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Exploiting unassigned codons in *Micrococcus luteus* for tRNA-based amino acid mutagenesis

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ABSTRACT

An alternative to suppression of stop codons for the biosynthetic insertion of non-natural amino acids has been developed. *Micrococcus luteus*, a Gram-positive bacterium, is incapable of translating at least two codons. One of these unused codons was inserted in a gene to act as a nonsense site. An aminoacylated tRNA was synthesized which was complementary to this codon. The gene containing the missing codon was expressed *in vitro* in a *M.luteus* transcription/translation system. Read-through of the missing codon occurred only when the complementary tRNA was included. The results demonstrate that *M.luteus* can be used for incorporation of amino acids via synthetically prepared aminoacylated tRNAs. The use of a *M.luteus* translation system provides a method for incorporation of non-natural amino acids which avoids the use of stop codons.

INTRODUCTION

Site-directed mutagenesis is one of the most powerful tools for studying structure–function relationships in proteins. The utility of this technique is impressive. Nevertheless, expansion of the repertoire of amino acids that can be incorporated into proteins beyond the 20 coded amino acids is desirable to incorporate novel residues and several biosynthetic methods have been developed for this purpose (1–3). However, each of these techniques has limitations which affect the usefulness of the systems to varying degrees. We now describe a strategy for biosynthetic incorporation of non-coded amino acids which overcomes some of the limitations of these methods. This system avoids suppression of stop codons and has the potential to introduce as many as six different site-specific mutations per protein.

Research by Hecht and co-workers has demonstrated that chemically aminoacylated tRNAs are functional for ribosomal peptide synthesis (4 and references therein). This work set the foundation for the only currently available method for ribosome-based, site-specific incorporation of non-coded amino acids which shows broad utility. The aminoacylated tRNA is designed to recognize the amber stop codon (UAG) and to deliver the attached amino acid to the growing peptide chain, thus 'suppressing' the normal function of this termination signal. The technique allows previously unobtainable changes to be made site specifically in a protein and has found use in a number of investigations (5).

There are intrinsic restrictions on the efficiency of this method however. The misacylated tRNA must compete with release factor RF1, which binds to the UAG stop codon and initiates hydrolytic cleavage of the growing peptide from the attached tRNA. As a result, the efficiency of read-through may be reduced by this native process of the host (6). Jim Wells at Genetech has observed that release is so effective at some sites that the natural function of the amber stop codon is not suppressed even when the suppressor tRNA is charged with the amino acid that normally appears at that point in the wild-type protein. This has been ascribed to a context effect. Context effects at both the nucleotide and amino acid levels have been found to affect the efficiency of termination (7–9) and regulate *in vivo* suppression of stop codons used to insert selenocysteine (10). Similar effects may be operating to mediate read-through of the amber stop codon in *in vitro* systems. Alternatively, it is possible that the nonsense mutations accelerate decomposition of the mRNA (11). The development of more efficient and widely applicable strategies is desirable in order to mitigate these limitations.

As alternatives to suppression of nonsense sites, low usage and frameshift codons (12) and unnatural nucleoside bases (13) have been tested as sites for recognition by complementary tRNAs. While novel and potentially useful in selected cases, these methods are unlikely to be used routinely to replace the suppression of a stop codon.

In order to avoid the use of stop codons as the nonsense site for insertion of an amino acid, we are investigating the potential of using other unassigned codons. Several organisms have lost the ability to translate one or more codons (14–16). These missing or unassigned codons may be useful as nonsense sites for non-natural amino acid mutagenesis, since they should alleviate poor suppression which results from competition with release factor. We have been investigating the use of unassigned codons in a *Micrococcus luteus* S-30 transcription/translation system (17). This method should provide a reliable system that will allow efficient incorporation of non-coded amino acids at any position in a protein.

Micrococcus luteus has desirable features which make it especially suitable for non-natural amino acid mutagenesis. In a sample of 5516 codons of the organism, six codons were reported to be absent (18). Isolation of all tRNAs from the organism showed that the complementary anticodons were also missing (19). Two of the missing codons (AGA and AUA) and a stop

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codon (UGA) were separately inserted into the middle of mRNA molecules coding for a peptide (20). The mRNA sequences were used as templates in *M.luteus in vitro* transcription/translation reactions. In each case the product was a truncated peptide produced by termination of ribosomal synthesis at the altered codon. This provided convincing proof that the codons are unrecognized by any available tRNAs. The behavior of the peptide formed by termination at the AGA codon was significantly different from the peptide formed by termination at the stop codon. Sucrose gradient centrifugation of the reaction mixtures showed that peptides terminating at the stop codon are released from ribosomes, whereas peptides terminating at the AGA codon remain associated with the ribosome. Release factors do not compete for the AGA codon and evidently no proteins have evolved to mimic the function of release factors at this unassigned codon. Cell-free extracts of the organism are capable of translating *Escherichia coli* genes containing the missing codons when supplemented with *E.coli* tRNAs (17).

Our investigations to date reveal that one of the missing codons in *M.luteus* can be used to insert non-natural amino acids. Further work will reveal if the other five missing codons can be used. This approach offers the possibility of performing suppression more efficiently and consistently than with stop codons. Furthermore, multiple distinct non-coded amino acids should be insertable site specifically into a protein.

MATERIALS AND METHODS

General

The phagemid pAX4a+ was purchased from US Biochemical, phagemid pALTER from Promega and plasmid pET14b from Novagen. *Micrococcus luteus* (IFO 3333) and the *E.coli* strain MRE600 were obtained from the ATCC. Restriction enzymes were purchased from Promega, US Biochemical and New England Biolabs. T4 DNA polymerase and T4 DNA ligase were from Promega and New England Biolabs. *Taq* polymerase was obtained from Stratagene and Boehringer-Mannheim. The Sequenase 2.0 kit including 7-deaza-dGTP and dITP reagents was obtained from US Biochemical. [^{35}S]Methionine and [3,4,5-^{3}H(*N*)]leucine were purchased from New England Nuclear. Transcription grade nucleotide triphosphate solutions were obtained from Pharmacia and deoxynucleotide triphosphate solutions were from New England Biolabs. Oligonucleotides were synthesized by Integrated DNA Technologies Inc. Peptides were synthesized and analyzed at the W.M.Keck Biotechnology Resource Center at Yale University. Chemicals were of molecular biology or enzyme grade and were obtained from Sigma and Fluka. Acrylamide and bis-acrylamide were from BioRad. HPLC grade acetonitrile and trifluoroacetic acid were purchased from Fisher. Dialysis tubing (12 000–14 000 mol. wt cut-off) was from SpectraPor. Centricon spin filters were obtained from Amicon. Ultrafree spin filters (45 µm) were purchased from Millipore. Liquid scintillation counting was performed using Packard Optifluor scintillant and samples were counted on a Beckman LS5801 counter. Peptides were analyzed on a Dynamax 300 Å, 4.6 mm i.d. × 25 cm C-18 column. *Micrococcus luteus* cells were lysed with a BraunSonic 2000 sonicator. The plasmid pAR1219 containing the gene for T7 RNA polymerase was obtained as a gift from W.Studier (Brookhaven National Laboratory). T7 RNA polymerase was purified from this clone in *E.coli* strain BL21(DE3) as described (21). Total *M.luteus* tRNA was isolated following a procedure described by Osawa on a Rainin 7 µm PureGel strong anion exchange column (19).

Template synthesis

The gene containing the codon AGA was prepared by solid phase synthesis. In order to add restriction sites for cloning and to provide more material the sample was amplified by PCR. The PCR product was digested with *Nco*I and cloned into the vector pAK321. pAK321 consists of the *Bgl*II–*Eco*RV fragment of pET14b cloned into phagemid pAX4a+ with the *Nde*I fragment removed. This construct lacks the *lac* promoter region as well as a major portion of the β-galactosidase gene.

Desired transformants were screened by digestion with *Nru*I and verified by dideoxy sequencing. Mutation of the AGA codon to a TAG nonsense codon was performed using single-stranded DNA and a single mutagenic primer. Mutant colonies were identified by sequencing.

Construction of aminoacylated tRNAs

DNA with the sequence of the desired amber suppressor tRNA containing an upstream T7 promoter was synthesized by solid phase methods. The DNA was cloned into pSP64 for transcription. For mutagenesis of the sequence the DNA was subcloned into pALTER and mutagenesis was performed with the Altered Sites mutagenesis procedure as described by the manufacturer. The DNA templates in pSP64 were digested with *Bst*XI and blunt ended with T4 DNA polymerase in order to reduce template-dependent addition of extra bases during transcription with T7 RNA polymerase (22,23). The transcription products were purified on a denaturing acrylamide gel. The size of the correct transcript was determined by performing controlled hydrolysis of 5′-end-labeled material (24,25). Ligation of the dinucleotide, which had been previously aminoacylated with Phe, was performed with T4 RNA ligase as described (26). Gel electrophoresis indicated almost quantitative conversion to the ligated product.

Preparation of the *M.luteus* S30 extract

Cells were harvested in mid log to late log phase; corrected OD_{600} readings were 3–6 units. The flasks were chilled to 4°C and cells were harvested by pelleting in centrifuge buckets which had been peroxide treated (8000 *g*, 15 min). The collected cells were washed once with a large volume of S30 buffer (20 mM Tris–acetate, pH 8, at room temperature, 97.5 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.125 mM EDTA, pH 8.0, 1 mM DTT) and stored at –80°C overnight.

The cells (13 g) were thawed at 4°C and resuspended in S30 buffer in a 50 ml Falcon tube at a final concentration of 1 ml/g. Lysozyme was added to a concentration of 2.5 mg/ml and the sample was incubated at 15°C for 3 min. The cells were lysed with 7–10 bursts of sonication (output 37 W) for 10 s each, spaced at 1 min intervals. The tube was kept on ice during the procedure.

Following lysis, DTT and AEBSF were added to final concentrations of 1 mM each. The lysate was centrifuged twice in peroxide-treated Oakridge tubes to remove cellular debris (27 000 *g*, 35 min). The clear yellow supernatant was collected and dialyzed against S30 buffer (3 × 200 ml) at 4°C with 1 h between changes. The sample was centrifuged (27 000 *g*, 15 min)

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                ATG GGC TGG ATC GCG ATG GGC TAC GCC CTG ATC GGG NNN GCT GTA CGG CCT GTG ATC TGA CCA TGG
RT              Met Gly Trp Ile Ala Met Gly Tyr Ala Leu Ile Gly Phe Ala Val Arg Pro Val Ile *
FS              Met Gly Trp Ile Ala Met Gly Tyr Ala Leu Ile Gly Phe Leu Tyr Gly Leu *
TR              Met Gly Trp Ile Ala Met Gly Tyr Ala Leu Ile Gly *
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Figure 1. DNA templates used for incorporation of amino acids in *M.luteus* cell-free extracts. The NNN codon is either TAG or AGA. The amino acid sequences of the possible translation products are shown below. RT, read-through product; FS, frameshift product; TR, termination product. If the nonsense (TAG) or unassigned (AGA) codon is translated in-frame the stop codon (double underlined) is used. If frameshifting occurs the out of frame stop codon (single underline) is used.

and the supernatant (9 ml) collected. The sample was concentrated over a Centricon filter (10 000 mol. wt cut-off) to a final volume of 1.3 ml and stored in aliquots at –80°C.

Translation reactions and analysis of peptides

Following ligation of the dinucleotide or acylated dinucleotide to the truncated tRNA, the sample was divided and the portions were ethanol precipitated. Each of the resulting pellets was dried. Transcription/translation reactions were set up in duplicate in a total volume of 40 µl. Each reaction contained 12 µl S30 extract, 10 µg synthetically prepared tRNA, 35 mM Tris–OAc, pH 8, 2.2 mM DTT, 2 mM ATP, pH 7, 0.5 mM CTP, pH 7, 0.5 mM GTP, pH 7, 0.5 mM UTP, pH 7, 0.5 mM each of 19 amino acids (no methionine), 0.24 µM [^{35}S]methionine (11 µCi), 20 mM phosphoenolpyruvate, pH 7, 1 mM cAMP, pH 7, 0.02 mg/ml folinic acid, 10 mg/ml *M.luteus* tRNAs (deacylated), 27 mM NH_4OAc, 27 µM NADP, 0.10 mM pyridoxine–HCl, 24 µM FAD, 63 µM *p*-aminobenzoic acid, 84 mM potassium glutamate, 0.05 mg/ml rifampicin, 1.5 µg template DNA and 700 U T7 RNA polymerase. Magnesium concentrations were optimized for each synthetic tRNA with magnesium acetate. Optimum concentrations were typically between 9 and 13 mM.

Each reaction was incubated at 37°C for 1 h on an orbital shaker and quenched with 660 µl 1 M NaOH. After 2 min incubation at room temperature carrier peptides (20 µg each) were added and the solution was extracted three times with 250 µl $CHCl_3$:CH_3CN (1:1). The sample was centrifuged to break up emulsions (10 000 *g*, 15 min). The organic layers and the white solid which formed at the interface of the solvents were combined and the solvent was evaporated under a stream of nitrogen.

The samples were dissolved in trifluoroacetic acid (100 µl), water (50 µl) and acetonitrile (50 µl). The sample was spin-filtered and heated to 55–65°C for 2 min immediately before analysis. HPLC separation was performed on a C18 column with an elution gradient of from 20% CH_3CN/0.4% TFA in H_2O/0.5% TFA to 100% CH_3CN/0.4% TFA over 53 min at a flow rate of 1 ml/min. Elution profiles were monitored at 220 nm and fractions were collected at 1 min intervals. The total radioactivity of each fraction was determined by counting 750 µl aliquots.

RESULTS AND DISCUSSION

To determine if unassigned codons in *M.luteus* are suitable for incorporation of amino acids with a synthetically aminoacylated tRNA we prepared two DNA templates (Fig. 1). The templates contain a T7 RNA polymerase promoter followed by sequences which are identical except for codon 13. In one of the templates this position is a TAG nonsense codon. The other template contains an AGA (Arg) codon at the same position. The AGA codon is unrecognized in *M.luteus*. Two aminoacylated tRNAs,

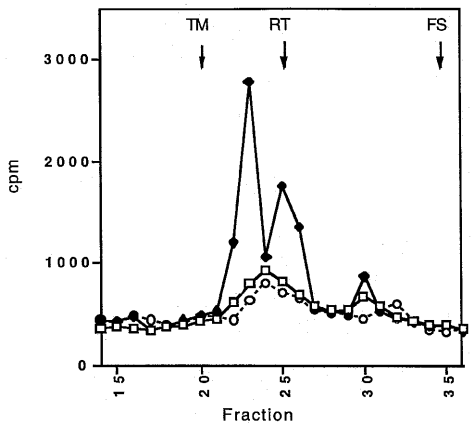

Figure 2. Insertion of Phe at a TAG nonsense codon in *M.luteus*. ○, no tRNA; □, unacylated tRNA; ◆, acylated tRNA. Arrows indicate elution fraction of synthesized peptides added as carrier. HPLC analysis: C18 column, linear gradient of 20–100% CH_3CN, 0.5%TFA in H_2O over 53 min, flow 1 ml/min.

each containing an anticodon complementary to the nonsense codon in one of the templates, were synthesized by ligation of the product from run-off transcription of a DNA template with a chemically synthesized dinucleotide aminoacylated with phenylalanine. The tRNA sequence is based on the sequence of $tRNA^{Ala}$. Changes in the anticodon loop were made to complement the UAG or AGA codons and to make the tRNA more efficient during translation (27). A G-U base pair in the acceptor stem was also changed to eliminate charging of the tRNA with alanine by an aminoacyl synthetase (28,29).

The *M.luteus* transcription/translation system was prepared by modifying previously described procedures (20,30). The DNA templates were added to *M.luteus* transcription/translation reaction mixtures containing T7 RNA polymerase and either no tRNA, unacylated tRNA or aminoacylated tRNA complementary to the altered codon. [^{35}S]Methionine was used to label peptide products. After termination and addition of previously synthesized carrier peptides the reactions were extracted with 1:1 $CHCl_3$:CH_3CN and analyzed by reverse phase high performance liquid chromatography. With either template the read-through peptide is produced only when acylated tRNA is added to the reaction mixture, indicating that Phe has been incorporated (see Figs 2 and 3).

The amount of labeled material synthesized in these reactions was compared with the amount of labeled protein synthesized from the AGA-containing template when the *M.luteus* extract was complemented with *E.coli* tRNAs. *Escherichia coli* tRNAs are capable of translating the codons which are unrecognized in *M.luteus* (30), thus producing read-through peptide. The sup-

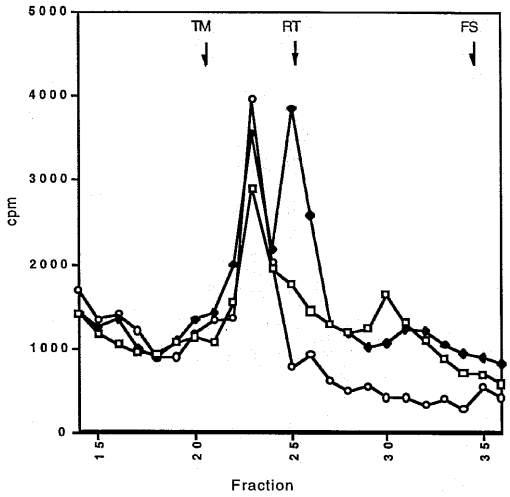

Figure 3. Insertion of Phe at a AGA codon in *M.luteus*. ○, no tRNA; □, unacylated tRNA; ◆, acylated tRNA. Arrows indicate elution fraction of synthesized peptides added as carrier. HPLC conditions as in Figure 2.

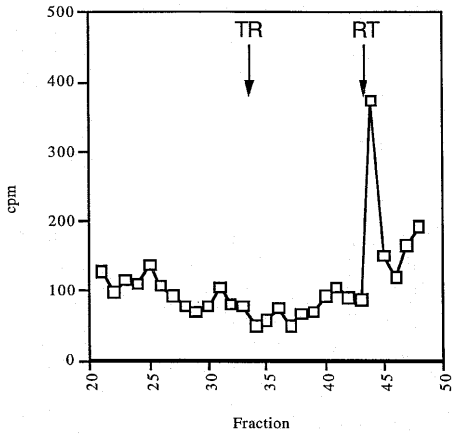

Figure 4. Incorporation of [^{3}H]leucine into peptide in *E.coli* transcription/translation extracts. Arrows indicate elution fraction of synthesized peptides added as carrier. HPLC analysis: C18 column, linear gradient of 20–100% CH_3CN, 0.5%TFA in H_2O over 103 min, flow 1 ml/min.

pression efficiencies of the AGA and TAG codons were 72 and 53% respectively.

In each case additional peaks, which do not correspond to any of the expected peptide products, are observed in the HPLC profile. Background incorporation of methionine into pre-exisiting proteins by a ribosome-independent process has been observed in other *in vitro* translation systems (31). The level of expression in these systems is highly dependent on salt concentrations, in particular the concentration of magnesium. The magnesium concentration in the reactions was optimized for each DNA template. The intensity of background peaks may be due to variations in the effective magnesium concentration, which is affected by the amount of magnesium in the buffer, the DNA template and the tRNA species.

To determine whether the additional peaks are unique to the methionine label two experiments were performed. The template containing the arginine codon AGA was used in *E.coli* transcription/translation reactions. One experiment used [^{35}S]methionine, as had been the case in the *M.luteus* system. A second reaction was run with [^{3}H]leucine as the radiolabeled amino acid. When [^{35}S]methionine was used three radioactive peaks eluted during the HPLC run (data not shown). In contrast, when the experiment was repeated with [^{3}H]leucine only a single radioactive peak co-eluting with carrier read-through peptide was observed (see Fig. 4). These results demonstrate that additional products are isolated when [^{35}S]methionine is used as the radiolabel, as has been observed in rabbit reticulocytes.

Although peaks corresponding to the termination peptide were not observed this may be due to low yields or to rapid degradation of the smaller peptide. We have found that translated peptides undergo rapid degradation in this and other cell-free systems. Because the lower specific activity of [^{3}H]leucine provides insufficient dynamic range for quantitative assays, future work will be directed to expression of larger proteins that can be analyzed by gel electrophoresis or by catalytic activity.

The results indicate that the AGA codon can be utilized for insertion of amino acids with a *M.luteus* expression system. The efficiency of suppression of TAG codons depends on a number of factors, including the sequence of the suppressor tRNA and the structure of the amino acid to be inserted (32). In order to determine the relative suppression efficiency at TAG and AGA codons a variety of natural and non-natural amino acids and tRNA sequences will be tested. Background degradation of the peptides in the translation mixture precludes using them in quantitative studies. We are now designing an assay using larger proteins that are stable in *in vitro* translation systems in order to quantitate insertion of natural and non-natural amino acids at these codons. These studies will allow us to provide more definitive evidence as to the efficiency of the AGA codon as well as other unused codons from *M.luteus*.

We have demonstrated that it is possible to use synthetically aminoacylated tRNAs to recognize an unassigned codon in *M.luteus*. The insertion of an amino acid at the AGA codon is more efficient than at the TAG codon. Future work will determine whether multiple and different non-natural amino acids can be inserted as well as to investigate the possibility of incorporating amino acids at nonsense sites in proteins where suppression of stop codons fails completely. *Micrococcus luteus* may also prove to be a good system in which to perform *in vivo* non-natural mutagenesis. The efficiency and applicability of this system for routine introduction of non-natural amino acids into proteins is under investigation.

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