Site-Specific Insertion of Spin-Labeled L-Amino Acids in *Xenopus* Oocytes†

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Received August 27, 2003; Revised Manuscript Received April 27, 2004

ABSTRACT: Site-specific insertion of modified amino acids in proteins expressed in living cells is an emerging field holding great promise for elucidating protein structure−function relationships, expression levels, localization, and activation states in a complex milieu. To evaluate the efficiency of amino acids modified to carry either a nitroxide spin probe or a fluorescence probe, we have developed a screen using the levels of functional luciferase protein expressed in *Xenopus* oocytes. Natural and modified amino acids were targeted to position 14 in firefly luciferase using an amber mutation or introducing the four-codon nucleotide GGGU. Using the amber stop codon, the incorporation efficiencies of injected tRNA charged with the native phenylalanine residue, a fluorescent NBD-alanine, or nitroxide-labeled cysteine and tyrosine amino acids ranged from 1% to 18%. While the NBD-amino acid derivative gave higher incorporation levels, the EPR signals from the spin-labeled amino acids allow for the direct assessment of aminoacylation extent and stability. Applying the four-base codon for the first time in *Xenopus* oocytes, we found the incorporation efficiencies were significantly lowered compared to results using the three-base amber codon. The studies presented here provide quantitative assessment of protein expression levels when using nonsense suppression to site-specifically label proteins with spectroscopic probes in oocytes. Finally, the effect of a 77-base RNA aptamer known to inhibit the eucaryotic release factor of protein synthesis was tested for its influence on nonsense incorporation in *Xenopus* oocytes. The combination of A34 and charged suppressor tRNA produced a 3-fold increase in the expressed TAG^{14}-luciferase level, compared to the use of charged suppressor tRNA alone.

Because of the high reactivity and specificity of sulfhydryl chemistry, conventional site-directed labeling of proteins most commonly relies on cysteine substitution of targeted residues. These experiments are carried out on a protein background where any native positions containing a reduced cysteine side chain have been replaced by a nonreactive side chain, usually serine or alanine. Following purification of the recombinant protein, the cysteine residues are modified with a reporter label probe and examined by spectroscopy or microscopy. Unfortunately, this widely used and productive approach is limited in its application. The requirement of purification prior to label attachment makes the examination of the labeled protein in situ difficult, since the reconstitution of a complex biological context (e.g., a native biological membrane) may not be practical. Furthermore, the silencing of native Cys residues may affect protein function, or be too laborious in very large proteins.

The use of mutant tRNA containing a CUA anticodon for suppression of the UAG amber stop codon has developed into the established approach of tRNA-mediated protein engineering (TRAMPE)[1] (for a review see ref *1*). TRAMPE is a powerful approach that can facilitate the examination of protein structure and function in the context of a cell environment, involving a single side chain modification to the protein molecule. The use of TRAMPE for the site-specific insertion of unnatural amino acids into proteins has been achieved using in vitro translation systems, including bacterial S-30 extracts (*2*−*4*), wheat germ extract (*5*, *6*), and rabbit reticulocyte lysate (*7*−*9*).

However, due to complications from membrane trafficking and insertion, in vitro systems are problematic for the study of integral membrane proteins. For membrane proteins modification by TRAMPE has been achieved in live *Xenopus* oocytes. *Xenopus* oocytes are advantageous for TRAMPE, because exogenous tRNA and mRNA can be conveniently injected into these cells. The large surface area of oocytes also facilitates the functional assessment of heterologous proteins involved with, or coupled to, transport or conductance events. Furthermore, the ability of oocytes to synthesize, fold, traffic, and provide posttranslational modifications

† This work was supported by a grant from the UC Davis Health System Research Fund, and in part from a March of Dimes research award to J.C.V. K.H. acknowledges the support of grants from the Hungarian National Research Foundation (OTKA T34307 and M 028226).

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[1] Abbreviations: CUA-tRNA, tRNA containing the anticodon for the amber stop mutation; CUA-$tRNA^{xxx}$, tRNA containing the anticodon for the amber stop mutation charged with the indicated amino acid; ACCC-$tRNA^{xxx}$, tRNA containing the anticodon for the GGGU mutation charged with the indicated amino acid; ^{UAG14}mRNA, mRNA transcript containing the UAG mutation at position 14; ^{GGGU14}mRNA, mRNA transcript containing the GGGU mutation at position 14; TRAMPE, tRNA-mediated protein engineering; DCM, dichloromethane; DAP, diaminopropionic acid, EPR, electron paramagnetic resonance; TLC, thin-layer chromatography. A34, aptamer 34; NVOC-Cl, 6-nitroveratryloxycarbonyl chloride.

10.1021/bi035542i CCC: $27.50
Published on Web 06/08/2004

makes them suitable for the examination of membrane proteins. Applications include the incorporation of biotinylated and other unnatural amino acids for probing binding and topology in the nicotinic acetylcholine receptor membrane (*10*, *11*) and ion channels (*12*−*14*). And in the substance K receptor, fluorescence spectroscopy has been used to examine the protein containing fluorescent amino acids, with the protein residing in native oocyte membranes (*15*, *16*).

To address the limitations of TRAMPE in proteins, we have made a quantitative assessment of factors that may affect the efficiency of incorporation for amino acids containing biophysical probe side chains. These factors include the influence of release factors on nonsense stop codons (UGA, UAA, UAG). Termination of protein synthesis is manifested by a release factor known as the eukaryotic class 1 release factor (eRF1) in eukaryotes and RF1 and RF2 in prokaryotes (for a review see ref *17*). The crystal structure of human eRF1 strongly resembles that of a charged tRNA, and it is thought that the release factor is able to fit into the A site of the ribosome, causing hydrolysis of the peptide bond and deacylation of the charged tRNA (*18*). Carnes et al. (*19*) have developed a small RNA molecule known as aptamer 34 (A34), using selection amplification, and have demonstrated that the aptamer has a selective affinity for eRF1 (*20*−*22*). The aptamer was shown to block the function of the release factor as judged by increases of amber readthrough of a message translated in vitro. Here, using the *Xenopus* oocyte expression system, we evaluate the ability of A34 to inhibit the action of release factor at engineered stop codons in vivo. Our results indicate the aptamer may be useful in improving the application of TAG/suppressor technology (*23*−*28*). These findings also suggest small aptamers such as A34 may be useful for other in vivo applications, such as the suppression of naturally occurring nonsense mutations.

MATERIALS AND METHODS

Collagenase and bacitracin were purchased from Sigma. The protease inhibitor cocktail was from Calbiochem. NAP-5 desalting columns were purchased from Amersham. Acid phenol chloroform (5:1) was from Ambion. NBD-F was purchased from Molecular Probes. L-Cysteine methyl ester hydrochloride, L-tyrosine methyl ester hydrochloride, and 4,5-dimethoxy-2-nitrobenzyl chloroformate (NVOC-Cl) were purchased from Aldrich. All restriction enzymes and T4 RNA ligase were acquired from New England Biolabs.

Mutagenesis of Luciferase. pSP6.LUC (Promega) was used as a template in a PCR to remove the luciferase gene (*Photinus pyralis*) using the primers tccaccatggaagacgccaaaaacataaagaaaggcc and cactctcgagttacaatttggactttccgccc. The PCR fragment was cut with *Nco*I and *Xho*I and cloned into the transcription vector pAMV (kind gift of the Cesar Labarca) to give pAMV.LUC. To introduce an amber mutation, the codon TTC encoding for phenylalanine at position 14 was mutated to TAG using the primers gaaaggcccggcgccaTAGtatcctctagaggatg and catcctctagaggataCTAtggcgc-cgggcctttc, where the uppercase letters denote the nonsense mutation. Mutagenesis was performed in accordance with the Quickchange manual (Stratagene). For the luciferase gene encoding the four-base codon at position 14, GGGT was introduced as described above for the amber mutation. Three natural GGGT sequences were first removed by silent mutagenesis. All PCR steps were verified by DNA sequencing. The pAMV constructs were linearized with *Not*I, purified by phenol chloroform isoamyl alcohol, 25:24:1 (pH 6.7), ethanol precipitated, resuspended to a concentration of 1 μg/μL in DEPC-treated water, and stored until use for transcription at −20 °C.

Preparation of Aptamer DNA for Transcription. The plasmid PSZA34 and the random pool PCR template were kind gifts of Jason Carnes, as well as the primers required to make a PCR template that would be used to make batches of DNA for transcription. To make a random pool DNA template for transcription, the PCR DNA template was amplified using the primers previously described (*19*). To generate the DNA template used for transcription of aptamer 34 (A34), pSZA34 was digested with *Bam*HI and *Hin*dIII. This DNA fragment was gel purified and then amplified by PCR using the primers above. Both the PCR reactions for the random pool and the A34 were directly precipitated with 0.1 volume of 3 M NaOAc (pH 5) and 2 volumes of ethanol at −20 °C for 10 min, centrifuged, and dried. The pellet was then resuspended in DEPC-treated water and stored until use at −20 °C.

Preparation of Suppressor tRNA and tRNA$_{ACCC}$. The gene for the natural amber suppressor from *Tetrahymena thermophila* (tRNA^{Gln}) was provided in the pTHG73 plasmid (kind gift of Henry Lester). To create an anticodon with four bases, ACCC, the CUA anticodon found in the suppressor tRNA^{Gln} gene was mutated to CCC and an extra A was inserted just 5′ of the first C by a single round of mutagenesis using Quikchange to generate the construct ptRNA$_{ACCC}$ (see Figure 5), and the mutation confirmed by DNA sequencing. Both pTHG73 and ptRNA$_{ACCC}$ were linearized with either *Bsa*I or *Fok*I and purified as described for the pAMVLUC constructs. These templates can be used to transcribe full-length (using *Bsa*I) suppressor tRNA or the transcript missing the last two bases (−CA, using *Fok*I).

Generation of Aptamer, tRNA, and mRNA Transcripts. A34 and random pool of RNA aptamers and tRNA were generated by transcribing the templates described above using the T7 megashortscript kit (Ambion). The transcription reactions were carried out according to the manufacturer's instructions. The products were purified by phenol/chloroform, 5:1 (pH 4.5), extraction and ethanol precipitation followed by desalting on NAP-5 columns. The RNA was then aliquoted into 10 μg amounts, lyophilized, and stored at −80 °C. Luciferase mRNA was transcribed using the mMessage Machine (Ambion), purified using the RNeasy minikit (Qiagen), desalted, and stored as described above. The RNeasy minikit was not used for the smaller RNA because it binds only RNA 200 bases or larger in size.

Synthetic Acylation of the Suppressor tRNA. Synthesis of *N*-(6-nitroveratryloxy)-L-phenylalanine was carried out as previously described with some modifications (*16*, *28*). Briefly, 3 mmol (495 mg) of L-phenylalanine and 3 mmol of Na_2CO_3 (317 mg) were dissolved in 10 mL of water. In an equal volume (10 mL) of anhydrous dioxane (preheated to 37−40 °C), 3 mmol of NVOC-Cl (825 mg, 1 equiv) was dissolved completely. The organic phase was slowly dissolved in the aqueous phase and the resulting solution stirred at ambient temperature overnight. The reaction was diluted with 1 volume dichloromethane (DCM) and 1 volume of 1

N sodium bisulfate, extracted with equal volume DCM washes, and dried in vacuo. The product was then crystallized in ethyl acetate/hexane (1:1) to give a yield of 28%.

To activate the carboxyl group with a cyanomethyl ester, 610 μmol of *N*-(6-nitroveratryloxy)-L-phenylalanine (250 mg) was dissolved in 38 mL (610 mmol, 1000 equiv) of chloroacetonitrile. To this mixture was added 3.42 mL of dry triethylamine (24.4 mmol, 40 equiv). The reaction was stirred at ambient temperature for 30 h. The reaction was dried in vacuo and then redissolved in an equal volume of DCM and water. The organic phase was washed extensively with water and dried over sodium sulfate. The product was evaporated to a minimal volume and purified using flash silica chromatography using a 1−5% gradient of acetone in DCM. The product was confirmed by proton NMR with a total yield of 91%.

Synthesis of the Fluorescent Amino Acid (NBD-Alanine). To remove the Fmoc group from 2-Fmoc-3-tBOC-diaminoproprionic acid (DAP), exactly 870 mg (2 mmol) of 2-Fmoc-3-tBOC-DAP was stirred at ambient temperature into 10% piperdine in DMF for 4 h. The reaction was complete as determined by thin-layer chromatography (TLC solvent system: 1% AcOH, 10% methanol in DCM) when the starting compound was no longer present. The reaction was diluted with 3 volumes of water and dried overnight. The white powder was rinsed with 100−200 mL of methylene chloride to remove trace Fmoc impurities for an 84% yield. Proton NMR confirmed complete removal of the Fmoc group to give 3-tBOC-DAP.

Addition of the NVOC (6-nitroveratryoxycarbonyl) protecting group to the α amine group of DAP was achieved by combining 6 mL of 0.3 M Na_2CO_3 (1 equiv) with 1.68 mmol of 3-TBOC-diaminoproprionic acid. In 5.5 mL of preheated dioxane (42 °C), 465 mg (1 equiv) of NVOC-Cl was dissolved. The organic phase was slowly added to the aqueous mixture, and the reaction was stirred until completion as judged by the loss of ninhydrin staining by TLC (solvent system: 1% AcOH, 10% methanol in DCM). The reaction was diluted with 3 volumes of DCM and acidified with 1 volume of $NaHSO_4$. The aqueous phase was washed three times with an equal volume of DCM. The organic phase was pooled, dried with sodium sulfate, and lyophilized. The dry white powder was dissolved in a minimal volume of DCM and purified by preparative TLC (solvent system: 10% MeOH in DCM). The product was confirmed by proton NMR with a total yield of 27%.

To remove the *t*BOC group, exactly 1974 μmol of 2-NVOC-3-tBOC was dissolved in minimal TFA for 2 min, precipitated in excess ether, and filtered to give a yield of 99%.

The addition of NBD to the β amine group of 2-NVOC-L-DAP was carried out by partially dissolving ~104 mg (289 μmol) of 2-NVOC-L-DAP in 3 mL of DMF. The solid was fully dissolved by adding dropwise 100 mM sodium borate (pH 9, ~4.5 mL of total buffer). In 2 mL of acetonitrile, 75 mg (1.4 equiv, 405 μmol) of NBD-fluoride was dissolved. The organic phase was added to the aqueous phase and the mixture stirred for 1 h at ambient temperature. The reaction was completely lyophilized and diluted in 40 mL of 1 M $NaHSO_4$, and the product was extracted with three washes of DCM (40 mL), dried over $NaSO_4$, and purified by flash silica chromatography (1−10% MeOH in chloroform). Fractions were pooled, and the product was confirmed by proton NMR for a total yield of 37%.

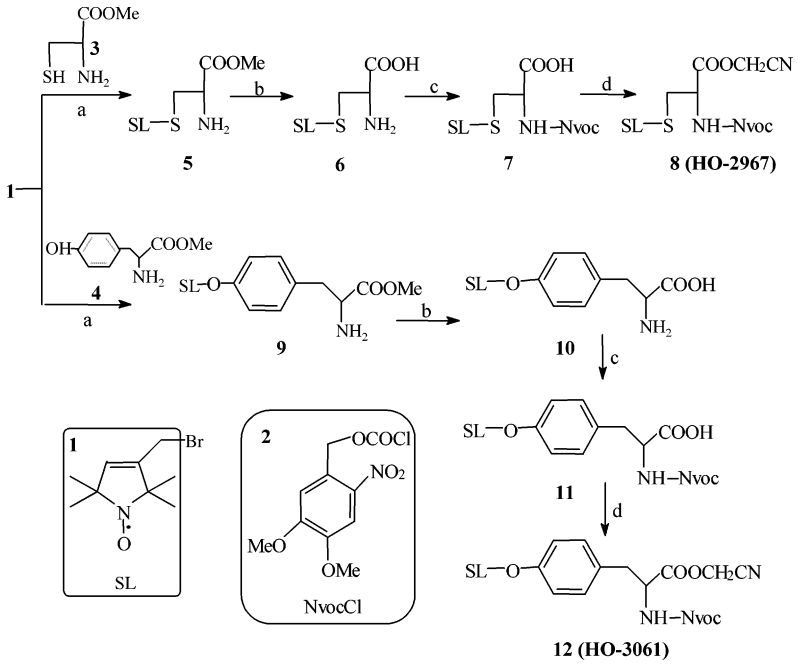
Synthesis and purification of 2-NVOC-3-NBD-L-DAP acid cyanomethyl ester (NBD-NVOC-DAP-CME) were carried out in the manner described for the synthesis of NVOC-PHE-CME, giving a total yield of 90%. The product was confirmed by proton NMR.

Synthesis of Spin-Labeled Amino Acids. Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on a Carlo Erba EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a VG TRIO-2 instrument in the EI mode (70 eV, direct inlet) or with a thermospray technique. Samples were analyzed in the bypass mode. The sample solution in CH_3OH (10 μL) was introduced via the thermospray interface. The mobile phase was CH_3OH/H_2O, 1:1 solution containing 0.1 M NH_4OAc. The capillary tip temperature was 230 °C, the electrode voltage was 180 V, and the source temperature was 210 °C. EPR spectra were obtained from 10^{-5} M solutions ($CHCl_3$), using a Bruker ECS-106 spectrometer. Preparative flash column chromatography was performed on Merck Kieselgel 60 (0.040−0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF_{254}. All monoradicals exhibited three equally spaced lines with a_N = 15.1−15.5 G. Compound **1** (see Scheme 1) was prepared according to a published procedure (*29*), and compound **8** (*4*) was published as an end product without synthetic details.

Synthesis of 2-Amino-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethylsulfanyl)propionic Acid Methyl Ester (5) and 2-Amino-3-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethoxy)phenyl]propionic Acid Methyl Ester (9). To a stirred solution of L-cysteine methyl ester hydrochloride (1.71 g, 10.0 mmol) or L-tyrosine methyl ester hydrochloride (2.31 g, 10.0 mmol), 2.76 g (20.0 mmol) of K_2CO_3, and 18-crown-6 (100 mg, 0.37 mmol) in dioxane (20 mL) was added compound **1** (2.33 g, 10.0 mmol), and the mixture was stirred and refluxed for 6 h. The inorganic salts were filtered off, the dioxane was removed under reduced pressure, the residue was dissolved in $CHCl_3$ (40 mL) and washed with brine (20 mL), the organic phase was separated, dried ($MgSO_4$), and filtered, and the organic solvent was evaporated. The residue was purified by flash column chromatography ($CHCl_3/Et_2O$) to give the title compound **5** (1.67 g, 58%) as an oil. Anal. Calcd for $C_{13}H_{23}N_2O_3S$: C, 54.33; H, 8.07; N, 9.75; S, 11.16. Found: C, 54.20; H, 7.99; N, 9.57; S, 11.01. MS: *m*/*z* (rel intens) 287 (M^+, 4), 257 (9), 138 (20), 88 (100). Compound **9** was obtained as an oil (1.91 g, 55%). Anal. Calcd for $C_{19}H_{27}N_2O_4$: C, 65.67; H, 7.84; N, 8.07. Found: C, 65.49; H, 7.77; N, 8.00. TSP $[M + H]^+$: *m*/*z* 348.

Synthesis of 2-Amino-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethylsulfanyl)propionic Acid (6) and 2-Amino-3-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethoxy)phenyl]propionic Acid (10). A mixture of compound **5** (2.0 g, 6.95 mmol) or compound **9** (2.41 g, 6.95 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (2.24 g, 7.1 mmol) in MeOH (35 mL) was refluxed for 3 h. Then the solution was neutralized by dry ice, the inorganic salt was filtered off, the filtrate was evaporated, and the residue was purified by flash column chromatography ($CHCl_3$/MeOH) to yield free

Scheme 1: Summary of Spin-Labeled l-Amino Acid Synthesis[a]



[a] Following the addition of the nitroxide moiety to the side chain, the α amino groups are protected using NVOC-Cl to generate the Cys (HO-2967) or Tyr (HO-3061) versions: (a) K_2CO_3, 18-crown-6 (catalytic), dioxane, 80 °C, 6 h, yields 58% for **5** and 55% for **9**; (b) MeOH, $Ba(OH)_2 \cdot 8H_2O$ reflux 3 h, then CO_2, yields 39% for **6** and 45% for **10**; (c) TEA, NVOC-Cl, acetonitrile, 0 °C → rt, 1 h, yields 68% for **7** and 73% for **11**; (d) $ClCH_2CN$, TEA, acetonitrile, rt, 24 h, yields 48% for **8** and 56% for **12**.

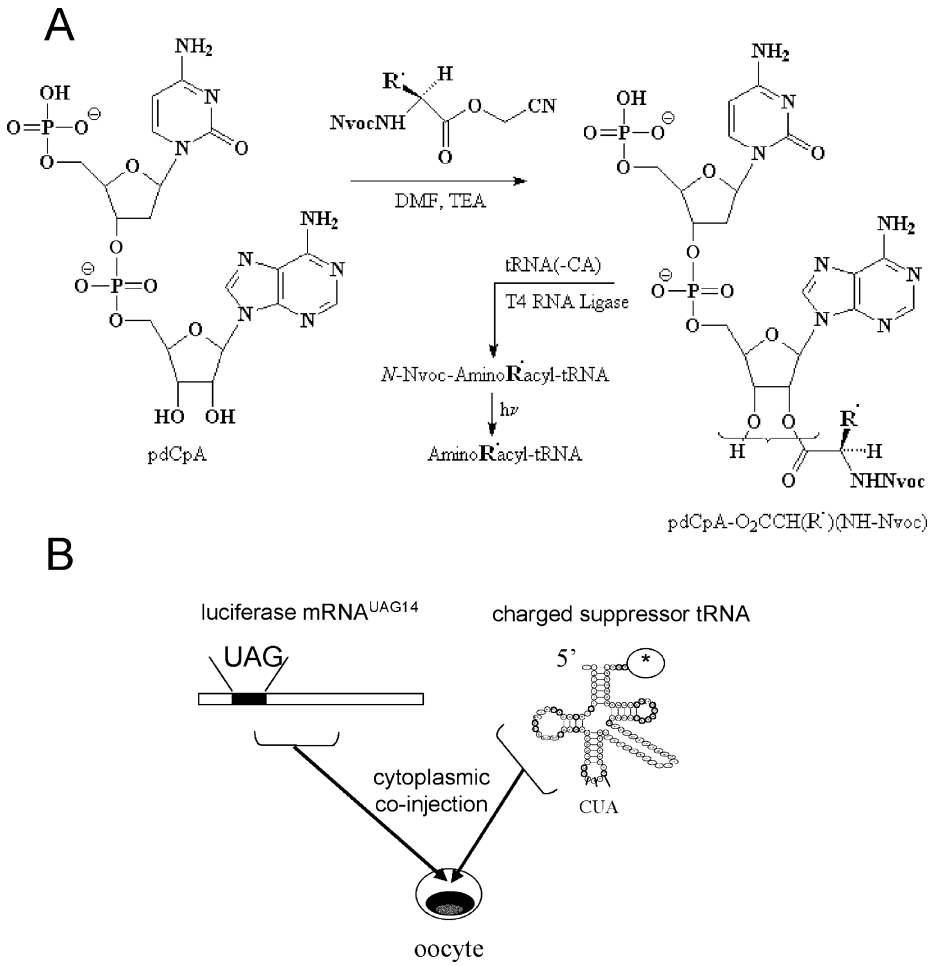
amino acid **6** (spin-labeled cysteine) **(**740 mg, 39%) or **10** (spin-labeled tyrosine) **(**1.04 g, 45%). The following are data for **6**. Mp: 101−103 °C. Anal. Calcd for $C_{12}H_{21}N_2O_3S$: C, 52.73; H, 7.74; N, 10.25; S, 11.73. Found: C, 52.65; H, 7.86; N, 10.20; S, 11.55. TSP $[M + H]^+$: *m*/*z* 274. The following are data for **10**. Mp: 186−188 °C. Anal. Calcd for $C_{18}H_{25}N_2O_4$: C, 64.84; H, 7.56; N, 8.40. Found: C, 69.00; H, 7.55; N, 8.42. TSP $[M + H]^+$: *m*/*z* 334.

Synthesis of 2-(4,5-Dimethoxy-2-nitrobenzyloxycarbonylamino)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethylsulfanyl)propionic Acid (7) and 2-(4,5-Dimethoxy-2-nitrobenzyloxycarbonylamino)-3-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethoxy)phenyl]propionic Acid (11). To a stirred solution of amino acid **6** (1.36 g, 5.0 mmol) or **9** (1.66 g, 5.0 mmol) and Et_3N (555 mg, 5.5 mmol) in anhydrous acetonitrile (20 mL) was added dropwise at 0 °C NVOC-Cl (1.51 g, 5.5 mmol) dissolved in acetonitrile (10 mL), and then the mixture was stirred at room temperature for 1 h. The acetonitrile was evaporated off, and the residue was dissolved in $CHCl_3$ (20 mL) and washed with brine (10 mL). The organic phase was then dried ($MgSO_4$), filtered, and evaporated, and the *N*-protected amino acids were purified by chromatography. For **7** ($C_{22}H_{30}N_3O_9S$) the yield was 1.74 g (68%). Mp: 68−70 °C. Anal. Calcd for $C_{22}H_{30}N_3O_9S$: C, 51.56; H, 5.90; N, 8.20; S, 6.26. Found: C, 51.50; H, 5.81; N, 8.13; S, 6.10. TSP $[M + NH_4]^+$: *m*/*z* 530. For **11** ($C_{28}H_{34}N_3O_{10}$) the yield was 2.08 g (73%). Mp: 160−162 °C. Anal. Calcd for $C_{28}H_{34}N_3O_{10}$: C, 58.73; H, 5.99; N, 7.34. Found: C, 58.68; H, 5.96; N, 7.22. TSP $[M + NH_4]^+$: *m*/*z* 590.

Synthesis of 2-(4,5-Dimethoxy-2-nitrobenzyloxycarbonylamino)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethylsulfanyl)propionic Acid Cyanomethyl Ester (8) and 2-(4,5-Dimethoxy-2-nitrobenzyloxycarbonylamino)-3-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethoxy)phenyl]propionic acid Cyanomethyl Ester (12). To a stirred solution of compound **7** (1.02 g, 2.0 mmol) or compound **11** (1.14 g, 2.0 mmol) and Et_3N (222 mg, 2.2 mmol) in anhydrous acetonitrile (10 mL) was added chloroacetonitrile (166 mg, 2.2 mmol), and the mixture was stirred at ambient temperature for 24 h. The solvents were evaporated off, the residue was dissolved in DCM (10 mL), and the organic phase was washed with brine (5 mL), separated, dried ($MgSO_4$), filtered, and evaporated. The residue was purified by flash column chromatography (hexane/EtOAc) to give 529 mg of compound **8** (48%). Mp: 49−51 °C. Anal. Calcd for $C_{24}H_{31}N_4O_9S$: C, 52.26; H, 5.66; N, 10.16; S, 5.81. Found: C, 52.14; H, 5.70; N, 10.02; S, 5.72. TSP $[M + NH_4]^+$: *m*/*z* 569. The yield for **12** was 685 mg (56%). Mp: 76−78 °C. Anal. Calcd for $C_{30}H_{35}N_4O_{10}$: C, 58.91; H, 5.77; N, 9.16. Found: C, 58.85; H, 5.75; N, 9.13. MS: *m*/*z* (rel intens): 611 (M^+, 20), 581 (40), 406 (20), 342 (78), 328 (100).

Synthesis of Acylated Dinucleotide. The dinucleotide pdCpA was synthesized by Dharmacon. Acylation of the dinucleotide was carried out as previously described (*16*, *28*). Briefly, 3 μmol of any of the natural or unnatural esterified amino acid derivatives was dissolved in 40 μL of dry *N*,*N*-dimethylformamide (DMF). The dissolved amino acid was then added to 1 μmol of the tetra-*n*-butylammonium salt of pdCpA. The reaction was stirred at ambient temperature under argon for 24 h and purified by HPLC (column: Keystone scientific 250/10/5 nucleosil C18, 2.5 mL/min, gradient of acetonitrile in 50 mM ammonium acetate, pH 4.5, detection at 260 and 350 nm). The acylated dinucleotide was then desalted using a gradient of acetonitrile in 0.5 mM magnesium and 0.5 mM sodium acetate, pH 4.5, lyophilized, and stored at −80 °C. The yields for the acylated dinucleotides ranged from 10% to 30%. The product was confirmed using the A_{260}/A_{350} ratio (3.6/1) of the peak purity spectra

Scheme 2: (A) General Synthesis Strategy for Synthetic Aminoacylation of tRNA[a] and (B) Overview of the Biosynthetic Incorporation of Modified Amino Acids into *Xenopus* Oocytes[b]



[a] The dinucleotide pdCpA is coupled to the amino acid and then enzymatically ligated to the truncated (−CA) tRNa using T4 RNA ligase. The NVOC group protecting the α amine is removed by UV irradiation (350 nm). [b]Luciferase ^{UAG14}mRNA containing a UAG (or GGGU codon) at position 14 and charged suppressor tRNA-CUA (or tRNA-ACCC) are co-injected into oocytes. Microinjection is carried out on the vegetal (yellow) pole of the oocyte.

generated from the diode array detector as well as by the ability of the product to functionally be incorporated into luciferase as described in the Results.

Ligation of Acyl-pdCpA. Ligation was carried out essentially as previously described (*16*). Briefly, to 25−30 nmol of aminoacylated pdCpA were added 4 μL of DMSO, 10 μL of 4× ligation buffer (220 mM Hepes Na^+ (pH 7.5), 80 μg/mL BSA, 1 mM ATP, 60 mM $MgCl_2$), and 21 μL of DEPC-treated water. The mixture was added to solubilize 0.38 nmol (10 μg) of lyophilized truncated tRNA ($tRNA^{Gln}$ or $tRNA^{gln}_{ACCC}$). To the mixture was then added 100 units (5 μL) of T4 RNA ligase, and the ligation was allowed to proceed for 10 min at 37 °C. The reaction was immediately quenched with the addition of 100 μL of 0.42 M, pH 4.5, sodium acetate and then purified by adding an equal volume of acid phenol/chloroform (5:1, pH 4.5), followed by ethanol precipitation. The pellet was dried in vacuo and stored at −80 °C.

Deprotection of Acyl-tRNAs. To remove the photolabile protecting group (NVOC) located on the α amine group of the unnatural or natural amino acids, the following deprotection strategy was followed as described previously but with some modifications (*16*). To the dried acyl-tRNA was added 20 μL of 1 mM potassium acetate (pH 4.5), the solution was transferred to a small 6 × 50 mm glass test tube (Kimax), this test tube (sealed with Parafilm) was inserted into a 10 mL glass test tube (Pyrex), and the Pyrex tube was packed with ice. The tubes were then wedged directly between two 15 W, 350 nm bulbs powered by a transilluminator (UVP Inc., model TM 36). The samples were illuminated for 10 min and then quenched with 80 μL of 0.3 M sodium acetate. The sample was ethanol precipitated, washed with 70% ethanol (in 100 μM potassium acetate, pH 4.5), dried in vacuo, and stored at −80 °C just prior to use.

Preparation of Oocytes. Oocytes were isolated from mature female *Xenopus laevis* frogs by ovarectomy, and washed with oocyte ringer (OR2) solution without Ca^{2+} (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 2.5 mM $NaHCO_3$, and 5 mM HEPES, pH 7.4). The follicular layer was removed by gently rotating the oocytes in a 50 mL plastic test tube at room temperature in a Ca^{2+}-free OR2 supplemented with 2 mg/mL collagenase type IA for 60−80 min. The oocytes were then rinsed with OR2 until the rinse was clear. Stage V1 oocytes were manually sorted under a microscope from the smaller oocytes, transferred to a modified L15 medium (50% Leibovitz's L15 medium (Gibco), 0.5 mM L-glutamine, 25 units/mL penicillin, 25 μg/

mL streptomycin, 50 μg/mL gentamycin, 0.25% chick ovalbumin, and 15 mM HEPES, pH 7.4), and allowed to recover for 18 h at 17 °C. See Scheme 2.

Microinjection. A 2−25 ng sample in 100 μM potassium acetate (pH 4.5) of wild-type luciferase mRNA or UAG luciferase mRNA (mRNA^{UAG14}) was injected into the vegetal pole of the 30 oocytes with a glass pulled pipet (Sutter Instruments) using a picospritzer pressure device (General Valve Corp). Varying amounts (0.01−50 ng) of tRNA^{Gln} or tRNA$_{ACCC}$ were co-injected with ^{UAG14}mRNA or ^{GGGU14}mRNA. For the aptamer studies, varying amounts of A34 and random pool aptamers and tRNA^{Gln} were co-injected with fixed or varying amounts of ^{UAG14}mRNA. Oocytes were incubated for 1−24 h postinjection for the time course experiments, and 2 h for all other experiments. Experiments were performed at minimum in triplicate. Error bars for all bar graphs represent the standard error of the mean with the exception of the time course experiments.

Luciferase Assay. Oocyte extracts were obtained as previously described with some modifications (*30*). Briefly, 30 oocytes were homogenized using 75 μL of cold oocyte homogenization buffer containing 75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1 mM EDTA, 30% sucrose benzamidine (0.15 mg/mL), and 0.1 mg/mL bacitracin with protease inhibitor cocktail added just prior to homogenization. Oocytes were quickly homogenized at room temperature with a 200 μL pipet tip using 50 pipet motions until the oocytes were homogeneous green milky mixtures and then immediately placed on ice (prechilled oocytes are difficult to homogenize). The samples were then centrifuged at 10000*g* at 4 °C for 10 min and placed on ice. Then 50 μL of the lysate from each sample was transferred to a new tube, taking care not to transfer the top milky yellow layer. An equal volume of Bright Glow luciferase assay reagent (Promega) was added to a 96-well luminometer plate, and the samples were mixed with the assay reagent immediately before reading by the luminometer (ML3000, Dynatech Laboratories). To estimate the number of moles of luciferase/oocyte, a standard curve was constructed by adding 1 μL containing 0.1−100 fmol of purified luciferase (Promega) to 49 μL of oocyte extract and quantifying as described above.

RESULTS

Suitability of Position 14 for Nonsense Suppression. Endogenous stop codon suppression can occur through natural suppressor tRNAs (for a review see ref *31*), with an efficiency dependent on the position and context of the mutation. Because of its proximity to the beginning of the transcript, and the large volume of the native (Phe) side chain at this position, we choose position 14 in firefly luciferase to evaluate the incorporation of modified amino acids. To determine whether codon 14 is an appropriate target for amino acid incorporation using the amber (TAG) codon, the functional expression of luciferase was determined from oocytes injected with 2 ng of wild-type mRNA or message containing the UAG amber stop mutation at position 14 (^{UAG14}mRNA). Two hours postinjection, the measured difference between the wild type and the mutant luciferase was 5 orders of magnitude (Table 1). The low level of endogenous readthrough at position 14 allows for the use of this site to monitor incorporation of amino acids coupled to nonsense suppressor tRNAs.

Table 1: Range of the Amount of Functional Luciferase Made per Oocyte for Control Injection Experiments Using Either Wild-Type (wt) Message or mRNA Containing the Nonsense Codon at Position 14

injection sample	amount of luciferase expressed (fmol)
2 ng of wt Luc mRNA	13
2 ng of Luc ^{UAG14}mRNA	7.8×10^{-4}
2 ng of Luc ^{UAG14}mRNA and 25 ng of CUA-tRNA	6.5×10^{-3}
2 ng of Luc ^{UAG14}mRNA and 25 ng of CUA-tRNA^{Phe}	1.22

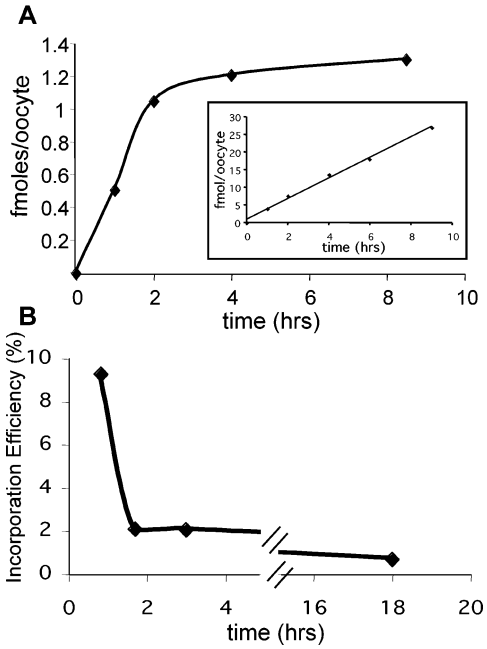


FIGURE 1: (A) Time course for phenylalanine incorporation at position 14 of luciferase through nonsense suppression. Suppressor tRNA (25 ng) charged with phenylalanine was co-injected with 2 ng of luciferase message (^{UAG14}mRNA), and the functional expression levels were determined at the indicated times. The inset shows the time course for expression of wild-type luciferase mRNA. (B) Efficiency of Phe incorporation over time at the UAG codon using charged suppressor tRNA. The percent efficiency was calculated by dividing the expression of wild-type luciferase into the expression of luciferase obtained by incorporation of phenylalanine through nonsense suppression of the amber mutation (UAG) at position 14 in the luciferase gene.

Expression from Luciferase Message Containing the UAG^{14} Mutation. As shown in Table 1, luciferase is expressed from the ^{UAG14}mRNA transcript when oocytes are co-injected with suppressor tRNA synthetically charged with phenylalanine (CUA-tRNA^{Phe}), whereas only a trace amount of luciferase function is detected from oocytes co-injected with uncharged suppressor tRNA (CUA-tRNA). Previous studies in which unnatural amino acids have been incorporated into ion channels (*11*−*13*, *16*) or a G-protein-coupled receptor (*11*−*13*, *16*) have typical incubation times of 18−24. However, the results in Figure 1A demonstrate suppression of the amber stop (TAG) codon at position 14 of luciferase by aminoacylated CUA-tRNA^{Phe} saturates by 3 h. When evaluated for efficiency, the incorporation of phenylalanine in TAG^{14}-luciferase is ∼9%, on average, 50 min postinjection (Figure 1B). These results varied according to the oocyte batch, with some batches providing an

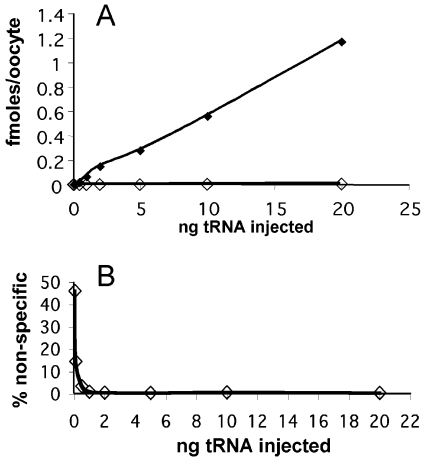
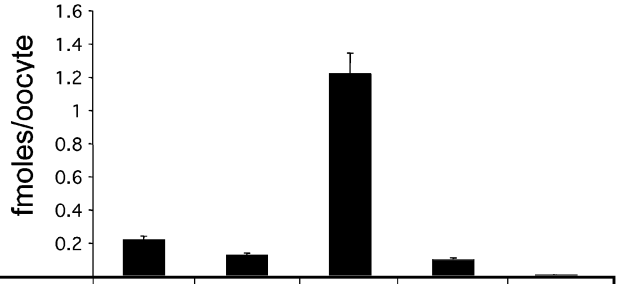


FIGURE 2: TAG^{14}-luciferase expression depends on the levels of charged suppressor tRNA. (A) The number of femtomoles of luciferase expressed from incorporation of phenylalanine after 3 h of incubation (solid tilted squares) is shown as compared to that of luciferase expressed from injection of uncharged tRNA (open tilted squares). (B) The percent of nonspecific acylation of uncharged tRNA and subsequent nonspecific readthrough rapidly decreases in the presence of increasing amounts of suppressor tRNA charged with phenylalanine. The percent nonspecific acylation was calculated by dividing the luminescence derived from co-injection of uncharged suppressor tRNA and ^{UAG14}mRNA by the luminescence obtained from co-injection of CUA-tRNA^{Phe} and ^{UAG14}mRNA. The standard error is within 10% for each data point.



aminoacylated CUA-tRNA	NBD-Ala	SL-Cys	Phe	SL-Tyr	uncharged
% IE	1.7	1.0	9.0	0.70	0.10
% NS	2.9	5.2	0.50	6.5	100

FIGURE 3: Expression levels by site-specific incorporation of synthetically charged phenyalanine (Phe), spin-labeled cysteine (SL-Cys), spin-labeled tyrosine (SL-tyr), or NBD-alanine (NBD-Ala) by nonsense suppression of UAG at position 14 in luciferase. Also shown for each experiment are the incorporation efficiencies (IEs) and the specificity of the incorporation as determined by the percent of nonspecific signal (NS). The IE is obtained by calculating the ratio of expression through the suppression to the expression level of wild-type luciferase. The NS is determined by dividing the signal obtained from uncharged tRNA by the signal from oocytes injected with synthetically charged CUA-tRNA. For each experiment, oocytes were injected with 2 ng of UAG mRNA and 10 ng of charged or uncharged suppressor tRNA, and assayed 3 h post-injection. Error bars represent the standard error of the mean from triplicate experiments.

efficiency of incorporation as high as 18%, a level comparable to what has been reported using in vitro translation extracts (*9*). By 100 min the efficiency decreases to ~2% and then decreases to less than 1% after 18 h. Notably, the diminished incorporation efficiency corresponds to the time period where the expression level by nonsense suppression plateaus, indicating that while wt message is competent for protein synthesis over several hours (Figure 1A, inset), luciferase expression dependent on the presence of CUA-tRNA^{Phe} does not appreciably continue after 2 h (Figure 1A).

Effect of Charged Suppressor tRNA Levels on TAG^{14}-Luciferase Functional Expression. To optimize expression levels of TAG^{14}-luciferase, increasing amounts of CUA-tRNA^{Phe} were co-injected with ^{UAG14}mRNA, and the luciferase activity was measured 1 h later (Figure 2A). Each experiment contained 2 ng of injected message, since expression levels do not significantly improve when more than 2 ng of ^{UAG14}mRNA is used (not shown). We observe a linear relationship between the amount of CUA-tRNA^{Phe} injected and the expression of TAG^{14}-luciferase. At tRNA amounts higher than 20 ng, the increased viscosity of the injection solution leads to clogging of the microinjection needles, and thus, experiments with higher tRNA levels gave inconsistent results. The maximum UAG^{14}-luciferase expression for each oocyte injected with CUA-tRNA^{Phe} was approximately 1.2 fmol, which corresponds to a cellular concentration of 1.2 nM, assuming an average oocyte volume of 1 μL in ref *32*. In the absence of charged suppressor tRNA, the levels of luciferase produced by nonspecific acylation of uncharged tRNA or amber codon readthrough is minimal (<1%, Figures 1A and 2). This level of nonspecific expression is similar to earlier findings using the *T. thermophila* suppressor tRNA (*33*).

Monitoring the Incorporation of Unnatural Amino Acids into Luciferase. As reported in Figure 3, a spin-labeled cysteine residue is incorporated into luciferase by means of amber suppression in oocytes with an efficiency of 1%, compared to the wild type message after 3 h. This corresponds to a spin-labeled luciferase concentration of 35 pM in the oocyte. We achieve a high specificity for the incorporation of the spin-labeled cysteine, with the background signal obtained from the injection of uncharged CUA-tRNA providing only 1/15 of the luciferase signal generated by CUA-tRNA$^{SL\text{-}Cys}$. In a similar experiment, we evaluated the incorporation levels of spin-labeled tyrosine. As shown in Figure 3, co-injection of CUA-tRNA$^{SL\text{-}Tyr}$ with ^{UAG14}mRNA provides slightly less expression (~0.1 fmol/oocyte). Finally, we measured the incorporation of a fluorescent NBD-alanine amino acid following co-injection of CUA-tRNA$^{BD\text{-}Ala}$ with ^{UAG14}mRNA (Figure 3). Of the three residue probes measured, NBD-Ala incorporation is the highest, providing 0.2 fmol/oocyte.

Stability of the Aminoacyl-tRNA. Interestingly, the EPR spectrum of CUA-tRNA aminoacylated with spin-labeled Tyr is dramatically different from the version charged with Cys. The broader spectrum from CUA-tRNA$^{SL\text{-}Tyr}$ indicates this amino acid is more restricted in its mobility, suggesting an interaction of its aromatic ring with the tRNA molecule. Although the EPR spectrum of CUA-tRNA$^{SL\text{-}Cys}$ displays a shorter correlation time, an increase in its mobility is readily seen with conditions that promote hydrolysis of the aminoacyl ester bond (*34*) (pH > 6, Figure 4). Since the EPR spectra can be used to distinguish the free amino acid from the amino acid attached to the tRNA (Figure 4), the spin-labeled amino acids provide a convenient method for monitoring hydrolysis rates of the aminoacyl ester bond. On the basis of the estimated cytoplasmic pH of 7.3−7.6 (*35*, *36*) for an oocyte, it can be assumed that more than half of the aminoacyl-tRNA injected in these studies has hydrolyzed after 2 h.

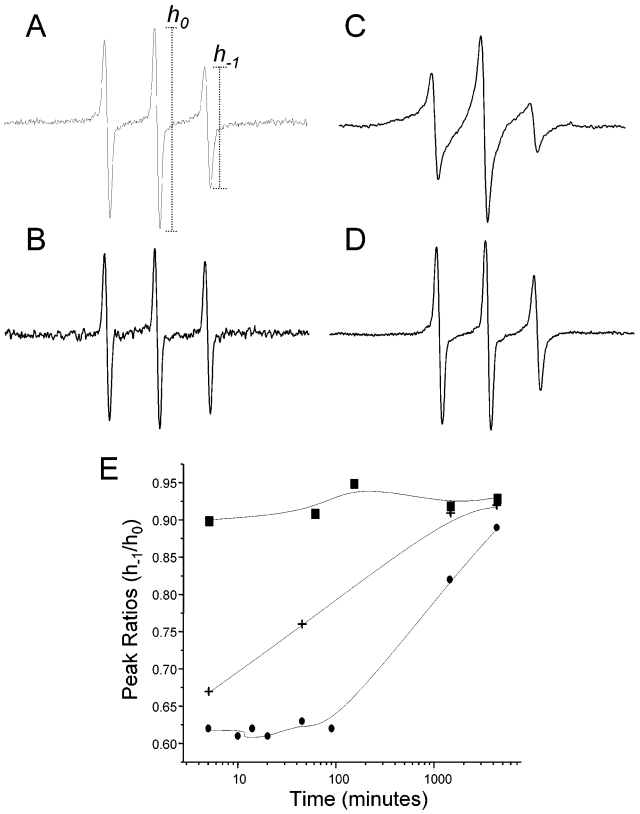


FIGURE 4: Sensitivity of the EPR spectrum to the spin-labeled amino acid's tRNA attachment. The spectrum for the tRNA-conjugated spin-labeled Cys (CUA-tRNA$^{SL\text{-}Cys}$; A) shows more anisotropy than the spectrum for the free SL-Cys amino acid (B). The spectrum of CUA-tRNA$^{SL\text{-}Tyr}$ in (C) is broader than that of CUA-tRNA$^{SL\text{-}Cys}$. Panel D shows the spectrum of the SL-Tyr coupled to the dinucleotide, which is similar to that of the free amino acid (not shown). In (E) the loss of spectral anisotropy from the CUA-tRNA$^{SL\text{-}Cys}$ sample over time provides hydrolysis rates for aminoacylated tRNA held at pH 5.5 (circles), 7.5 (plus signs), or 8.8 (squares).

Incorporation Efficiencies of Unnatural Amino Acids As Measured by Luciferase Expression Using a Four-Base Codon. Another variety of TRAMPE utilizes a four-base codon/anticodon pair instead of the canonical three-base codon/anticodon pair (for a review see ref *37*). Previous efforts in cell-free expression assays have shown efficient incorporation of amino acids at a GGGU codon in mRNA (*38*, *39*). We therefore explored the potential of the four-codon approach in live oocytes using the ^{GGGU14}mRNA and ACCC-tRNA pair (Figure 5). The luciferase gene naturally contains three GGGT sequences. To avoid incorporation of unnatural amino acids at nontarget GGGU sites in the transcript, silent mutagenesis was carried out on the native luciferase gene to alter the three GGGT sequences without disruption of the protein sequence. The 3 h postinjection expression levels from this "GGGU-silent" transcript were similar to those of the native luciferase transcript, producing 3.45 ± 0.345 fmol of luciferase/oocyte, vs 3.10 ± 0.248 fmol of luciferase/oocyte from the transcript containing the native sequence.

As shown in Figure 6, the use of ACCC-tRNA^{Phe} in oocytes leads to a much lower (0.04 fmol/oocyte compared to 1.2 fmol/oocyte) incorporation efficiency of phenylalanine at position 14 of luciferase than what is obtained from the amber ^{UAG14}mRNA and CUA-tRNA^{Phe} pair. For the spin-labeled amino acids, four-codon expression levels were not substantially higher than levels of nonspecific incorporation obtained with uncharged ACCC-tRNA. The fluorescent amino acid NBD-alanine appears to be moderately specific in terms of incorporation, with the nonspecific signal accounting for only 27% of the total signal obtained through incorporation at the GGGU codon. These results suggest the oocyte translational machinery poorly tolerates the use of four-base codons as cognate acceptors of tRNAs designed to recognize the four-base codon, or that the suppressor tRNA from *T. thermophila* is a poor template for engineering a tRNA intended to recognize the four-base codon.

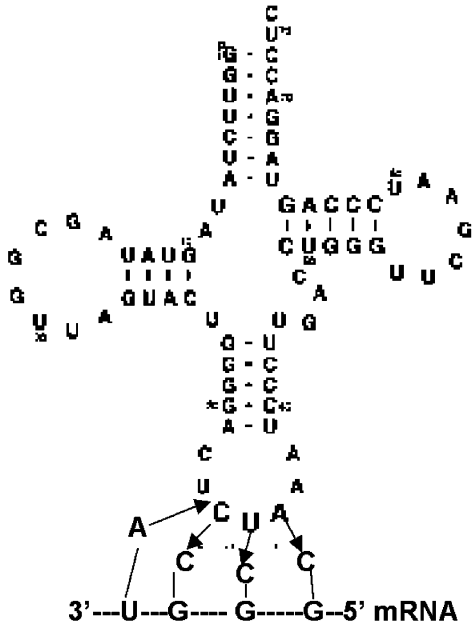
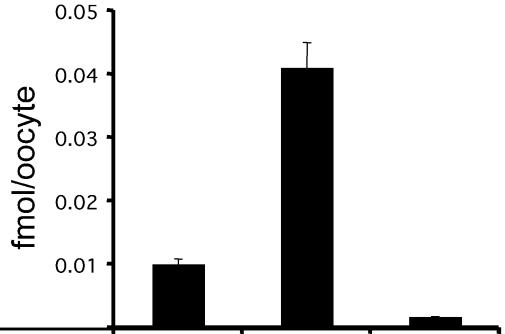


FIGURE 5: Design of the four-base codon ACCC-tRNA.The anticodon to the amber suppressor tRNA form *T. thermophila* was modified to ACCC using site-directed mutagenesis. The new ACCC-tRNA was designed to recognize the four-base codon GGGU, which was substituted in place of the UAG codon at position 14 within the luciferase gene.



aminoacylated ACCC-tRNA	NBD-Ala	Phe	uncharged
% IE	0.37	1.45	0.10
% NS	27	6.4	100

FIGURE 6: Incorporation levels of modified amino acids at position 14 encoded by the GGGU four-base codon. Expression levels are shown for the GGGU^{14} incorporation of Phe and NBD-Ala from injection of synthetically charged ACCC-tRNA. IEs and the percent NS are also provided. In each experiment, oocytes were injected with 2 ng of mRNA and 10 ng of the indicated tRNA. Error bars represent the standard error of the mean from triplicate experiments.

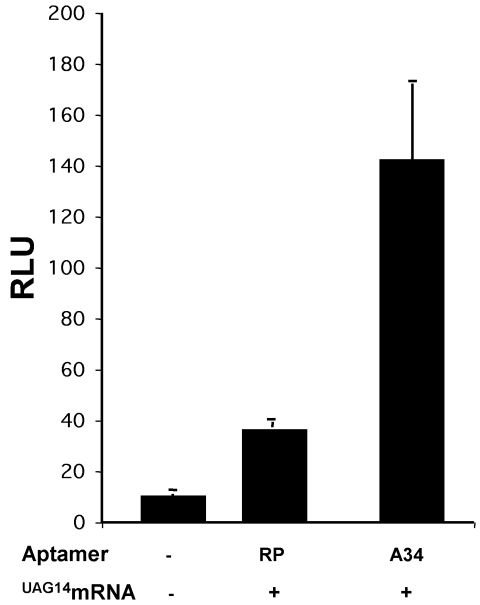


FIGURE 7: Effect of aptamer A34 on readthrough of a UAG stop codon introduced at position 14 in luciferase. Batches of 30 *Xenopus* oocytes were injected with 2 ng of luciferase ^{UAG14}mRNA with or without 50 ng of A34 aptamer. Luciferase activity, given in relative light units (RLUs), was measured 2 h postinjection as described in the Materials and Methods. To obviate uncertainty due to injection tip variability, each experiment analyzed three batches of oocytes, where each batch was injected using a new micropipet tip. Error bars represent the standard error of the mean for four separate experiments.

Effect of Release Factor Inhibitor on the Suppression of Stop Codons in Xenopus Oocytes. Release factor participates in the termination of protein synthesis through its putative recognition of stop codons (*22*, *40*). Previous in vitro work with the A34 aptamer demonstrated the molecule's ability to attenuate the signal strength of stop codons through a direct inhibition of release factor (*19*). To evaluate the effect of RNA aptamer A34 in vivo, the Luc14 → UAG mRNA (^{UAG14}mRNA) was co-injected into *Xenopus* oocytes with either A34 or a random pool of aptamers. Following a 2 h incubation, the levels of functional luciferase were measured (Figure 7). The expression of luciferase in the presence of A34 was 3.9-fold higher compared to that of the extract from oocytes injected with a random pool of aptamers. This demonstrates that, in live cells, inhibition of release factor increases levels of endogenous readthrough at nonsense mutations.

To address the time dependence of nonsense codon suppression by A34, the suppression of the Luc14 → TAG mutation by A34 was measured after different postinjection incubation periods. Figure 8 shows expression levels of luciferase become saturated between 2 and 3 h. Surprisingly, saturation of luciferase expression occurs much sooner for the oocytes injected with or without the aptamer as compared to saturation of expression for the wild-type luciferase, which saturated after 24 h (not shown).

Since the effect of the A34 aptamer is to effectively decrease the quantity of available release factor, a direct competition between the release factor and a suppressor tRNA should be apparent by analyzing the amount of CUA-tRNA suppression in the presence and absence of release factor. As shown in Figure 9A, A34 suppression levels are

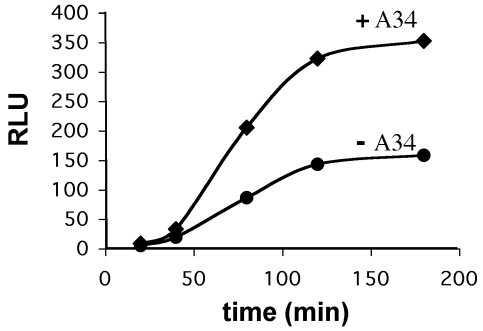


FIGURE 8: Time course of suppression enhancement by A34. Luciferase activity was measured over time following injection of 2 ng of ^{UAG14}mRNA with (tilted squares) or without (circles) co-injection of 50 ng of A34. Measurements were made from the bioluminescence in the extract from two separately injected batches of 30 oocytes. Each point represents the average from three independent experiments.

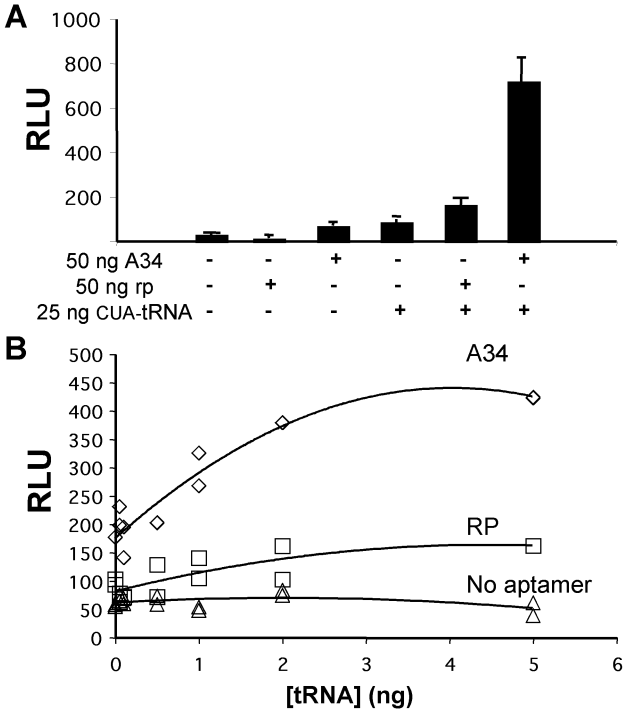


FIGURE 9: (A) Effects of co-injecting suppressor tRNA and/or aptamers with 2 ng of ^{UAG14}mRNA. Measurements were made from the bioluminescence in the extract from three separately injected batches of 30 oocytes. Each point represents the average from three independent experiments. Error bars represent the standard error of the mean (SEM). (B) Aptamer 34 elevates expression in the presence of increasing amounts of uncharged suppressor tRNA. The production of active luciferase as a function of CUA-tRNA concentration was measured in the presence of 20 ng of A34 (tilted squares) or random pool (squares) aptamer, or in the absence of aptamer (triangles). Each point represents duplicates of 30 oocytes injected with 2 ng of ^{UAG14}mRNA, the indicated aptamer, and the indicated amount of CUA-tRNA. An average error of ±12% was observed due to variation in microinjection volumes.

increased more than 7-fold with the injection of exogenous uncharged suppressor CUA-tRNA, suggesting the effects of the aptamer could be limited by the endogenous supply of suppressor tRNAs. While CUA-tRNA has been engineered to undergo minimal aminoacylation by endogenous synthetases (*33*), these results are in line with previous observations showing the modified suppressor tRNA does display a low level of re-acylation by endogenous oocyte synthetases

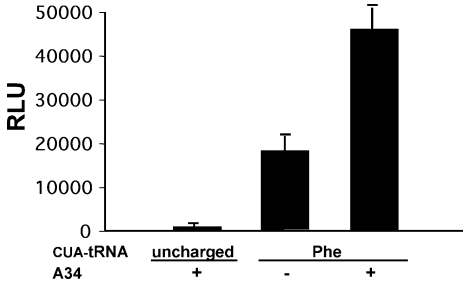


FIGURE 10: Aptamer 34 increases the site-specific incorporation at TAG^{14} by suppressor tRNA charged with phenylalanine. The addition of 50 ng of A34 causes a 2.5-fold increase in the expression of luciferase when oocytes are co-injected with 10 ng of CUA-$tRNA^{Phe}$ as compared to those lacking A34. When uncharged tRNA is co-injected with A34, only background readthrough is observed. Measurements were made from the bioluminescence in the extract from three separately injected batches of 30 oocytes. Each point represents the average from three independent experiments. Error is expressed as the SEM.

(*33*). Compared to the eggs injected with the random pool of aptamers, the presence of A34 increased the expression levels 7-fold, which again indicates the increase is linked to the inhibition of the release factor.

To further address the possibility that A34 and CUA-tRNA act in a synergistic manner, we then measured the expression levels of luciferase in the presence of increasing levels of tRNA. The suppression shown in Figure 9B is dependent on the availability of suppressor tRNA, providing further evidence that exogenous A34 and CUA-tRNA act in a synergistic manner to increase suppression. More importantly, eggs injected with A34 and tRNA have almost a 10-fold increase in expression over the eggs injected with A34 and mRNA (Figure 9A). The substantial increase in Luc14 → TAG expression levels upon the introduction of CUA-tRNA, which presumably is re-acylated with a low efficiency, suggests that injection of fully charged CUA-tRNA along with aptamer should lead to an increased level of incorporation of the amino acid. Thus, CUA-tRNA was synthetically charged with phenylalanine, the natural amino acid found at position 14 in luciferase. Compared to the co-injection of ^{UAG14}mRNA and uncharged CUA-tRNA, injection of CUA-$tRNA^{Phe}$ with ^{UAG14}mRNA results in greater than a 20-fold increase in the amount of expressed protein (Figure 10). In addition, the suppression of the stop codon by CUA-$tRNA^{Phe}$ is further enhanced (~3-fold) when the A34 aptamer is also present (Figure 10).

DISCUSSION

The approach described here allows for a sensitive, convenient, and reliable method for detecting levels of expressed protein containing unnatural amino acids, and is therefore useful in the screening of variables that are likely to affect incorporation efficiency. We describe a system for rapidly screening the in vivo incorporation of amino acids modified to serve as biophysical probes for reporting protein structure and function. The use of luciferase as a reporter of incorporation efficiencies provides a sensitive (as low as 10^{-18} mol) method that is independent of the signaling machinery in the oocyte, providing high reproducibility. These features are especially salient with use of the oocyte system since we, as others (*41*), find a 2−4-fold variability in expression levels with oocytes derived from different specimens. Thus, wild-type luciferase expression levels provide a useful screen for oocyte batch competence, for their use in unnatural amino acid incorporation studies. We chose position 14 in luciferase for residue probe incorporation because the native residue (Phe) is large and hydrophobic, and is not essential for structure or function (*42*). Luc14 → TAG expression is 5 orders of magnitude lower than for the wild-type protein. Thus, position 14 provided high sensitivity for analyzing inhibition of termination and suppression of the nonsense mutation by exogenously added suppressor tRNAs. Furthermore, locating the stop codon close to the N-terminus ensures that the terminated translation product does not have any function. Finally, expressed luciferase is highly stable. We note no decrease in the activity of luciferase when the enzyme is incubated with oocyte cell extract over a 1 h period at room temperature.

Our kinetic data would suggest that the majority of labeled luciferase protein is made within the first 6 h. As previously observed (*43*), the expression of soluble proteins in oocytes does not saturate the translational and trafficking machinery as rapidly as occurs for integral membrane proteins. At 24 h postinjection, we observed expression levels exceeding 100 fmol of luciferase/oocyte, whereas the 24 h expression levels for equivalent amounts of mRNA encoding either the substance K receptor or the acetylcholine receptor drop to 10 and 5 fmol/oocyte, respectively (*16*, *44*).

Yet, even with the ability to predict how well an amino acid analogue is tolerated by the translational machinery, we cannot rule out or predict mRNA-sequence-specific context effects that may influence incorporation efficiency and subsequent expression levels. Although exhaustive studies using *E. coli* extracts show that amino acid identity has more weight on the efficiency of incorporation than codon context (*4*, *45*), as our data also suggest, the rules for how context governs suppression efficiencies are mixed and at times contradictory (for a review see ref *31*). However, it is well established that suppression efficiency drops off the closer the stop codon is placed near the end of the message (*15*, *46*−*49*). A growing body of evidence also shows that natural suppression in eukaryotes and prokaryotes by normal cellular tRNAs can be greatly enhanced by the context of nucleotides just 3′ of the UAG codon (*50*−*52*), yet in the study by Turcatti et al., the author aligned 12 out of 90 UAG-containing sequences of the substance K receptor which had successfully incorporated the fluorescent amino acid used in this study and found no underlying nucleotide context motif near or surrounding the UAG codon (*15*). Perhaps future studies in oocytes using our luciferase system will help to resolve whether the context rules that apply to natural suppression of UAG codons also contribute to the overall incorporation efficiency of unnatural amino acids in oocytes. Because our data are based on the use of one position, the amino acid identity is clearly the determining factor for incorporation efficiency.

The time course in Figure 1A shows that the overall rate of protein synthesis as well as maximum expression of the luciferase synthesized through the incorporation of phenylalanine at position 14 plateaus at 2−3 h, much sooner than the established protein synthesis rate for exogenous mRNA translated in oocytes (6 h) (*53*), a time course consistent with our measurements of wild-yype luciferase. This suggests that the availability of $mRNA^{UAG14}$ or charged tRNA is a limiting

factor in the protein synthesis. For example, while most normal transcripts are stable in oocyte for greater than 24 h (*54*), a rapid degradation of UAG-containing transcripts is documented in other eukaryotic systems (*55*). Since injecting higher concentrations of $mRNA^{UAG14}$ with a fixed amount of $CUA\text{-}tRNA^{Phe}$ does not result in a marked expression level increase, it appears that $mRNA^{UAG14}$ is not the limiting substrate. Because of the labile nature of the aminoacyl ester bond (*34*), it is difficult in oocytes to address the levels of tRNA synthetically charged with unnatural amino acids. When suppressed with charged suppressor tRNA, Luc14 → TAG expression plateaus within 2−3 h. This correlates with the half-life of the aminoacyl ester−tRNA bond measured by EPR at a comparable pH. Thus, we identify the availability of charged suppressor tRNA as a primary factor in the protein levels obtained by nonsense suppression in oocytes.

The lack of an observed saturation within the 20 ng range of injected $CUA\text{-}tRNA^{Phe}$ is in contrast with nonsense suppression studies on the nicotinic acetylcholine receptor (*33*). In this case, current activity saturates at 4 ng of $CUA\text{-}tRNA^{Tyr}$; however, the saturation of the current signal using the two-microelectrode voltage clamp may not correspond to a saturation of protein expression. We have observed this saturation effect when incorporating the NBD-alanine amino acid into the substance P receptor, and monitoring its function in oocytes by the two-microelectrode technique (unpublished results).

To quantitate the oocyte incorporation efficiency of amino acids modified to contain biophysical probes, we examined UAG^{14}-luciferase expression levels using CUA-tRNA synthetically charged with either NBD or nitroxide side chains. Suppression with NBD-alanine results in a luciferase level of 0.2 fmol/oocyte, whereas CUA-tRNA synthetically charged with spin-labeled cysteine or tyrosine produced approximately half this amount. The structure of the nitroxide radical moiety may be a factor contributing to this lower incorporation level, by hindrance to ribosome docking or the binding of the aminoacyl-tRNA to elongation factor (*39*, *56*). Though molecules with ring structure similar to that of the spin-labeled tyrosine such as *p*-biphenylalanine or *p*-benzoylphenylalanine could be incorporated into streptavidin at efficiencies greater than 40% (*39*). Lysine represents a potential amino acid for nitroxide modification, since when labeled with BODIPY, a large tricyclic fluorescent compound also containing methyl groups, the modified amino acid is efficiently incorporated into proteins using a mammalian in vitro translation system (*57*).

For cytosolic proteins, spin-label reduction must be considered in attempts to observe nitroxyl amino acids incorporated by the TRAMPE method. Within in live cells nitroxide labels are reduced to the EPR-silent diamagnetic species, but reoxidize to the paramagnetic species when exposed to air or mild oxidants (*58*). This would not be a concern in in vitro systems as long as reductants are not added. The convenient oocyte−luciferase screen described here can identify factors important to unnatural amino acid incorporation before attempts are made with costly in vitro systems.

Our conventional X-band (9.5 GHz) EPR configuration can acquire a spectrum from 1×10^{12} free nitroxide spins in 3 μL of solution. The levels of 0.1−0.2 fmol of spin-labeled amino acid/oocyte correspond to $\sim 1 \times 10^{8}$ spins. Certainly, this approach will benefit from recent progress in developing imaging systems for unpaired spins and more sensitive spectrometers. The higher intrinsic sensitivity achieved with increasing frequency allows for the detection of fewer spins (*59*). For example, point sensitivity is reported to improve by a factor of 1000 at W-band (95 GHz) in comparison to X-band measurements (*60*). In addition, other developments in detecting electron spin transitions show promise of single-molecule sensitivity (*61*). One such technology is magnetic resonance force microscopy, where signal from as few as two polarized electron spins has been measured (*62*). Thus, the continuing advances in magnetic resonance instrumentation coupled with molecular approaches that increase the incorporation efficiency of modified amino acids in vivo (see below) should soon make practical the examination of proteins containing incorporated spin-label side chains.

Significant efforts have been made to improve the incorporation efficiencies of unnatural amino acids into proteins (*63*). One of the major improvements was the use of a tRNA that decodes a four-base codon instead of the canonical three-base codon found in mRNA (*38*). Incorporation efficiencies exceeding 80% were realized with the incorporation of certain unnatural amino acids, with the GGGU codon providing the best incorporation efficiency in an *E. coli* extract system (*38*). Readthrough in this approach is less of a concern, since its occurrence would lead to a frame shift. For example, if Gly (GGG) is inserted into the ^{GGGU14}mRNA of luciferase, the resulting frame shift introduces a stop codon three residues downstream. However, our data suggest that the four-base codon designed for *E. coli* is not effective in the oocyte. The tRNA itself may not tolerate the insertion of an additional base, leading to improper folding. The GGG codon in oocytes is used with about the same frequency as the other codons encoding for glycine, and thus, readthrough may result in a significant lowering of protein yield via the competition between endogenous $CCC\text{-}tRNA^{Gly}$ and the ACCC-tRNA (*38*). Considering this possibility, GCGU may be more suitable since GCG has the lowest frequency (∼0.05) in genes found in oocytes.

The ease of delivery of exogenous molecules into oocytes by microinjection provides a convenient in vivo system for studying the effects of the aptamer on the suppression of an engineered amber mutation. Introduction of A34 results in a 2-fold increase in luciferase production over that of oocytes injected with mRNA alone. Furthermore, this effect was maintained 3 h postinjection, 3 times longer than was reported by Carnes et al. for the in vitro system. Elevated temperatures can increase nonnatural amino acid incorporation by as much as 11-fold in *E. coli*-derived S-30 extracts containing temperature-sensitive release factors (*64*, *65*). Here, as in previous studies with rabbit reticulocyte lysate systems (*19*, *40*), we show competition with the eRF1 release factor by increasing amounts of suppressor tRNA results in a parallel increase of readthrough at the Luc14 → TAG stop codon. However, due to the low amount of aminoacylation of the engineered CUA-tRNA molecules (*33*), only a slight increase in readthrough at Luc14 → TAG is seen upon injection of uncharged suppressor tRNAs, and the CUA-tRNA effect saturates at ∼1 ng. The low level of suppression seen with uncharged tRNA is not solely limited by the

availability of its aminoacylated form, since Luc14 → TAG expression in the presence of both A34 and CUA-tRNA is more than 3-fold greater than the sum of the levels generated in the presence of either species alone. Since A34 effectively lowers the amount of release factor competent to bind UAG codons, the synergistic effect suggests the release factor may have a higher affinity for the stop codon than does CUA-tRNA. The observation that injection of charged suppressor tRNA does not lead to restoration of wild-type levels of expression further supports this notion. Clearly, other factors may also contribute to the low efficiency of amino acid incorporation at amber codons, such as the rapid decay of UAG-containing transcripts (*55*) and/or a low binding affinity of the charged tRNA to elongation factor (*56*). Nevertheless, our results suggest that titration of eRF1 by A34 increases the opportunity for aminoacylated CUA-tRNA to interact with the UAG codon.

New efforts hold continued promise for the use of TRAMPE, specifically with the development of the 21st aminoacyl synthetase/tRNA pair, which provides the ability to recycle the tRNA through specific re-acylation with an unnatural amino acid both in vivo (*66*−*68*) and in vitro (*69*). A possibility is the stable integration of an orthogonal synthetase/tRNA pair into the genome of *X. laevis* that allows for the expression of the pair during the oocyte stage of embryogenesis. In this case, a modified amino acid (recognized by the engineered synthetase) could be co-injected with $mRNA^{UAG}$, leading to a continuous charging of the target tRNA. Since our results indicate hydrolysis of the amino ester bond is the significant limiting factor with nonsense suppression in oocytes, this direction may expand the applicability of in vivo probe incorporation. Furthermore, enzymatic regeneration of the aminoacyl-tRNA alleviates complicated chemical synthesis. The use of the luciferase system described here will be appropriate to evaluate these emerging approaches.

ACKNOWLEDGMENT

We thank Mária Balog (University of Pécs) for technical assistance and József Jekô (ICN, Hungary) for mass spectral measurements.

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BI035542I