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Genetics

Construction of two *Escherichia coli* amber suppressor genes: $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$

(gene synthesis/amino acid substitution/nonsense suppression)

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ABSTRACT **Amber suppressor genes corresponding to *Escherichia coli* $tRNA^{Phe}$ and $tRNA^{Cys}$ have been constructed for use in amino acid substitution studies as well as protein engineering. The genes for either $tRNA^{Phe}_{GAA}$ or $tRNA^{Cys}_{GCA}$ both with the anticodon 5′ CTA 3′ were assembled from four to six oligonucleotides, which were annealed and ligated into a vector. The suppressor genes are expressed constitutively from a synthetic promoter, derived from the promoter sequence of the *E. coli* lipoprotein gene. The $tRNA^{Phe}$ suppressor ($tRNA^{Phe}_{CUA}$) is 54–100% efficient *in vivo*, while the $tRNA^{Cys}$ suppressor ($tRNA^{Cys}_{CUA}$) is 17–50% efficient. To verify that the suppressors insert the predicted amino acids, both genes were used to suppress an amber mutation in a protein coding sequence. NH_2-terminal sequence analysis of the resultant proteins revealed that $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$ insert phenylalanine and cysteine, respectively. To demonstrate the potential of these suppressors, $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$ have been used to effect amino acid substitutions at specific sites in the *E. coli lac* repressor.**

Nonsense suppressors are alleles of tRNA genes altered in the anticodon, resulting in insertion of an amino acid in response to a termination codon. These suppressors have been important tools in bacterial genetics (1) as well as in the study of recognition of tRNA by aminoacyl tRNA synthetase (2). The use of nonsense suppressors also offers exciting possibilities for protein engineering, since numerous altered proteins with known amino acid changes can be generated easily. By this method, genes containing amber, ochre, or opal mutations (resulting in UAG, UAA, or UGA chain-terminating codons, respectively) are expressed in mutant strains that produce suppressor tRNAs capable of inserting an amino acid in response to the nonsense mutation. Many single amino acid substitutions at specified positions in proteins have already been made. For example, nonsense suppression has been used to create close to 400 mutant *lac* repressor proteins, each carrying a known amino acid replacement (3). Unfortunately, the range of amino acid exchanges made possible by nonsense suppression has been limited by the relatively small set of nonsense suppressors available. Until recently, only five different amino acids could be inserted at an amber codon by suppressor tRNAs. The amber suppressor tRNAs that have been generated *in vivo* (*supD, supE, supF*, and *supP*), insert serine, glutamine, tyrosine, and leucine, respectively, in response to UAG codons. In addition, Murgola and coworkers have described a glycine-inserting amber suppressor derived from a series of *in vivo* genetic manipulations (4). [Ochre suppressors operating at reduced efficiencies can insert additional amino acids—for instance, *supG* directs the insertion of lysine at UAA and UAG codons (5), but the inefficient suppression greatly limits their utility for generating altered proteins.] With the exception of the glycine suppressor described by Murgola, the existing amber suppressor alleles arose from a single base change, resulting in the anticodon 5′ CUA 3′, which recognizes the amber codon 5′ UAG 3′ (6–10). Creating new suppressor alleles by standard genetic means is unlikely, as virtually every other tRNA requires more than a single base change to yield a CUA anticodon. However, advances in oligonucleotide synthesis (11) have made possible the *in vitro* construction of new suppressor tRNAs, which permits us to greatly extend the utility of this approach. To expand the available collection of amber suppressors in *Escherichia coli*, we have synthesized the genes for several tRNAs, altering the anticodon to enable recognition of UAG (amber) codons. Here we describe the construction of the genes for two such suppressor tRNAs, which insert phenylalanine and cysteine at high efficiency, and we demonstrate their utility in amino acid substitution studies of the *E. coli lac* repressor.

MATERIALS AND METHODS

Bacteria, Bacteriophage, Media, and Reagents. *E. coli* strain XAC-1 is F′ $lacI_{373}lacZ_{u118_{am}}proB^+/F^-\ \Delta(lacproB)_{x111}$ *nalA, rif*, $argE_{am}$, *ara*. The F1 phage, IR1, was obtained from E. Meyerowitz. Indicator plates used for transformations were minimal M9 glucose (12) supplemented with ampicillin (100 μg/ml) and the indicator dye 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (40 μg/ml). Folic acid and methotrexate were purchased from Calbiochem and ICN, respectively.

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. Three to five A_{260} units of oligonucleotide were purified by electrophoresis through a 20% acrylamide/7 M urea gel, visualized by UV shadowing, excised, and eluted in 0.3 M NaCl/10 mM Tris·HCl, pH 7.4/1 mM EDTA/1% phenol, at 37°C for 18 hr. The DNA was precipitated and washed with ethanol, dried, and resuspended in distilled H_2O.

Gene Synthesis. Each oligonucleotide (2 μg) was phosphorylated in 70 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/5 mM dithiothreitol/100 μM ATP, with 4 units of polynucleotidekinase for 1 hr at 37°C. The phosphorylated oligonucleotides (80 pmol each) were mixed together in 100 mM NaCl, heated to 80°C for 5 min, then allowed to cool to room temperature over a period of 3 hr. The entire mixture was combined with vector PGFIB-I (previously cleaved with *Eco*RI and *Pst* I) to give a mass ratio of 10:1 (insert/vector). Subsequent ligation was carried out in 50 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/20 mM dithiothreitol/50 mM NaCl/1 mM ATP/bovine serum albumin (50 μg/ml), with 1 unit of T4

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Abbreviations: DHFR, dihydrofolate reductase; bp, base pair(s).

DNA ligase at 15°C for 12 hr. The ligation mixture was used to transform competent *E. coli* XAC-1 cells. From purified colonies exhibiting the suppressor phenotype on indicator medium, single-stranded DNA was made by using the F1 phage IR1 as a helper (13) and sequenced by the chain-termination method of Sanger *et al.* (14).

Protein Purification. Purification of mutant dihydrofolate reductase (DHFR) protein was carried out by a combination of the methods of Baccanari *et al.* (15–17) and is briefly described here. *E. coli* XAC-1 carrying plasmid pDa3-12:Phe was cultured in 6 liters of minimal M9 glucose medium supplemented with ampicillin (50 μg/ml) at 37°C with aeration for 18 hr. Cells were harvested and lysed (15) and the crude lysate was extracted with ammonium sulfate to 90% saturation (16). The precipitate was brought to a protein concentration of 20 mg/ml, 3.2 ml of methotrexate Sepharose was added, and the mixture was dialyzed for 18 hr (16). The resin was batch-washed (17) and the DHFR was eluted with folic acid (17). At this point, the DHFR was 99% pure as judged by silver staining (18) of sodium dodecyl sulfate/polyacrylamide gels (19). To remove folate and nucleic acid, peak fractions were first dialyzed against 50 mM potassium phosphate, pH 8.0/1 mM dithiothreitol, and then applied to a DEAE-Sephacel (Pharmacia) column (protein, 1 ml/mg) equilibrated with the same buffer. DHFR was eluted in a linear gradient of 0–0.4 M KCl in 50 mM potassium phosphate, pH 8.0/1 mM dithiothreitol. Peak fractions were pooled, dialyzed against water, and lyophilized.

Protein Sequencing. Mutant DHFR protein was sequenced on an Applied Biosystems Model 470A gas-phase protein sequenator (20) by the USC Microchemical Core Laboratory. Mutant β-galactosidase was purified (21) and sequenced by A. Fowler (University of California at Los Angeles).

RESULTS

Gene Construction. The sequences of *E. coli* tRNA$^{Phe}_{GAA}$ and tRNA$^{Cys}_{GCA}$ with the alterations necessary to convert them to amber suppressor tRNAs are shown in Fig. 1. The synthetic genes for tRNA$^{Phe}_{CUA}$ and tRNA$^{Cys}_{CUA}$ are depicted in Fig. 2. Each gene is constructed from a set of 4–6 complementary oligonucleotides ranging in size from 23 to 46 nucleotides, with 7-base-pair (bp) overlaps at the junctions. Each tRNA gene sequence is flanked at the 5′ and 3′ ends by *Eco*RI and *Pst* I restriction endonuclease site cohesive ends, respectively. Since the *E. coli* cellular nucleases that process tRNA precursors recognize conserved structural elements within the tRNA and not surrounding sequences (25), we expected that these constructs would produce fully processed functional tRNAs.

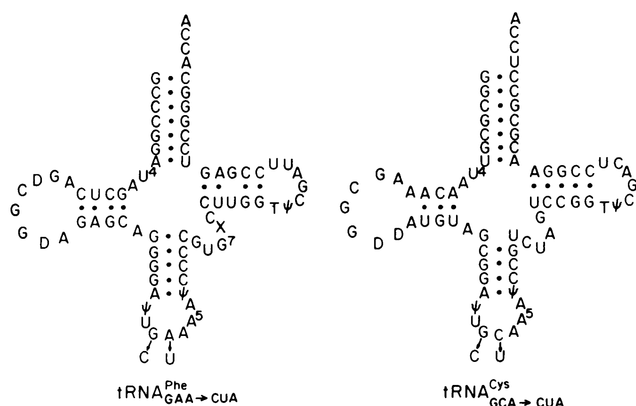

FIG. 1. RNA sequences of *E. coli* tRNA$^{Phe}_{GAA}$ (22) and tRNA$^{Cys}_{GCA}$ (23) with the base changes that would enable the tRNAs to recognize amber codons.

The oligonucleotides were annealed and ligated into the *Eco*RI/*Pst* I sites of plasmid pGFIB-I as described in *Materials and Methods*. In this vector (Fig. 3), the tRNA gene is expressed constitutively from a strong synthetic promoter, based on the promoter sequence of the *E. coli* lipoprotein gene *lpp* (26). Distal to the promoter and restriction site polylinker is a transcription terminator, which we have synthesized based on the termination sequence of the ribosomal RNA operon *rrnC* (27–28). Plasmid pGFIB-I also carries the F1 intergenic region, which in the presence of F1 helper phage allows the production of single-stranded DNA (13), thus facilitating dideoxy sequencing of the synthetic gene.

To clone active suppressors, the ligation mixtures pGFIB:Phe and pGFIB:Cys were transformed into *E. coli* strain XAC-1. This strain carries an F′ *lacproB* episome with an amber mutation early in the *lacZ* gene. In an Su^- strain, virtually no active β-galactosidase (the *Z* gene product) is formed. Suppression of the amber mutation should yield functional β-galactosidase, resulting in blue colonies on medium containing the indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. When XAC-1 was transformed with either pGFIB:Phe or pGFIB:Cys, on average, 60% of the transformants were blue. To confirm that this phenotype was plasmid encoded and not due to spontaneous reversion of the amber mutation, plasmid DNA was isolated from blue colonies and used to retransform XAC-1. Subsequently, single-stranded DNA was isolated from transformants that gave blue colonies and sequenced to ensure that there were no sequence anomalies in the synthetic construct.

Determination of Suppression Efficiency. We have exploited the properties of a *lacI–Z* fusion system to measure the efficiency of suppression. In this system, the *lacI* and *Z* genes are fused, resulting in a hybrid protein with full β-galactosidase activity (29). The NH_2-terminal, *I*-encoded portion of the fusion is not required for β-galactosidase activity, although continued transcription and translation through this portion of the fusion is necessary. Thus, the introduction of nonsense mutations that interrupt translation of the *I* portion of the message results in negligible β-galactosidase synthesis, while suppression of these nonsense mutations restores β-galactosidase synthesis. As β-galactosidase activity is independent of the nature of the amino acid being inserted into the *I*-encoded portion of the hybrid protein and dependent only on the degree of transmission through the translation block, the level of β-galactosidase activity is a direct measure of the efficiency of suppression. Fig. 4 depicts this fusion system. Previous studies of suppression at different nonsense sites have shown that the efficiency of suppression is dependent on the sequence immediately surrounding the nonsense site; the two bases 3′ to the nonsense site are especially important (29, 30). Generally, amber codons followed by an A or G are suppressed more efficiently than those followed by a C or U, although codons followed by the sequence CU are well-suppressed.

We have examined tRNA$^{Phe}_{CUA}$ and tRNA$^{Cys}_{CUA}$ against a set of ambers in the *lacI–Z* fusion by assaying β-galactosidase activity of the suppressed fusion protein (12). Table 1 shows the relative efficiency of the new suppressors as well as those for other suppressors. tRNA$^{Phe}_{CUA}$ has a suppression efficiency of 54–100%, while the efficiency of tRNA$^{Cys}_{CUA}$ ranges from 17% to 50%, depending on the context of the amber codon.

Determination of Suppressor Specificity. A single change in the anticodon of *E. coli* tRNA^{Trp} results in mischarging by the glutamine aminoacyl tRNA synthetase (31). Similarly, alteration of the anticodon of *E. coli* tRNA^{fMet} results in mischarging by glutamine synthetase (32–34). Given these precedents, we designed an assay to show directly that the new suppressors actually insert the predicted amino acid. tRNA$^{Phe}_{CUA}$ and tRNA$^{Cys}_{CUA}$ were each used to suppress an amber mutation in

FIG. 2. Synthetic genes for $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$. The first and last nucleotides encoding each tRNA (indicated by a dot) are immediately flanked by the cohesive ends of *Eco*RI and *Pst* I restriction endonuclease sites, respectively. Arrows indicate the oligonucleotide junctions, and the altered anticodon is underlined. Note that the $tRNA^{Phe}_{CUA}$ gene does not encode the 3'-terminal C and A residues. These residues are added *in vivo* by tRNA nucleotidyltransferase (24).

a protein-coding sequence. The resulting altered proteins were subsequently isolated and subjected to NH_2-terminal sequence analysis to determine which amino acid(s) had been inserted in response to the amber codon.

For determination of the specificity of $tRNA^{Phe}_{CUA}$, we constructed an amber mutation via oligonucleotide-directed mutagenesis, in the 10th residue (valine) of the *E. coli fol* gene encoding DHFR (35) (Fig. 5). DHFR was chosen because it can be purified in virtually one step by methotrexate affinity chromatography. Furthermore, the structure of DHFR has been well-delineated by x-ray crystallography (39–41). The tenth residue of this protein is not highly conserved throughout phyla and is not in close proximity to the active site. Hence, we predicted that substitutions at this site would not significantly alter the protein's activity or its behavior during purification. We also converted the 11th residue from Asp to Asn. The purpose of this GAT → AAT conversion was to provide a better context for the amber mutation. Subsequently, we constructed a plasmid (pDa3-12:Phe) that carries both the *fol* amber gene under the control of the *tac* promoter (38) and $tRNA^{Phe}_{CUA}$ flanked by the lipoprotein promoter and *rrnC* terminator (Fig. 5).

DHFR was isolated from XAC-1 cells harboring this plasmid and subjected to Edman degradation on an automated protein sequenator (20). In Fig. 6, the picomolar yields of phenylthiohydantoin-derivatized phenylalanine, valine, asparagine, and aspartic acid are plotted versus the residue number. It should be noted that the wild-type copy of the *fol* gene is also present in the host chromosome. Under control of the *tac* promoter, the plasmid-borne *fol* gene overproduces DHFR ≈100-fold above the level produced from the chromosomal copy. This should result in minimal levels of Val-Asp at positions 10 and 11 (compared with Phe-Asn) in the presence of a strong suppressor such as $tRNA^{Phe}_{CUA}$. The major amino acid at position 10 is indeed phenylalanine, and no other amino acids are detected at a significant level, including valine or glutamine. In other studies, we have been able to detect 10% insertion of an amino acid at residue 10 (28). Therefore, to our limits of detection, phenylalanine is the sole amino acid inserted by the new suppressor. Residue

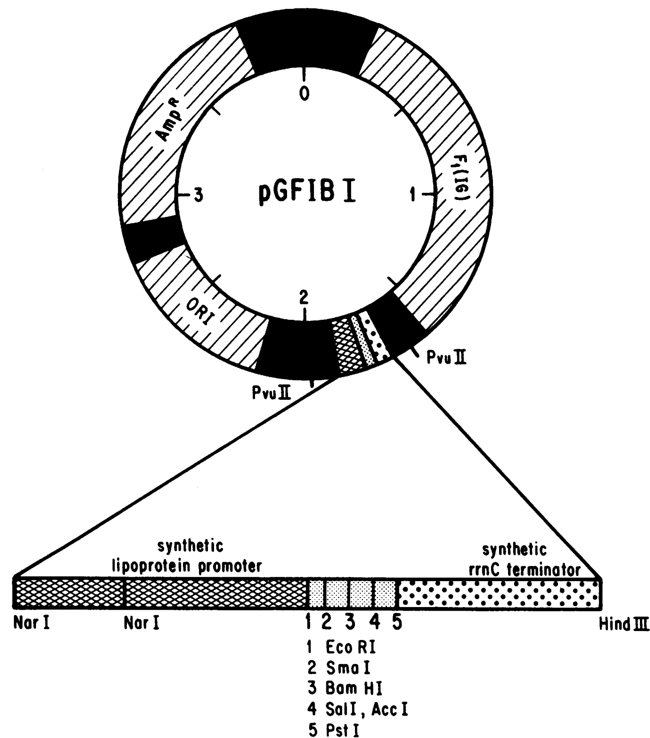

FIG. 3. Plasmid pGFIB-I. This vector is a derivative of pEMBL8+ (13). We have replaced the *lac* promoter with a synthetically constructed promoter (J.-M.M. and J.H.M., unpublished results) based on the promoter sequence of the *E. coli* lipoprotein gene *lpp* (26). Two complementary oligonucleotides encoding a transcription termination sequence from the *E. coli rrnC* operon (27) have been ligated into the *Pst* I and *Hin*dIII site of the polylinker (28). The oligonucleotides encoding the tRNA genes are ligated into the *Eco*RI and *Pst* I sites of the polylinker.

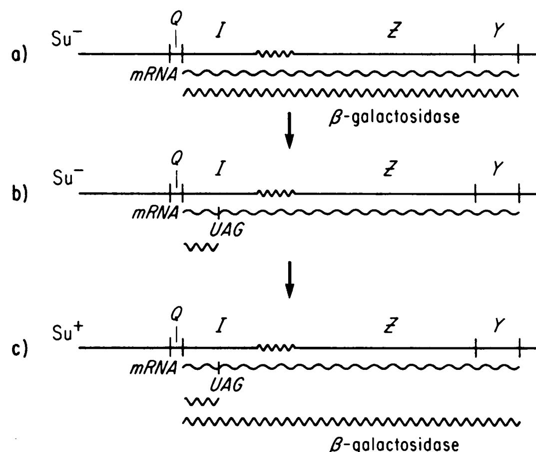

FIG. 4. Use of *lacI–Z* fusion strains to measure suppression efficiency. *lacI–Z* gene fusions are expressed under the control of the I^Q promoter (*a*). They can be used to measure the efficiency of suppression when an amber mutation is present in the *I* sequence (*b*). In the absence of suppression, a truncated polypeptide without β-galactosidase activity is produced (*b*). With suppression, a full-length polypeptide with β-galactosidase activity is synthesized (*c*).

Table 1. Suppression of amber mutations in a *lacI–Z* fusion

	β-Galactosidase activity, % of wild type						
Suppressor	O21c	A30	O28c	A26	A16	O17c	O13c
$tRNA^{Phe}_{CUA}$	79	100	78	100	78	54	84
$tRNA^{Cys}_{CUA}$	34	53	50	51	35	17	35
supD	27	54	32	25	21	6	26
supE	15	26	10	11	10	0.8	11
supF	34	67	62	100	43	11	46
supP	72	70	80	100	62	30	58

All assays were carried out at 37°C and were determined in duplicate (12). Values are given as the percentage of the wild-type fusion in the respective suppressor strains. Values for *supD*, *-E*, *-F*, and *-P* are taken from Miller and Albertini (29).

11 is predominantly asparagine, with a trace amount of aspartic acid. This may be the result of deamidation of asparagine or due to wild-type background.

The specificity of $tRNA^{Cys}_{CUA}$ was determined by A. Fowler in a similar fashion (personal communication). The synthetic $tRNA^{Cys}_{CUA}$ gene was used to suppress an amber mutation located at residue 17 in the *lacZ* gene. The resulting β-galactosidase was purified and subjected to NH_2-terminal sequence analysis. Cysteine is the major amino acid inserted at this site, and again glutamine is not detected at a significant level (data not shown). $tRNA^{Phe}_{CUA}$ was also used to suppress the *lacZ* amber, and the mutant β-galactosidase was purified and sequenced by A. Fowler. The results are similar to those obtained with the *fol* amber (data not shown).

Substitutions in the *lac* Repressor. To demonstrate the potential of the new suppressors for structure function studies, we have used them to generate specific amino acid replacements in the *E. coli lac* repressor. The *lac* repressor is especially suited to amino acid substitution studies as its properties are well-understood, its activity is readily assayed, and a large number of amber mutations in the repressor gene, *lacI*, have been generated (42). We have previously described the use of suppressed nonsense mutations to generate altered *lac* repressor molecules (3). Close to 90 different nonsense sites in the *lacI* gene have been described, and the ability to add different amino acids by suppression offers the opportunity to study a hierarchy of amino acid substitutions at each site. Table 2 shows several positions in the repressor that are particularly sensitive to certain amino acid substitutions. These data can yield useful information regarding the nature of the amino acid being inserted by the new suppressors. For instance, glutamine is the wild-type residue at positions 18 and 248. From Table 2 it is evident that these sites specifically require glutamine among the amino acids examined so far. Exchanges at position 18 affect operator binding, and sub-

```
           2                                          12
DHFR wt:   Ile Ser Leu Ile Ala Ala Leu Ala Val Asp Arg
           ATC AGT CTG ATT GCG GCG TTA GCG GTA GAT CGC

DHFR am:   ... ... ... ... ... ... ... ... ... am Asn ...
           ... ... ... ... ... ... ... ... ... TAG AAT ...
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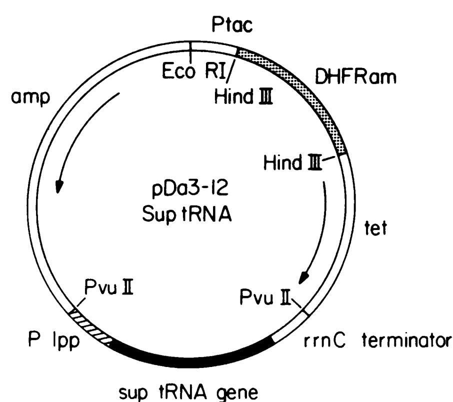

FIG. 5. Construction of plasmid pDa3-12. Oligonucleotide-directed mutagenesis (36) was carried out on the *E. coli fol* gene (35) encoded by a 1-kilobase fragment contained in the vector M13 mp8 (37). Plasmid pDa3-12:sup tRNA, in this case pDa3-12:Phe, was constructed by a series of subcloning steps. The 247-bp *Eco*RI/*Hin*dIII fragment of pTAC12H (38) containing the *tac* promoter was ligated into the *Eco*RI/*Hin*dIII sites of pBR322 to create pTAC10. Subsequently, the DHFR amber encoded by a 1057-bp *Hin*dIII fragment, was subcloned into the *Hin*dIII site of pTAC10, which yielded pDa3-12. The ≈350-bp *Pvu* II fragment from pGFIB:Phe containing the lipoprotein promoter, $tRNA^{Phe}_{CUA}$, and the *rrnC* terminator was ligated into pDa3-12 to yield pDa3-12:Phe.

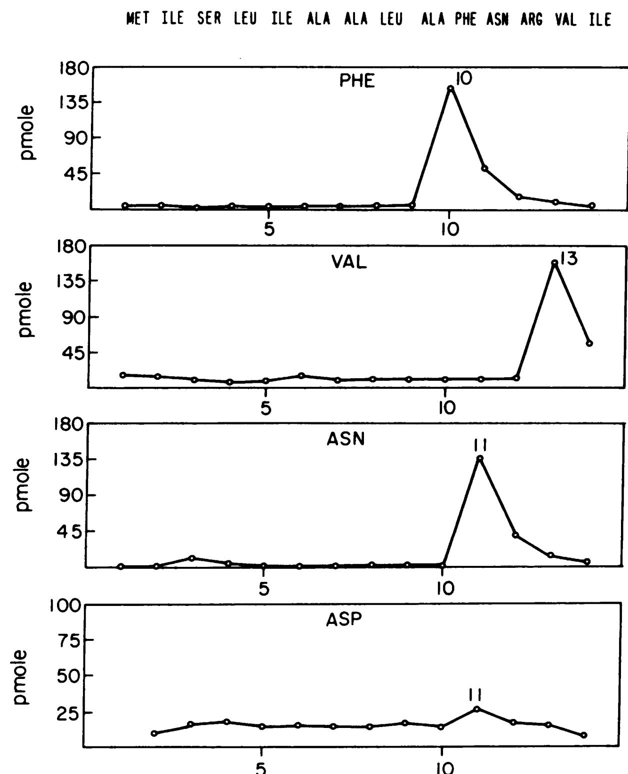

FIG. 6. The picomolar yields of phenylthiohydantoin-derivatized phenylalanine, valine, asparagine, and aspartic acid have been plotted versus the residue number for DHFR purified from *E. coli* XAC-1 cells carrying pDa3-12:Phe.

Table 2. Amino acid substitutions in the *lac* repressor

Wild-type residue	Replacement by suppression					
	Ser	Gln	Tyr	Leu	Phe	Cys
Tyr-17	±	∓	+	∓	∓	∓
Tyr-47	–	–	+	–	±	–
Tyr-282	–	–	+	+	+	–
Trp-201	–	–	+	–	+	–
Phe-293	±	±	s	s	+	s
Gln-18	–	+	–	–	–	∓
Gln-248	s	+	s	s	s	s

+, Normal activity; s, DNA binding activity retained but not inducibility; –, no activity, presumably because of defects in folding, aggregation, or DNA binding. Partial activities: ± indicates a less severe defect than ∓.

stitutions at position 248 affect inducer binding (the I^s phenotype). These two sites can be used to determine whether a suppressor is inserting glutamine. Also, the phenylalanine at position 293 is not easily replaced without affecting the activity of the protein. Note that the phenylalanine-inserting suppressor restores the wild-type character, reinforcing the protein chemistry results for this suppressor. In addition, the tyrosines at positions 17, 47, and 282, and the tryptophan at position 201 are sensitive to substitution. Only certain hydrophobic amino acids are allowed at positions 201 and 282, and the phenylalanine-inserting suppressor does restore activity in both cases. None of the previous substitutions for tyrosine at position 47 could restore even partial activity. However, as can be seen from Table 2, the phenylalanine-inserting suppressor can substitute reasonably well. Possibly, the ring moiety of the tyrosine is crucial at this position, and the phenylalanine ring fulfills a similar function. We note that phenylalanine cannot substitute for tyrosine at position 17, a residue that has been implicated in operator binding mediated by hydrogen bonding (43). This would be expected, since the phenylalanine ring lacks the hydroxyl side chain of tyrosine that could participate in hydrogen bonding.

DISCUSSION

Nonsense suppressors can be generated by altering the anticodon of a tRNA to allow recognition of chain-termination codons. This approach has already met with success in several cases via oligonucleotide-directed mutagenesis (44–47).

We have described the construction of two amber suppressor tRNA genes, $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$, which insert the predicted amino acid at high frequency. By using the approach of total gene synthesis, we have been able to construct these suppressor genes very rapidly. Furthermore, the manner in which the gene constructs are designed—i.e., without their natural 5′ and 3′ flanking sequences—does not preclude the synthesis of a functional tRNA. We have subsequently assembled the genes for several other suppressor tRNAs in an effort to create as large a "bank" of amber suppressors as possible (unpublished results). One potential obstacle to creating a complete collection of amber suppressors is the possibility that the anticodon of certain tRNAs is crucial for recognition by its cognate aminoacyl tRNA synthetase. We therefore developed an assay that would enable us to determine whether the anticodon had affected the specificity of the tRNA in question, by showing directly which amino acid it inserts into an amber site of a protein coding sequence. From these protein chemistry studies, it is evident that the new suppressors insert the original amino acids, phenylalanine and cysteine, at least to the limits of detection of the protein sequencing systems. Data from amino acid replacement studies in the *lac* repressor, utilizing $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$, support this conclusion. To extend the sensitivity of detection beyond the limits of protein sequencing, genetic systems similar to the one featured in Table 2 can be developed further.

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