be expressed as a positive control.

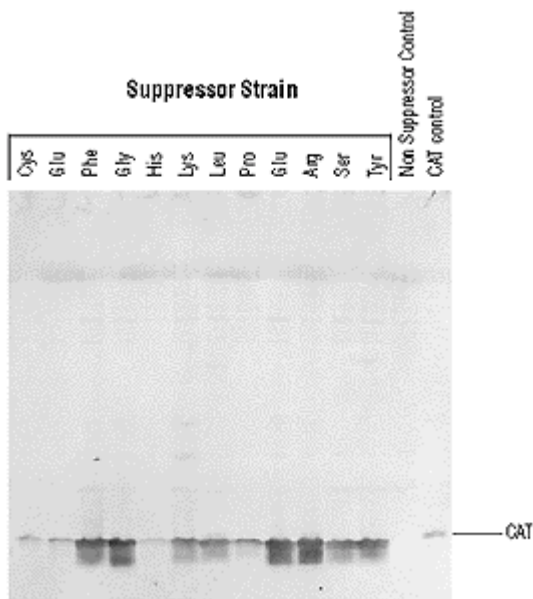
Mutational analysis using amber suppressors

To measure the effect of a particular substitution on protein activity, an amber mutant is generated at the location of interest by site-directed mutagenesis. Subsequently, the plasmid containing this amber mutant is transformed into the various suppressor bacterial strains. Protein activity is then assayed in each of the strains to determine the effect of each amino acid substitution.

Promega's Altered Sites® II-*Ex2 in vitro* Mutagenesis System (Cat.# Q6080) is compatible with the suppressor ColE1 replicons and contains the *tac* promoter for expression in *E. coli*. A second round of mutagenesis is easily accomplished with this system which is useful for confirming suppression results. Restriction sites often can be introduced when making site-directed mutations by taking advantage of the degeneracy of the genetic code and incorporating these sites in the mutagenic oligonucleotide. Incorporation of these restriction sites can serve as a quick means to confirm the identity of amber mutants. The amber stop codon (TAG) can be incorporated into a variety of restriction sites including *Bfa* I (CTAG), *Nhe* I (GCTAGC), *Spe* I (ACTAGT), *Xba* I (TCTAGA), and *Avr* II (CCTAGG).

Mutational analysis of chloramphenicol acetyltransferase

Amber suppression analysis of the chloramphenicol acetyltransferase (CAT) gene was performed utilizing a construct which replaces the threonine at amino acid position 10 with an amber codon. This construct was transformed into each suppressor strain for measurement of CAT activity. [Figure 1](#) is an immunoblot using an antibody to detect CAT protein in the suppressor strains from an amber mutant at position 10 in the CAT protein. In the absence of an amber suppressor (control strain) full-length protein is not observed. Full-length protein is observed with each suppressor strain from this construct. The efficiency of amino acid insertion at the amber site can be determined by measuring the relative levels of truncated to full-length protein. The truncated fragment in this case is too small to be observed on the blot. The level of each protein is also a function of its stability in the cell. Some site-dependent variation in suppression efficiency is also evident with these strains.



1.

Figure 1. Expression of chloramphenicol acetyltransferase from the Amber Suppressor Control Plasmid. The Amber Suppressor Control Plasmid was transformed into each of the INTERCHANGE Amber Suppressor Strains. Clones were picked and grown in 5ml of LB broth containing the appropriate antibiotic. Cultures were incubated overnight at 37°C, centrifuged and resuspended in TBS to a concentration of 10 OD600/ml (1 OD600 unit is equivalent to 1ml of culture at an OD600 of 1.0). SDS-PAGE sample buffer was added and samples were heated at 95°C for 3 minutes. The equivalent of 0.1 OD600 was loaded and run on a 12% polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane and immunodetection was performed using chicken anti-CAT antibody and goat anti-chicken conjugated to alkaline phosphatase. Western Blue(TM) Stabilized Substrate for Alkaline Phosphatase was used for color development. The lane marked "CAT Control" contained 20ng of Chloramphenicol Acetyltransferase (Cat.# E1051).

With CAT, enzyme activity can be measured simply by assaying the chloramphenicol resistance phenotype. [Figure 2](#) shows a simple experiment demonstrating the effect of various substitutions for phenylalanine at position 25 on CAT activity. Amino acid 25 lies in the active site of CAT (5) and would be expected to be sensitive to amino acid substitution. Plasmids containing an amber mutation for phenylalanine at position 25 were transformed into the 12 suppressor strains. Variation in enzyme activity is observed as a change in zone of inhibition surrounding the discs of antibiotic. The results shown in [Figure 2](#) demonstrate that substitution with the wild-type amino acid (Phe) generates an active enzyme resistant to chloramphenicol. [Table 2](#) summarizes the *in vivo* phenotype of the various substitutions at position 25. Substitution with phenylalanine, histidine, tyrosine or leucine results in a chloramphenicol resistant phenotype.

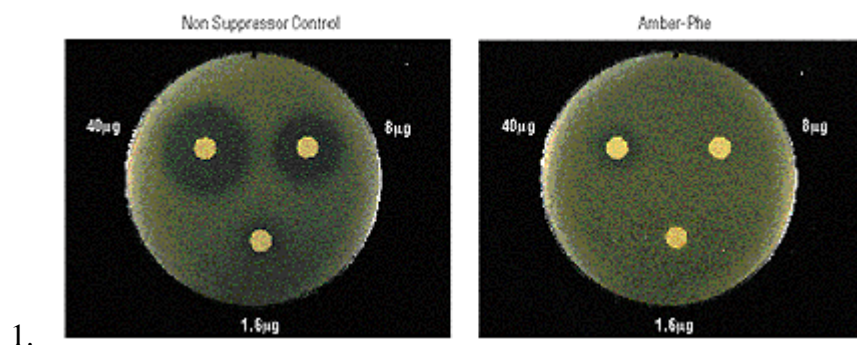


Figure 2. Suppression of an amber mutation at position 25 of CAT. A derivative of pACYC184 containing the CAT gene with an amber mutation at amino acid 25 was transformed into the Amber-Phe and Non Suppressor Control Strains and selected on plates containing tetracycline (12.5µg/ml) and ampicillin (100µg/ml) (Amber-Phe) or tetracycline only (Non Suppressor Control Strain). Cultures from each were grown overnight in LB broth containing the appropriate antibiotics. An aliquot (50µl) of the respective overnight cultures was added to 4ml of top agar and poured onto plates containing tetracycline and ampicillin (Amber-Phe) or tetracycline only (Non Suppressor Control Strain). After the top agar had solidified, filter discs containing 40, 8, or 1.6µg of chloramphenicol were placed on top of the plates. After overnight growth at 37°C, zones of growth inhibition were detected around antibiotic discs. Zones of growth inhibition indicate sensitivity to chloramphenicol.

The chloramphenicol resistance phenotype is dependent upon both the specific activity of the mutant enzyme and the stability of that mutant in the cell. Western analysis indicates that some variability is observed in enzyme levels. Specific activity is a better measure of the effect of the substitution. Mutant CAT was purified from each of the suppressor strains and the enzymatic activity determined. The purification consists of an affinity binding step to a chloramphenicol-coupled resin. The results of the specific activity determination are shown in [Table 2](#). These results indicate that the substitutions can be divided among several types. One class of substitutions (cysteine, glutamic acid, glycine and proline) produces a CAT protein which is inactive and does not bind to the affinity resin. In this case, it appears that the substitution at position 25 alters the substrate binding pocket sufficiently to prevent binding of the chloramphenicol. A second class of substitutions (glutamine and serine) is catalytically inactive but still binds chloramphenicol with sufficient affinity to allow purification. A third class of substitutions (phenylalanine, histidine, leucine and tyrosine) are those which retain both binding activity and catalytic activity. Of these substitutions, phenylalanine (the wild-type amino acid) and histidine substitutions generate the most active enzyme. Substitution with the conservative amino acid tyrosine or with leucine yields an enzyme with greatly reduced activity, but one which is sufficient to produce the chloramphenicol resistant phenotype. This is an example of the expanded amount of information which can be obtained from a single mutation in conjunction with the amber strains.

Table 2. Suppression of Amber Mutation at Position 25 of Chloramphenicol Acetyltransferase.

Strain	Amino Acid	<i>in vivo</i> Phenotype ¹	Specific Activity ²
Amber-Cys	Cysteine	Cms	d.n.b.
Amber-Glu	Glutamic Acid	Cms	d.n.b.
Amber-Phe	Phenylalanine	Cmr	150,000 units/mg

Amber-Gly	Glycine	Cms	d.n.b.
Amber-His	Histidine	Cmr	215,000 units/mg
Amber-Lys	Lysine	n.d.	n.d.
Amber-Leu	Leucine	Cmr	20 units/mg
Amber-Pro	Proline	Cms	d.n.b.
Amber-Gln	Glutamine	Cms	< 5 units/mg
Amber-Arg	Arginine	n.d.	n.d.
Amber-Ser	Serine	Cms	< 5 units/mg
Amber-Tyr	Tyrosine	Cmr	500 units/mg
Non Suppressor	Stop	Cms	n.d.

¹Cmr = chloramphenicol resistant;

Cms = chloramphenicol sensitive.

²d.n.b. = protein did not bind to affinity resin.

n.d. = not determined.

Summary

Mutational analysis using the INTERCHANGE *in vivo* Amber Suppression Mutagenesis System provides a rapid method for screening the effect of amino acid substitutions to investigate protein structure and function. This system is particularly useful for identifying key residues in proteins that affect protein activity and stability. In many cases, significant amino acid sequence homology with known proteins can be utilized to identify potentially important amino acid residues. Amber suppression provides a convenient means of making both conservative and nonconservative amino acid substitutions within such regions of homology to test these hypotheses. Amber suppression is equally useful in those cases where very little is known about the protein structure: function relationships. A large number of substitutions easily can be produced for a systematic analysis of the entire protein. Once sites and substitutions have been identified as important for structure: function, the corresponding mutation can be made using a standard replacement codon or the amber plasmid simply can be maintained in the suppressor strain for expression.

References

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Ordering Information

Product

Cat.#

INTERCHANGE(TM) *in vivo* Amber Suppression
Mutagenesis System

Q5080

All the *E. coli* strains are supplied competent at an
efficiency of at least $10^3/\mu\text{g}$.

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