von Heijne, G. (1986) *Nucleic Acids Res. 14*, 4683-4690.
Vranckx, R., Savu, L., & Nunez, E. A. (1986) *FEBS Lett. 244*, 343-346.
Vranckx, R., Savu, L., Maya, M., Rouaze-Romet, M., & Nunez, E. A. (1990a) *Acta Endocrinol. 123*, 649-656.
Vranckx, R., Rouaze, M., Savu, L., Nunez, E. A., Beaumont, C., & Flink, I. L. (1990b) *Biochem. Biophys. Res. Commun. 167*, 317-322.
Young, R. A., Braverman, L. E., & Rajatanavin, R. (1982) *Endocrinology 110*, 1607-1612.
Young, R. A., Rajatanavin, R., Moring, A. F., & Braverman, L. E. (1985) *Endocrinology 116*, 1248-1252.
Young, R. A., Meyers, B., Alex, S., Fang, S. L., & Braverman, L. E. (1988) *Endocrinology 122*, 2318-2323.
Zinn, A. B., Marshall, J. S., & Carlson, D. M. (1978) *J. Biol. Chem. 253*, 6761-6767.

Site-Specific Incorporation of Nonnatural Residues during In Vitro Protein Biosynthesis with Semisynthetic Aminoacyl-tRNAs†

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ABSTRACT: A method is presented for the incorporation of nonnatural amino acids into proteins during in vitro cell-free translation. A combination of chemical synthesis and run-off transcription was employed to prepare a semisynthetic, nonhypermodified $tRNA^{Gly}$ nonsense suppressor acylated with L-3-[^{125}I]iodotyrosine. The presence of this synthetic tRNA during in vitro translation of mRNA containing a nonsense suppression site (e.g., a UAG termination codon) results in the incorporation of the nonnatural amino acid L-3-iodotyrosine into the polypeptide exclusively at the position corresponding to that site. Incorporation of the nonnatural amino acid L-3-[^{125}I]iodotyrosine into the model polypeptide was assessed by quantitative and unambiguous determination of suppression efficiency, read-through, and site specificity of incorporation. Minor modifications of the method employed in this initial experiment also allow the rapid analysis of unlabeled acylated tRNA analogues. Under optimum conditions, the unlabeled amino acid L-3-iodotyrosine was found to be incorporated with a suppression efficiency of 65%. Other nonnatural residues, including *N*-methylphenylalanine, D-phenylalanine, and phenyllactic acid, were tested in the assay under these same conditions. Suppression efficiencies for this series ranged from 0 to 72% depending on the structure of the residue incorporated. Several other aspects of this methodology, such as tRNA structure and context effects, are briefly discussed.

Site-specific mutagenesis is one of the most important experimental tools available for protein research, but the methodology suffers from the limitation that amino acid substitutions are restricted to the 20 primary amino acids. This drawback precludes the direct site-specific introduction of "designer" amino acids (e.g., detection, catalytic, linking, or cleaving residues) that could modify the function or activity of a protein in a novel way. One method to circumvent this problem has been to modify a specific residue in a protein posttranslationally. The exceptionally high degree of chemoselectivity required to modify a protein in this manner has limited the scope of this approach, although a few notable successes exist, as exemplified by work of Kaiser and coworkers (Hilvert et al., 1988). It seems likely, however, that a truly general method would require intervention during protein biosynthesis, i.e., during translation, if the innate selectivity problems associated with posttranslational chemical modification are to be avoided.

Methods to manipulate protein structure during biosynthesis have centered around incorporation of residues via an exogenous source of a "misacylated" tRNA or tRNA analogue, which have been prepared by various combinations of chemical and enzymatic methods. One of the earliest experiments in this area was the insertion of alanine at a cysteine codon by conversion of cysteinyl-$tRNA^{Cys}$ to alanyl-$tRNA^{Cys}$ through reductive desulfhydration with Raney nickel (Chapeville et al., 1962). This experiment was the first direct test of the "adaptor hypothesis" (Crick, 1958) and clearly established that recognition of each aminoacyl-tRNA by the ribosome is not dependent upon the amino acid itself, but rather upon structural elements of the tRNA to which it is attached, specifically the anticodon. Thus, a reasonable strategy for the introduction of nonnatural residues into proteins can be envisioned whereby a tRNA acylated with the desired residue is introduced into a protein expression system.

One successful approach for the formation of aminoacyl-tRNAs comes from the many reports from Hecht and coworkers, who have pioneered the area of "chemically misacylated" tRNAs (Pezzuto & Hecht, 1980; Heckler et al.,

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1984b, 1988; Payne et al., 1987; Roesser et al., 1989). Preparation of these misacylated tRNAs is accomplished by T4 RNA ligase mediated coupling of N-protected 2′(3′)-*O*-aminoacylated pCpA derivatives with tRNAs lacking the 3′-terminal cytidine and adenosine moieties, resulting in acylated tRNAs capable of dipeptide formation (Hecht et al., 1978; Heckler et al., 1983, 1984a; Roesser et al., 1986). Further innovations lead to the development of methods for preparation of charged tRNAs with a free amino group (Payne et al., 1987), thus allowing normal function in the ribosomal A site and biosynthetic incorporation of an amino acid into a protein. In one such example (Baldini et al., 1988), $tRNA^{Phe}$ misacylated with L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine was introduced into an in vitro expression system, resulting in the incorporation of this nonnatural amino acid into the translation product. However, insertion of the nonnatural residue occurred at all phenylalanine sites along the mRNA employed in this experiment—in competition with phenylalanine itself—which presents a problem if the desired goal is to site-specifically incorporate a nonnatural residue into a protein.

Such a competition would occur for all codons except the three termination codons (UAG, UGA, and UAA), for which there are normally no corresponding tRNAs. Thus, a conceptually simple solution to the problem of nonselective incorporation (i.e., at multiple sites) would be to provide the translation system with aminoacylated nonsense suppressor tRNAs capable of introducing any residue specifically at a *nonsense mutation site* (e.g., a UAG termination codon). This strategy is in fact a known biological mechanism, observed in *Escherichia coli*, by which selenocysteine is incorporated site-specifically during biosynthesis of formate dehydrogenase (Leinfelder et al., 1988; Söll, 1988). Recently, an analogous synthetic version of this theme was independently reported by us (Bain et al., 1989) and by Schultz and co-workers (Noren et al., 1989).[1] Our approach relied upon a combination of chemical synthesis and run-off transcription to prepare a semisynthetic, unhypermodified $tRNA^{Gly}$ nonsense suppressor acylated with L-3-[^{125}I]iodotyrosine, L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA.[2] The presence of this synthetic tRNA during in vitro translation of mRNA containing a nonsense suppression site (i.e., a UAG termination codon) results in the incorporation of the nonnatural residue L-3-[^{125}I]iodotyrosine into the polypeptide exclusively at the position corresponding to that site. The site specificity of incorporation was unambiguously demonstrated by careful analysis of the translation product, which was purified and sequenced. In addition, suppression due to the synthetic tRNA was quantified in relation to read-through—suppression by endogenous aminoacyl-tRNAs during in vitro translation—verifying that the observed suppression was due entirely to the added synthetic suppressor.

The in vitro expression of a model polypeptide allowed the rigorous and unambiguous characterization of the translation product. This choice, as an initial biosynthetic target, avoided the possible problems associated with quantification of suppression efficiency through the often tedious analysis from catalytic activity of enzymatic end products (Schimmel, 1989, 1990). Herein, we report a detailed analysis of this initial experiment and present a novel rapid assay method to efficiently examine a large array of acylated tRNA analogues. Additionally, a comparison of amber, ochre, and opal suppressors is presented, along with a discussion of termination attenuation due to context effects.

Experimental Procedures

Materials. The following biological products and reagents were employed: calf intestinal alkaline phosphatase and inorganic pyrophosphatase (Boehringer Mannhiem Biochemicals); RNasin (Promega Biotech); T4 polynucleotide kinase and T4 RNA ligase (New England Biolabs); T4 DNA ligase (Bethesda Research Laboratories); T7 RNA polymerase (New England Biolabs or United States Biochemicals); and restriction endonucleases *Eco*RI, *Eco*RV, *Hin*dIII, *Nco*I, *Pst*I, and *Xho*I (Bethesda Research Laboratories, Boehringer Mannhiem Biochemicals, or New England Biolabs). Plasmids used were either pIBI30 (International Biotechnologies Institute) or Bluescript M13$^+$ KS (Stratagene). *E. coli* TB-1 cells were a generous gift from Dr. M. Cumsky (UC Irvine). Benzoylated diethylaminoethylcellulose and Poly-Prep 2-mL disposable polypropylene columns (731-1550, Bio-Rad), Sephadex G-25 Select-D columns (5301-730608/725608, 5 Prime→3 Prime, Inc.), rabbit reticulocyte lysate (N.90, Amersham), and Geneclean (BIO 101, Inc.) were purchased from the manufacturers indicated.

Special care was taken whenever handling oligoribonucleotides due to the ubiquitous nature of RNA-destroying enzymes (Usman et al., 1987). Strictly aseptic techniques were used. All solutions were pretreated by the addition of 10% diethyl pyrocarbonate (v/v in absolute EtOH to a final concentration of 1%) for 24 h at room temperature and then sterilized by autoclaving at 125 °C and 18 psi for at least 30 min. Solutions for column chromatography were preserved by the addition of NaN_3 (0.001% final concentration) to preclude bacterial contamination. All glassware was treated with diethyl pyrocarbonate for at least 4 h and subsequently autoclaved; alternatively, glassware was heated to 250 °C for a minimum of 4 h. Glass pipets and minicolumns were siliconized with Sigmacote (Sigma) prior to sterilization.

The following chemical reagents were purchased: benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (Advanced ChemTech), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Calbiochem-Behring), tris(hydroxymethyl)aminomethane (Sigma), *N*-(9-fluorenylmethoxy)carbonyl-protected residues for peptide synthesis (Milligen/Biosearch or Bachem), trifluoroacetic acid (Pierce Chemical Co.), nucleotides, coupling, and oxidation reagents for oligonucleotide synthesis (Milligen/Biosearch), nucleotide triphosphates (Pharmacia), and P^1-5′-(7-methyl)guanosine-P^3-5′-guanosine triphosphate, dilithium salt (Boehringer Mannhiem Biochemicals). Carrier-free Na ^{125}I (~2500 Ci/mmol) was obtained from ICN Biomedicals, Inc., and L-[^{35}S]methionine (>800 Ci/mmol) and L-[3,4,5-^{3}H]leucine (143 Ci/mmol) were purchased from New England Nuclear

[1] This idea was apparently first suggested six years ago by Shih and Bayley (1985), on the basis of earlier extensive work by the Hecht group (Hecht et al., 1978; Pezzuto & Hecht, 1980; Heckler et al., 1983, 1984a,b, 1988; Roesser et al., 1986, 1989; Payne et al., 1987).

[2] Abbreviations: BD-cellulose, benzoylated diethylaminoethylcellulose; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; equiv, equivalent; Fmoc, (9-fluorenylmethoxy)carbonyl; Na$^+$/Hepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; HOBT, 1-hydroxybenzotriazole; PTH, phenylthiohydantoin; RT, room temperature; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran; THP, tetrahydropyran; $TIPDSCl_2$, 1,1,3,3-tetraisopropyldisiloxane; TMSCl, trimethylsilyl chloride; Tris, tris(hydroxymethyl)-aminomethane; $tRNA^{Gly}_{CUA}$-C_{OH}, tRNA in vitro suppressor run-off transcript, based upon *E. coli* $tRNA^{Gly3}$, which is missing the 3′-terminal cytidine and adenosine moieties; L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA, semisynthetic tRNA containing a deoxycytidine residue coupled to an adenosine moiety on the 3′ terminus and acylated with L-3-[^{125}I]iodotyrosine.

Research Products. Carrier-free L-3-[^{125}I]iodotyrosine was prepared by the method of Hadi et al. (1977).

General Methods. High-Performance Liquid Chromatography. HPLC was performed on a Waters system consisting of two 6000A pumps and U6K injector, a reversed-phase Vydac C-4 preparative column (5-μm packing, 10-mm i.d. × 250-mm length), an in-line Applied Biosystems 1000S diode array detector, and a LKB 2112 Redirac Fraction Collector.

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized on either a Milligen/Biosearch 8700 or an Applied Biosystems 380A synthesizer by use of 2-cyanoethyl phosphoramidite chemistry (Tanimura et al., 1988). Synthesis was carried out by O. Bates and K. Burke from the Protein/Nucleic Acid Analysis Lab (UC Irvine), B. Meeker from the Department of Molecular Biology (UC Irvine), or M. Lyttle (Milligen/Biosearch). Oligodeoxyribonucleotides (two to three A_{260} units of each) for gene construction were purified by electrophoresis through a 20% acrylamide/7 M urea gel, visualized by UV shadowing, and excised. The oligodeoxyribonucleotides were eluted twice from excised gel with 10 mM Tris·HCl, pH 8.0/300 mM NaCl/1 mM EDTA/1% phenol, at 37 °C for 6 h. The eluents were combined, and the DNA was EtOH-precipitated, washed with cold 70% EtOH, dried, and resuspended in distilled H_2O.

Chemical Peptide Synthesis and Purification. Peptide synthesis was performed on a semiautomatic low-pressure continuoues flow peptide synthesizer (Dryland & Sheppard, 1986) consisting of a Zenith Data Systems computer, a Fluid Metering Inc. RP-Sy-1CSC low-pressure continuous pump fitted with a R47a low-flow kit, Altex pneumatically activated valves, and an Altex ultraviolet spectrophotometer detecting at 366 nm, with *N*-(9-fluorenylmethoxy)carbonyl-protected residues (Carpino & Han, 1972; Chang & Meienhofer, 1978) and a polyamide–Kieselguhr solid support (Atherton et al., 1981). A 50-min synthesis cycle was employed, with a 5-min 20% piperidine/DMF treatment, 10-min DMF wash, 25-min acylation, and 10-min wash. The appropriate *N*-(9-fluorenylmethoxy)carbonyl residue (3 molar equiv) was activated just prior to use with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole dissolved in a 0.2 M *N*-methylmorpholine in DMF (Hudson, 1988) and delivered before the initiation of each cycle with a 1.0-mL disposable polypropylene syringe. Stepwise coupling yields were qualitatively analyzed by treatment of a few gains of resin with a 50-μL stock test solution (10 mg of picryl sulfonic acid dissolved in 1 mL of *N*-methylpyrrolidone) followed by heating to 50 °C for 1 min. The resin was washed with MeOH, dried in vacuo, placed in an appropriately sized test tube, and the peptide was deblocked and cleaved from the resin by adding a 5% $C_2H_5SCH_3$/TFA solution; the solution was allowed to stand with occasional mixing for 1.5–2 h. The supernatant was decanted and then diluted with Et_2O, yielding the TFA salt of the peptide as a white precipitate. The precipitate was washed twice with Et_2O and then dissolved in 77% formic acid. The solution was injected (1–2 mg of peptide) onto a Vydac C-4 column equilibrated in 0.1% TFA in H_2O/CH_3CN (1:1). Gradient elution with 0.1% TFA in CH_3CN (35–50% CH_3CN in 50 min) followed by ultraviolet detection at 214, 230, 260, and 280 nm resolved peptide peaks eluting at 40–45% CH_3CN. Isolated peaks were collected, pooled after several injections, and concentrated by lyophilization to a white fluffy powder. Purity was checked by thin-layer chromatography, performed on 0.25-mm E. Merck precoated silica gel plates (60 F_{254}) and eluted with EtOAc/$CHCl_3$ (1:4).

Residue Analysis. Samples (10% of peptide fraction from reverse-phase chromatography) were hydrolyzed in 6 N HCl for 22 h, dried, and derivatized by use of EtOH/Et_3N/H_2O/phenyl isothiocyanate (7:1:1:1) for 15 min at room temperature. The PTH derivatives were analyzed by reverse-phase HPLC on a Hewlett-Packard Model 1090 chromatograph (Heinrikson et al., 1987). Analysis was carried out by K. Burke from the Protein/Nucleic Acid Analysis Lab (UC Irvine).

Residue Sequence Determination. Sequence analysis was performed by use of either an Applied Biosystems 470A gas-phase or 477A pulsed-liquid sequencer. PTH derivatives (from the 470A) were identified by reverse-phase HPLC with a Hewlett-Packard Model 1084B chromatograph equipped with an Altex Ultrasphere ODS column (4.6 × 250 mm). The aqueous phase was 7.5 mM sodium phosphate, pH 5.5, and the PTH residues were eluted with a gradient of MeOH/CH_3CN in 17:3 ratio (v/v). The flow rate was 1.65 mL/min. Sequence determination was carried out by S. Disper from the Protein/Nucleic Acid Analysis Lab (UC Irvine).

Representative Preparation of Chemically Misacylated tRNAs. Construction of Unlabeled L-3-Iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA. Synthesis of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA was accomplished by ligation (Baldini et al., 1988; Heckler et al., 1984a) of tRNA$^{Gly}_{CUA}$-C_{OH} (200 μg) with 105 μg of 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′–5′)-[2′(3′)-*O*-(L-3-iodotyrosyl)adenosine] (Bain et al., 1991b) in a 400-μL reaction containing 55 mM Na^+/Hepes, pH 7.5/15 mM $MgCl_2$/250 μM ATP/8 μg of bovine serum albumin/10% DMSO, with 150 units of T4 RNA ligase. The mixture was incubated for 10 min at 37 °C and the reaction terminated by addition of 100 μL of a 250 mM NaOAc, pH 4.5/5 M NaCl/50 mM $MgCl_2$ buffer, followed by extraction once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), once with $CHCl_3$/isoamyl alcohol (24:1), and precipitation with 2.5 volumes of EtOH. The precipitate was dissolved in 10 mM NaOAc, pH 4.5/1 mM EDTA/100 mM NaCl (50 μL) and filtered through a Sephadex G-25 Select D column equilibrated with the same buffer. The initial isotope peak was immediately absorbed onto a BD-cellulose column (2 × 0.5 cm) that had been equilibrated at 4 °C with 50 mM NaOAc, pH 4.5/10 mM $MgCl_2$/1.0 M NaCl. Unreacted tRNA$^{Gly}_{CUA}$-C_{OH} was removed by eluting the column with 10 mL of the buffer. The column was then eluted with the buffer containing 25% EtOH to elute L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (Heckler et al., 1984a). The appropriate fractions were combined, and the tRNA EtOH-precipitated (2.5 volumes), washed with 70% EtOH, suspended in H_2O, lyophilized, and stored under argon at −20 °C as a fluffy white powder. The amber suppressors L-tyrosyl-tRNA$^{Gly}_{CUA}$-dCA, *N*-methyl-L-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, D-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, and L-phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA, in addition to the ochre suppressor L-3-iodotyrosyl-tRNA$^{Gly}_{UUA}$-dCA and opal suppressor L-3-iodotyrosyl-tRNA$^{Gly}_{UCA}$-dCA, were produced in an analogous fashion (Bain et al., 1991b).

Preparation of Chemically Misacylated L-3-[^{125}I]Iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA. Synthesis of L-tyrosyl-tRNA$^{Gly}_{CUA}$-dCA was accomplished as described above with 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′–5′)-[2′(3′)-*O*-(L-tyrosyl)adenosine]. Radioiodination (Scherberg et al., 1978) of the ligation product (10 μL) was accomplished by suspension in 70 mM sodium phosphate, pH 7.5, buffer (300 μL) containing 1.2 mCi of carrier-free Na ^{125}I. Chloramine-T (2 μL of a 35 mM solution in H_2O) was added and the mixture maintained at room temperature for 5 min, followed by addition of sodium bisulfite

(5 μL of a 0.1 M solution in H_2O) and NaCl (33 μL of a 3 M solution in H_2O) and precipitation with 2.5 volumes of EtOH. The precipitate was dissolved in 10 mM NaOAc, pH 4.5/1 mM EDTA/100 mM NaCl (50 μL) and filtered through a Sephadex G-25 Select D column equilibrated with the same buffer. The initial isotope peak was immediately absorbed onto a BD-cellulose column (2 × 0.5 cm) that had been equilibrated at 4 °C with 50 mM NaOAc, pH 4.5/10 mM $MgCl_2$/1.0 M NaCl. Unreacted $tRNA^{Gly}_{CUA}$-C_{OH} was removed by eluting the column with 10 mL of the buffer. The column was then eluted with the buffer containing 25% EtOH to effect elution of L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (Heckler et al., 1984a). The appropriate fractions were combined and the tRNA EtOH-precipitated (2.5 volumes), washed with 70% EtOH, suspended in H_2O, lyophilized, and stored under argon at −20 °C as a fluffy white powder.

Preparation of mRNA for Polypeptides. Method A: 5′-Capped mRNA. Four synthetic oligodeoxyribonucleotides were used to construct an insert containing the mRNA template. Synthesis and purification of the mRNA template was essentially the same as previously described for the $tRNA^{Gly}_{CUA}$-C_{OH} template (Bain et al., 1991b). A vector was prepared by digestion of plasmid M13$^+$ KS with *Eco*RI and *Hin*dIII. Plasmids were screened for the insert with *Nco*I (engineered into the insert)/*Hin*dIII restriction analysis. Template DNA was linearized by *Xho*I digestion of the plasmid. Capped mRNA transcripts (Contreras et al., 1982; Konarska et al., 1984) were produced by use of the linearized template (2 μg) in a 20-μL reaction containing 40 mM Tris·HCl, pH 8.0/20 mM $MgCl_2$/5 mM dithiothreitol/1 μg of bovine serum albumin/5 mM P^1-5′-(7-methyl)guanosine-P^3-5′-guanosine triphosphate, dilithium salt/1 mM NTP each, with 20 units each of RNasin and T7 RNA polymerase. The mixture was incubated for 1 h at 37 °C followed by addition of EDTA (10 μL of a 100 mM stock in H_2O), extracted once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), once with $CHCl_3$/isoamyl alcohol (24:1), and precipitated with 2.5 volumes of EtOH to yield 2.9 μg of transcript. Homogeneity of the product was confirmed by electrophoresis in a 1.5% agarose gel.

Method B: Uncapped mRNA. Synthesis, purification, and screening for the mRNA template was essentially the same as described above for 5′-capped mRNA. A vector was prepared by digestion of plasmid M13$^+$ KS with *Eco*RI and *Hin*dIII. Plasmids were screened for the insert with *Nco*I (engineered into the insert)/*Hin*dIII restriction analysis. A 500-base-pair DNA template was produced from digestion of the plasmid with *Pvu*II, which was isolated by electrophoresis in a 1.5% agarose gel and elution using Geneclean to yield the purified vector. The RNA transcript was produced in a 500-μL reaction containing 40 mM Tris·HCl, pH 8.1/20 mM $MgCl_2$/1 mM spermidine/5 mM dithiothreitol/25 μg of bovine serum albumin/5 mM NTP each, with 2 units of inorganic pyrophosphatase and 12 000 units of T7 RNA polymerase (Francklyn & Schimmel, 1989; Milligan et al., 1987). The mixture was incubated for 4 h at 37 °C followed by addition of EDTA (10 μL of a 100 mM aqueous solution), extracted once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), once with $CHCl_3$/isoamyl alcohol (24:1), and precipitated with 2.5 volumes of EtOH to yield 720 μg of transcript. Homogeneity of the product was confirmed by electrophoresis in a 1.5% agarose gel.

Protein Synthesis. Method A: Quantitative Evaluation of L-3-[^{125}I]Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA. Translation with rabbit reticulocyte lysate was performed as directed by the manufacturer's instructions (Amersham). Magnesium and potassium ion concentrations contained within the lysate were found to yield efficient translation, and no modifications were required. Optimal mRNA concentration was determined as per instructions. A typical reaction (10 μL) contained lysate (8 μL), L-[^{35}S]methionine (100 μCi), 5′-capped mRNA (1.0 μM), and L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (2.0 μM). The mixture was incubated for 1 h at 30 °C followed by addition of 1.0 M sodium hydroxide/hydrogen peroxide (0.5 mL) and warming to 37 °C for 10 min. Synthetically prepared polypeptide standards (see Chemical Peptide Synthesis and Purification), corresponding to the expected 8-mer and 16-mer products from the translation, were added (10 μL of a 0.5 mM solution in 77% formic acid), followed by addition of H_2O (200 μL) and extraction (3 × 250 μL) with CH_3CN/$CHCl_3$ (1:1). The organic layers were combined, concentrated in vacuo, suspended in 77% formic acid (100 μL), and immediately injected onto HPLC, as described in Materials and Methods, followed by radioisotopic detection by scintillation counting.

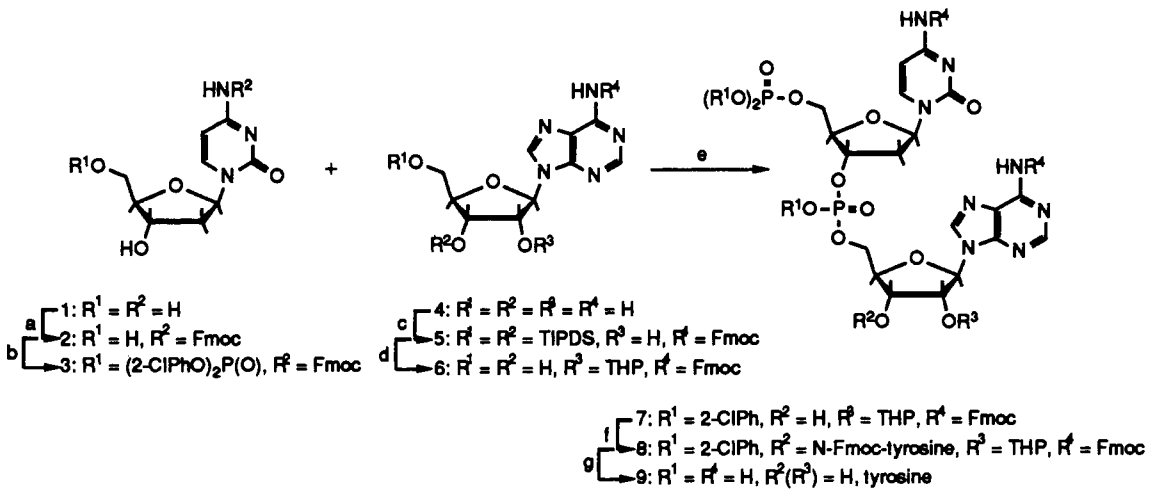
Method B: Rapid Screening of Unlabeled Nonnatural Residues. Translations containing unlabeled nonnatural residues with rabbit reticulocyte lysate were performed with a slightly modified procedure from manufacturer's instructions (Amersham). Magnesium ion and mRNA concentrations were determined as per instructions. A typical reaction (10 μL) contained lysate (9 μL), L-[^{35}S]methionine (15 μCi), L-[3,4,5-^{3}H]leucine (5 μCi), uncapped mRNA (2.0 μM), chemically misacylated tRNA (20 μM). The mixture was incubated for 1 h at 30 °C followed by addition of synthetically prepared polypeptide standards (see Chemical Peptide Synthesis and Purification), corresponding to the expected 8-mer and 16-mer products from the translation (10 μL of a 0.5 mM solution in 77% formic acid). The solution was immediately quenched with H_2O (1.0 mL), and the resulting precipitate was centrifuged, and the solvent was decanted. A cycle of resuspension in 77% formic acid (10 μL) followed by precipitation with H_2O (1.0 mL) was repeated twice. The resulting precipitate was dissolved in 77% formic acid (100 μL) followed by radioisotope detection by scintillation counting or injection onto HPLC, as described in Materials and Methods.

Results and Discussion

The biosynthetic production of modified proteins containing nonnatural residues would require (1) a specifically aminoacylated suppressor tRNA, (2) an appropriately designed mRNA containing a nonsense mutation site, (3) a translation system, and (4) a method for introduction of the exogenous tRNA and mRNA components. Additionally, a translation experiment was designed to allow the quantitative and unambiguous determination of suppression efficiency, read-through, and site specificity of incorporation. Also, the ability to rapidly analyze a large array of labeled or unlabeled acylated tRNA analogues was desired.

Preparation of Chemically Misacylated L-3-[^{125}I]Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA. In a previous report (Bain et al., 1989), L-3-[^{125}I]iodotyrosine was chosen as our first attempt at site-specific incorporation of this nonnatural residue during biosynthesis of a polypeptide. This particular residue was chosen for two reasons. First, previous attempts to incorporate either monoiodotyrosine or diiodotyrosine biosynthetically into proteins via in vitro rabbit reticulocyte lysate have failed when these residues are introduced into the system as the free acids (Scherberg et al., 1978). Secondly, this radiolabeled residue is easily synthesized with a specific activity within detectable levels for cell-free translation with rabbit reticulocyte lysate (Hadi et al., 1977).

Scheme I: Synthesis[a] of Acylated Dinucleotide 9



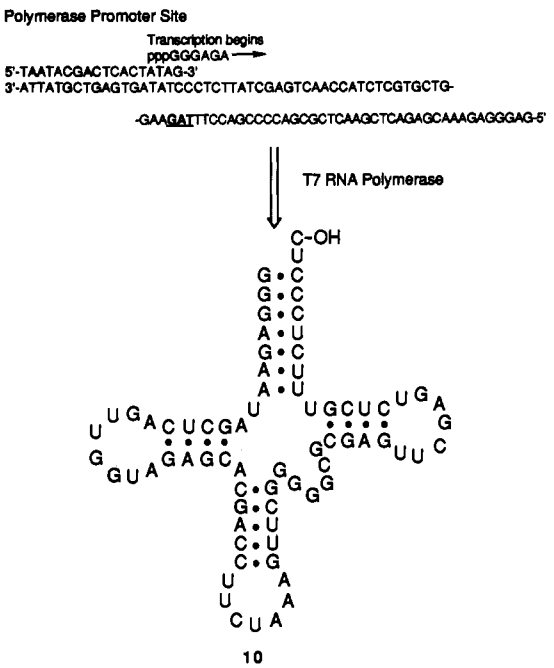
[a] Synthesis was carried out as follows: (a) (i) 2′-deoxycytidine monohydrochloride, TMSCl 4.1 equiv, pyridine, RT, 2 h; (ii) FmocCl 1.1 equiv, RT, 3 h; (b) **2**, $(2\text{-ClPhO})_2\text{P(O)Cl}$ 1.0 equiv, pyridine, 0 °C–RT, 1 h; (c) (i) adenosine, TIPDSCl_2 1.1 equiv, DMF/pyridine (1:1), RT, 1 h; (ii) TMSCl 2 equiv, RT, 1 h; (iii) FmocCl 1.1 equiv, RT, 3 h; (d) (i) **4**, 2,3-dihydropyran, *p*-toluenesulfonic acid 0.33 equiv, 3-Å molecular sieves, dioxane, RT, 1.5 h; (ii) TBAF 0.8 M, THF/pyridine/H_2O (8:1:1), RT, 1.5 h; (e) (i) **3**, $(2\text{-ClPhO})\text{P(O)(HOBT)}_2$ 1.1 equiv, THF, RT, 30 min; (ii) **6** 1.3 equiv, *N*-methylimidazole 0.6 equiv, RT, 30 min; (f) **7**, *N*-Fmoc-*O-tert*-butyltyrosine 3.0 equiv, *N*-methylimidazole 1.5 equiv, BOP 3.0 equiv, HOBT 3.0 equiv, DMF, RT, 30 min; (g) (i) **8**, 1,1,3,3-tetramethylguanidine 0.38 M, 4-nitrobenzaldoxime 0.33 M, CH_3CN, RT, 3 h; (ii) 80% formic acid, 0 °C, 30 min.

Chemical synthesis of the requisite aminoacyl dinucleotide (**9**) is based upon prior work developed by Chládek and coworkers (Hagen et al., 1988; Happ et al., 1987; Scalfi-Happ et al., 1987). However, 2′-deoxycytidine is employed rather than cytidine, resulting in the synthesis of a dinucleotide RNA/DNA hybrid (Scheme I)—the details of which are reported elsewhere (Bain et al., 1991b). The decision to employ a dinucleotide RNA/DNA hybrid in the construction of requisite nonsense suppressor leads to several advantages over a total RNA analogue in terms of synthetic simplicity and product stability, while maintaining compatibility with T4 RNA ligase (Snopek et al., 1976,; Sugino et al., 1977) and other enzymes involved in the biosynthesis of polypeptides (Chládek & Sprinzl, 1985; Khan & Roe, 1988). Accordingly, construction of the desired dinucleotide RNA/DNA hybrid was accomplished by condensation of the key intermediates N^4-[(9-fluorenylmethoxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidine (**3**) and N^6-[(9-fluorenylmethoxy)carbonyl]-2′-*O*-(tetrahydropyranyl)-adenosine (**6**) with the hydroxybenzotriazolyl phosphotriester method of Happ et al. (1987).

Acylation of the protected dinucleotide with *N*-(9-fluorenylmethoxy)carbonyl-*O-tert*-butyl-L-tyrosine, activated just prior to use with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole dissolved in a 0.2 M *N*-methylmorpholine in DMF (Hudson, 1988), was achieved in high yield. A two-step deprotection scheme developed by Chládek (Happ et al., 1987), followed by reverse-phase HPLC using gradient elution with CH_3CN (0–40% CH_3CN in 30 min) in 5 mM ammonium formate (pH 4.5) gave the fully deprotected acylated dinucleotide in 54% yield. This procedure represents an improvement over previous methods, which give a diacylated product requiring extra deprotection and purification steps (Baldini et al., 1988; Heckler et al., 1984a; Noren et al., 1989).

The final component to be synthesized was the 3′-truncated tRNA $\text{tRNA}^{\text{Gly}}_{\text{CUA}}\text{-C}_{\text{OH}}$ (**10**), prepared from a synthetic linear DNA template (Francklyn & Schimmel, 1989) featuring a double-stranded promoter region and a long 5′ overhang corresponding to the transcribed sequence (Scheme II). Run-off transcription of this template gives the expected 74-

Scheme II: Synthetic DNA Template[a]



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[a] Synthetic DNA template for run-off transcription of 3′-truncated tRNA $\text{tRNA}^{\text{Gly}}_{\text{CUA}}\text{-C}_{\text{OH}}$ (**10**). The $\text{tRNA}^{\text{Gly}}_{\text{CUA}}\text{-C}_{\text{OH}}$ template yields a 74-base run-off transcript containing the altered anticodon as indicated by underline. This template features a double-stranded promoter region and a long 5′ overhang corresponding to the transcribed sequence.

nucleotide product with a sequence corresponding to *E. coli* $\text{tRNA}^{\text{Gly3}}$ (Roberts & Carbon, 1975) except (1) the transcript contains no hypermodified bases, (2) the 3′ terminus lacks the normal cytidine and adenosine residues, and (3) the anticodon loop contains a CUA anticodon for suppression of amber nonsense suppression sites. Milligram quantities of this transcript are obtained in a single reaction and can be purified to single-nucleotide resolution on a 20% polyacrylamide preparative gel (Sampson & Uhlenbeck, 1988).

The T4 RNA ligase mediated coupling of 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′–5′)-[2′(3′)-*O*-(4-hydroxyphenylalanyl)adenosine] (**9**) with $\text{tRNA}^{\text{Gly}}_{\text{CUA}}\text{-C}_{\text{OH}}$ (**10**) to yield

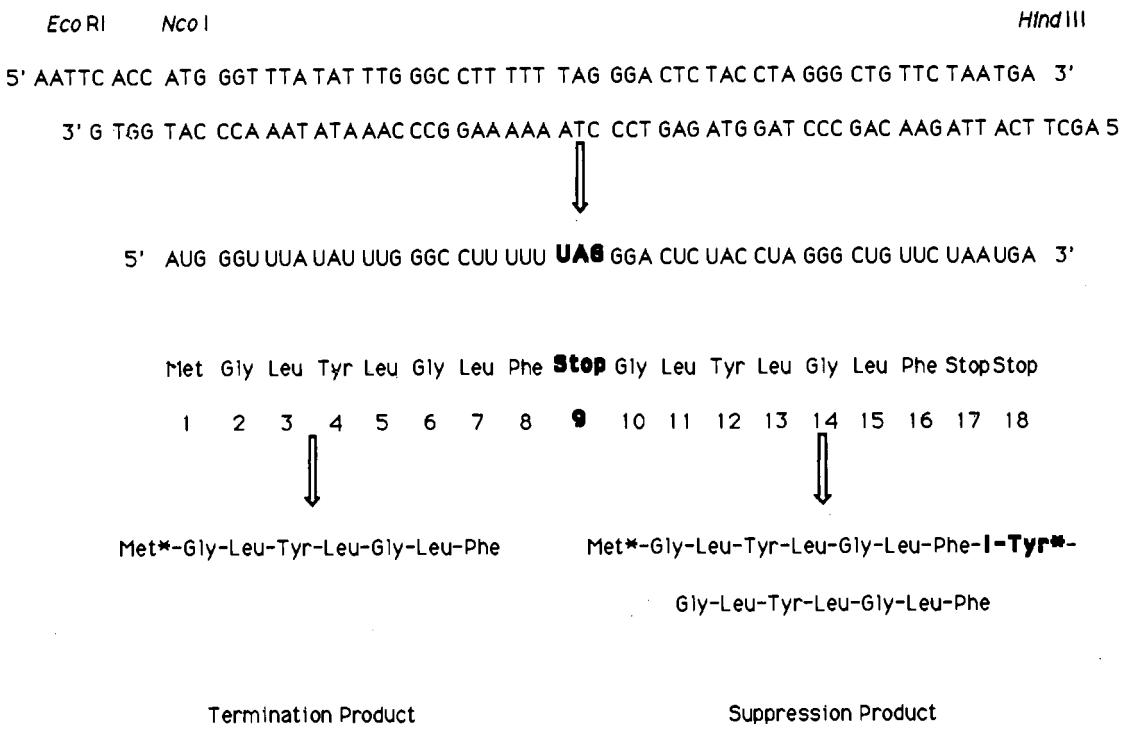

FIGURE 1: Termination and suppression products obtained from translation of mRNA. The mRNA template contains a 60-base leader sequence between the transcription start site and the initiation codon and a 50-base 3'-untranslated region for efficient interaction with the ribosome. The initiation codon is immediately flanked by a consensus sequence for optimum translation. In addition, a *Nco*I site was designed into the insert to facilitate screening of recombinants. Translation of the mRNA can yield either a termination (8-mer polypeptide) or L-3-iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA-suppressed (16-mer) product. Sequences for both polypeptides are as indicated in the figure, with position 9 corresponding to the amber site (i.e., the position where the nonnatural amino acid L-3-iodotyrosine is inserted in the suppression product).

tyrosyl-$tRNA^{Gly}_{CUA}$-dCA was carried out as described by Baldini et al. (1988) exept that the length of incubation was extended from 6 to 10 min to maximize the coupling yield. In addition, greater yields of the desired acylated tRNA were realized when the ligation was conducted in the presence of a large excess of the dinucleotide **9** (20 molar equiv). Following incubation, the excess dinucleotide is easily separated from the acylated tRNA by selective precipitation of tyrosyl-$tRNA^{Gly}_{CUA}$-dCA with 70% EtOH at −20 °C. Further purification of the crude precipitate was accomplished by $CHCl_3$/phenol extraction, which removed the T4 RNA ligase. The partially purified L-tyrosyl-$tRNA^{Gly}_{CUA}$-dCA was then radioiodinated with carrier-free Na ^{125}I under conditions that minimize radiolabeling of the tRNA (Scherberg et al., 1978). Final purification of L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA was accomplished by sequential elution through Sephadex G-25 and BD-cellulose (Heckler, 1984). Analysis of this purified product showed that 0.11 mCi of ^{125}I was incorporated into 9.6 μg of tyrosyl-$tRNA^{Gly}_{CUA}$-dCA, which resulted in a specific activity for L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA of 293 Ci/mmol.

Quantitative Evaluation of Read-Through, L-3-[^{125}I]-Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA Suppression Efficiency, and Site-Specificity of Incorporation into a Novel Synthetic Polypeptide during Biosynthetic Translation. A direct method for the determination of suppression efficiency, read-through, and site specificity of incorporation was desired for the initial translation experiment. Accordingly, biosynthesis was conducted with a cell-free rabbit reticulocyte lysate translation system to facilitate introduction of the exogenous acylated tRNA and mRNA. An appropriately designed mRNA containing a nonsense suppression site at position 9 was transcribed from a synthetic gene (Figure 1). Two possible polypeptides can be translated from this mRNA: an 8-mer and 16-mer. The 8-mer is the expected termination product, and the 16-mer would be produced if suppression of the nonsense site at position 9 were to occur. It should be noted that the polypeptide sequences were designed with amino acids mainly possessing hydrophobic side chains, allowing facile isolation of the expected translation products from rabbit reticulocyte lysate by simple extraction with organic solvents.

The DNA template for the mRNA was designed to expedite the preparation of the template and transcription of an appropriate RNA message. The DNA template for the mRNA contained a consensus sequence flanking the initiation codon for efficient translation of the RNA message (Kozak, 1984). The insert for the template was constructed from four synthetic oligodeoxyribonucleotides that were cloned into an appropriately prepared Bluescript M13$^+$ KS plasmid (Figure 1). Overproduction of the plasmid in TB-1 cells followed by purification, screening for recombinants by *Nco*I digestion (a unqiue restriction site was engineered into the gene to facilitate screening), and linearization of the plasmid by digestion with *Xho*I resulted in a linear DNA template with a 60-base leader sequence between the transcription start site and the initiation codon and a 50-base 3'-untranslated region. 5'-Capped mRNA transcripts were produced as previously described (Contreras et al., 1982; Konarska et al., 1984).

The translation experiment was conducted in the presence of L-[^{35}S]methionine and L-3-[^{125}I]iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (Figure 1). Following cessation of the translation reaction, nonradiolabeled polypeptide standards of the 8- and 16-mer were added as carriers to facilitate partitioning of the polypeptide products into the organic layer during extraction with $CH_3CN/CHCl_3$ (1:1). The extracts were evaporated to dryness, dissolved in 77% formic acid, and then fractionated by reverse-phase HPLC. Following isolation of the translation products, total suppression (i.e., that due to both read-through and nonsense suppression) was calculated from the relative levels of L-[^{35}S]methionine contained in the 8- and 16-mer polypeptide products (586 and 248 dpm, respectively) to give

Table I

experiment[b]	decays per minute (dpm)[a] 8-mer ^{35}S	8-mer ^{125}I	16-mer ^{35}S	16-mer ^{125}I
L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA	586	–	248	2120
control 1[c]	836	–	26	–
control 2[c]	863	–	37	–
control 3[c]	815	–	23	–

[a] Counts per minute have been converted into decays per minute (dpm) and are corrected for background. Dashes indicate no decays per minute above background levels. [b] In vitro rabbit reticulocyte lysate translation experiments were carried out as described in the text. Crude polypeptides were injected onto a Vydac C-4 column equilibrated in 0.1% TFA in H_2O/CH_3CN (1:1). Gradient elution with 0.1% TFA in CH_3CN (35–50% CH_3CN in 50 min) resolved the 8- and 16-mer eluting at 40–45% CH_3CN. [c] Controls were conducted in the absence of added L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA but were supplemented as follows: control 1, buffer alone; control 2, 0.5 μg of nonacylated tRNA; control 3, 0.5 μg of nonacylated tRNA plus 100 μCi of carrier-free L-3-[^{125}I]iodotyrosine.

a value of 30% suppression efficiency at the nonsense suppression site (Table I).

Suppression efficiency was further assessed by analyzing the L-3-[^{125}I]iodotyrosine to L-[^{35}S]methionine ratio in the 16-mer polypeptide to determine if read-through had contributed significantly to the isolated mass of the suppression product. Contamination of the 16-mer at positon 9 by amino acids other than the desired nonnatural residue would be represented by an L-3-[^{125}I]iodotyrosine to L-[^{35}S]methionine ratio other than 1:1 (after correction for specific activity differences). Specific activities of the radioisotopes were 34 and 293 Ci/mmol for L-[^{35}S]methionine and L-3-[^{125}I]iodotyrosine, respectively, to yield a ratio of 8.62. The observed L-3-[^{125}I]iodotyrosine (2120 dpm) to L-[^{35}S]methionine (248 dpm) ratio for the 16-mer suppression product was 8.54, which is identical with the value found in the lysate within the experimental error. This result clearly shows that insignificant amounts of read-through occur in the presence of the synthetic nonsense suppressor L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA. It was vitally important to quantify suppression with respect to this last calculation since preliminary experiments conducted by our lab (results not shown) demonstrated that read-through could account for as much as half of the suppression observed when minimal tRNA concentrations are employed (<10.0 μM).

The translation reaction containing L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA was conducted in parallel with several controls (Table I) to fully assess the suppression efficiency of the nonsense site by the chemically misacylated tRNA. The amount of read-through that would occur in the absence of exogenous tRNA was determined in control 1, which was conducted with only added mRNA and L-[^{35}S]methionine. Thus, any 16-mer produced is due to suppression at the nonsense site with endogenous aminoacyl-tRNAs. In control 2, the addition of nonacylated tRNA$^{Gly}_{CUA}$-dCA to the lysate was employed to rule out the possibility of enzymatic reacylation of the tRNA that is liberated after delivery of L-3-[^{125}I]-iodotyrosine to the ribosome. Finally, in control 3, confirmation that L-3-[^{125}I]iodotyrosine does not act as a substrate for any of the endogenous synthetases was obtained by the addition of only nonacylated tRNA$^{Gly}_{CUA}$-dCA and carrier-free L-3-[^{125}I]iodotyrosine to the lysate. If L-3-[^{125}I]iodotyrosine were to act as a substrate for one of the synthetases, the resulting acylated tRNA could be utilized as a substrate for protein biosynthesis under the conditions of the translation reaction. The observed results for the control experiments all closely correlate with one another within detectable levels, indicating that enzymatic acylation of tRNA$^{Gly}_{CUA}$-dCA does not occur nor does L-3-[^{125}I]iodotyrosine act as a substrate for the synthetases.

The final question to be addressed was the site specificity of L-3-[^{125}I]iodotyrosine incorporation during translation of the RNA message. Site-specific incorporation of L-3-[^{125}I]-iodotyrosine at the position of the UAG codon (position 9) was directly verified by sequencing of the 16-mer by Edman degradation and radiolabel detection of the resultant amino acid PTH derivatives for both L-[^{35}S]methionine and L-3-[^{125}I]iodotyrosine. The results clearly show that position 1 contained ^{35}S, position 9 contained ^{125}I, and all other positions had no activity above background levels. This result confirms unequivocally that L-3-[^{125}I]iodotyrosine is incorporated exclusively at the position of the UGA codon.

Development of a Rapid Assay Method for Determining Suppression Efficiencies. The experiments described above demonstrate that this strategy for incorporation of nonnatural amino acids into peptides is a workable one. A number of questions remained, however: How general is the process? Can it be made significantly more efficient? Are there specific, identifiable structural features of the aminoacyl-tRNA that affect the efficiency of incorporation? In order to answer these questions, the procedure was tested with several other nonnatural residues and tRNA modifications. Thus, rather than moving immediately to site-directed mutagenesis in enzymes, we opted to develop a rapid assay system to test these questions.

A simple method for determining suppression efficiency of synthetic acylated suppressors without requiring synthesis of radiolabeled nonnatural residues would be highly desirable. The translation system employed for characterization of L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA was also designed to provide suppression efficiencies in subsequent studies without the need to radiolabel each nonnatural residue to be tested. In order to do so, translation in the rabbit reticulocyte lysate is conducted as it was in the initial experiments except that *unlabeled* acylated tRNA is added, in conjunction with L-[^{35}S]methionine and L-[^{3}H]leucine.[3] As before, the termination product is an 8-mer polypeptide, while the suppression product is 16-mer, depending upon whether or not suppression occurs. Suppression efficiencies can then be determined simply by measuring the ratio of radiolabeled L-[^{35}S]methionine to L-[^{3}H]leucine: since the 8-mer contains three leucines and the 16-mer contains six, 0% suppression results in an ^{3}H:^{35}S ratio of 3:1 (corrected for specific activities), while 100% suppression would give a 6:1 ratio (Figure 1). Intermediate levels can thus be determined by simple interpolation. As the most rigorously studied example to date, L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA was employed to calibrate the assay and to act as a reference for comparison to all other acylated tRNA analogues. The lysates for individual translation experiments were each incubated for 1 h at 30 °C followed by addition of synthetically prepared polypeptide standards (Hudson, 1988), corresponding to the expected 8-mer and 16-mer products from the translation (10 μL of a 0.5 mM solution in 77% formic acid). The solution was immediately quenched with H_2O (1.0 mL), yielding a precipitate (i.e., the translation products) that was separated

[3] These ratios are derived from *probabilities* of incorporation; at the level of isotopic enrichment of the methionine and leucine (1 μCi/mM corresponds approximately to one radiolabeled atom in 10^7–10^8) it is very unlikely that any single peptide has more than one radiolabeled atom. Thus, averaged over all peptides produced, the 16-mer is twice as likely to contain tritium as the 8-mer.

Table II

entry	rapid assay conditions[a]	[^{3}H] in dpm	[^{35}S] in dpm	average % suppression[b]
1	with no added tRNA suppressor	245341	112541	0
		257113	124209	
		221475	104964	
2	with nonacylated tRNA suppressor	218512	101633	0
		239183	108719	
		263931	126282	
3	1.0 μM mRNA; 20.0 μM tRNA; 2.5 mM Mg	318494	115732	26
		289748	109463	
		323916	121317	
4	2.0 μM mRNA; 20.0 μM tRNA; 2.5 mM Mg	394733	146742	25
		408057	151214	
		413049	157837	
5	2.0 μM mRNA; 40.0 μM tRNA; 5.0 mM Mg	439126	157603	32
		442234	154911	
		429053	152003	
6	2.0 μM mRNA; 40.0 μM tRNA; 7.5 mM Mg	595052	171115	59
		592576	174593	
		545630	163127	
7	2.0 μM mRNA; 40.0 μM tRNA; 10.0 mM Mg	529517	149781	65
		613814	172063	
		557504	159337	
8	2.0 μM mRNA; 40.0 μM tRNA; 15.0 mM Mg	480323	147983	51
		493060	155748	
		523222	159650	

[a] A typical reaction (10 μL) contained lysate (9 μL), L-[^{35}S]methionine (15 μCi), and L-[3,4,5-^{3}H]leucine (5 μCi). The concentrations of mRNA, L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA, and magnesium ion are as indicated. [b] Values given are an average of three trials. Counts per minute have been converted into decays per minute (dpm) and are corrected for background and quenching for each individual trial prior to averaging. Average suppression values of individual trials were derived from the ratio of L-[^{35}S]-methionine to L-[^{3}H]leucine divided by the ^{3}H:^{35}S ratio obtained from entry 2. L-3-Iodotyrosine was employed as an internal standard in each assay to normalize interassay variances.

from lysate components by centrifugation. Again, the strategy to produce translation products that are hydrophobic in nature—which allows facile isolation from the lysate by extraction with organic solvents—also permits their isolation to be accomplished by a repeated cycle of resuspension in 77% formic acid (10 μL) followed by precipitation with H_2O (1.0 mL). After isolation, L-[^{35}S]methionine and L-[^{3}H]leucine radioisotope levels were detected by scintillation counting and the suppression efficiencies were determined after correction for background counts, quenching, and specific activities.

Optimization of Conditions. Translation conditions were first optimized with the unlabeled acylated tRNA suppressor L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA by varying the concentrations of mRNA, suppressor, and magnesium ion present during translation (Table II). As before, controls (entries 1 and 2) were conducted in parallel with the translation experiment containing the unlabeled acylated suppressor. The first control (entry 1) was employed to determine the observed ^{3}H:^{35}S ratio when translation of the mRNA is conducted in the absence of any suppressor tRNAs and thus is representative of the radiolabel ratio that would be expected for the 8-mer termination product only. Analogous with the initial experiments, a second control experiment (entry 2) contained nonacylated tRNA$^{Gly}_{CUA}$-dCA. As observed before with the experiments conducted with L-3-[^{125}I]iodotyrosine, identical results were obtained from controls within the experimental error (±5%).

Another issue to be addressed is the reliability of observed ^{3}H:^{35}S ratios to act as an *indirect* reporter of suppression efficiency for the unlabeled acylated tRNA. Thus, a translation experiment was conducted with the unlabeled acylated tRNA suppressor L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (entry 3) under the same reaction conditions previously employed for the analogous labeled acylated suppressor. The suppression efficiency (26%) derived from the ^{3}H:^{35}S ratio was indistinguishable from the value obtained in the initial experiments

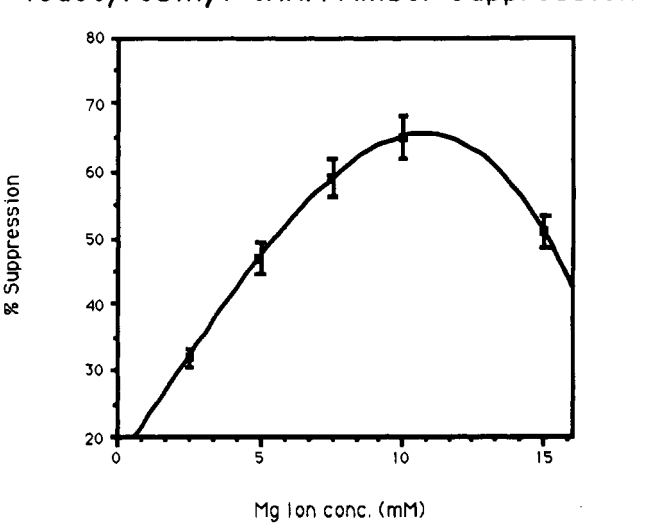

FIGURE 2: Magnesium ion concentration dependence upon suppression efficiency. Translation with rabbit reticulocyte lysate and determination of suppression efficiencies were performed as described in Table II legend. Error bars indicate an estimated uncertainty of ±5% for each of the averaged suppression values.

with L-3-[^{125}I]iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (30%) within experiment error (±5%). With this result in hand, further refinements of the optimum conditions for suppression efficiency could be determined.

The optimum mRNA and tRNA concentrations (entry 4) specific to the rapid assay conditions were determined in order to maximize both the suppression efficiency of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA and the relative amount of translation product produced. Optimization of mRNA concentration (based upon total L-[^{35}S]methionine incorporated into isolated translation products) revealed that the maximum amount of translation product occurs at 2.0 μM mRNA. Analogous optimization of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA revealed that both low (<10.0 μM) and high (>100.0 μM) concentrations of the acylated suppressor significantly reduced the amount of translation product produced (on the basis of total L-[^{3}H]leucine incorporated into isolated translation products). It was interesting to find that concentrations of the acylated tRNA greater than 100 μM actually inhibited the total amount of translation product, on the basis of incorporation of both ^{35}S and ^{3}H. This reduction may be due to competitive inhibition of the endogenous aminoacyl-tRNA by the nonsense suppressor at the ribosomal binding sites. Nevertheless, efficient production of translation products could be achieved with intermediate concentrations (10.0–100.0 μM) of acylated suppressor added to the lysates, with 20.0 μM suppressor being optimal. Overall, a significant increase (31%) in the relative amount of translation product produced could be achieved under optimum mRNA and suppressor tRNA concentrations (entry 4). However, the average suppression efficiency was unchanged under these optimized conditions when compared to the original conditions (entry 3) employed in the initial experiments.

Magnesium ion levels also proved to have a significant effect upon suppression efficiency of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (Table II, entries 4–8, and Figure 2). On the basis of L-[^{35}S]methionine incorporation, the relative amount of translation product (both 8-mer and 16-mer) produced gradually rose from its lowest level when no exogenous magnesium ion was added (entry 3; 2.5 mM Mg ion is the concentration in the lysate supplied by the vendor) to its highest when the magnesium ion concentration was at 7.5 mM (entry 6). A

similar trend was found when magnesium ion concentration was compared to the suppression efficiency of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA in the assay (Figure 2), except optimum suppression efficiency (65%; entry 7) was obtained at a slightly higher magnesium ion concentration (10.0 mM). The effect that magnesium ion concentration exerts upon the observed nonsense suppression levels may be due to a conformational change in the unhypermodified tRNA induced by the ion (Hall, et al., 1989). A similar magnesium ion dependence was found for an unhypermodified tRNA^{Phe} amber suppressor also employed for introduction of nonnatural residues into proteins (Noren et al., 1990).

Incorporation of Other Nonnatural Amino Acids. As stated previously, one major difference between the rapid assay and the methodology employed in the initial experiments with radiolabeled L-3-[^{125}I]iodotyrosine is that suppression values are calculated indirectly in the rapid assay. A comparison of the suppression efficiencies derived from these two different methodologies demonstrated that the rapid assay did in fact yield valid results for iodotyrosine. Further evidence to support the validity of the rapid assay method was obtained from additional experiments conducted with *N*-methylphenylalanine and D-phenylalanine. Accordingly, suppression experiments with these residues were repeated under conditions identical with those in the first rapid assay experiments (2.0 μM mRNA; 40.0 μM tRNA; 10.0 mM magnesium). Following incubation of the lysates, polypeptide standards (appropriate for the particular nonnatural residue being employed) were added and the lysates separated into two fractions. The first fraction was evaluated as before by direct scintillation counting and determination of suppression efficiencies derived from the ^{3}H:^{35}S ratios. The other fraction was applied directly to a reverse-phase column and fractionated by HPLC. The radioisotope levels of 8- and 16-mer translation products, after isolation of the two fractions by HPLC, were detected by scintillation counting. Suppression values were obtained by direct comparison of ^{35}S found in the two translation products. For both *N*-methylphenylalanine and D-phenylalanine, as well as an iodotyrosine control, both the normal rapid assay method and this direct analysis of the isolated HPLC fractions gave identical results within experimental error. Both methods show that *N*-methylphenylalanine is incorporated with a suppression efficiency of approximately 70% while D-phenylalanine is not incorporated. It is not yet clear whether the incorporation of N-methylated residues or the failure to incorporate a D-amino acid will prove to be general, but both cases merit further study.

This methodology was also applied in an attempt to introduce an ester linkage in place of the normal amide bond at position 9. Thus, phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA was prepared and tested in the rapid assay. The translation efficiency was found to be 46%, despite the substitution of a hydroxyl group for the normal α-amino moiety. Analysis of the translation products was complicated by the relative difficulty of synthesizing a polypeptide standard containing an ester linkage along the polyamide backbone. However, this particular experiment afforded an alternative method of verifying the site of incorporation of the ester residue: since the predicted suppression product contains a unique ester linkage (Figure 3), selective hydrolysis of the relatively labile ester group should result in cleavage at position 9, yielding two 8-mer polypeptide products. The N-terminal cleavage product would contain all the ^{35}S after hydrolysis. Accordingly, the lysate was fractionated by reverse-phase HPLC following cessation of the translation reaction. On the basis of L-[^{35}S]methionine levels,

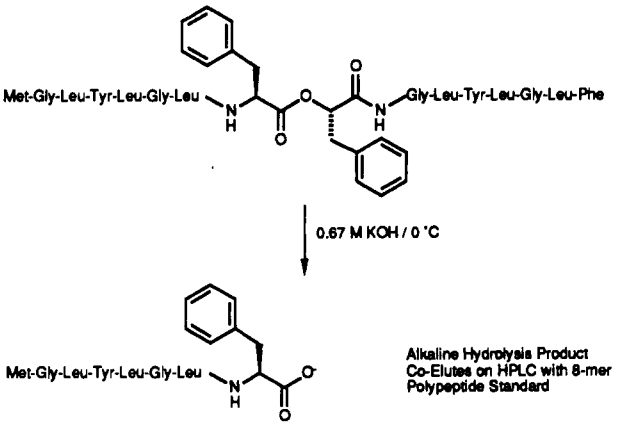

FIGURE 3: Verification that phenyllactic acid is site-specifically incorporated into a 16-mer suppression product. On the basis of detection of ^{35}S radiolabel, alkaline hydrolysis of the HPLC-purified suppression product was quantitatively converted to the expected cleavage product, which coelutes on HPLC with an 8-mer polypeptide standard. Crude polypeptides were injected onto a Vydac C-4 column equilibrated in 0.1% TFA in H_2O/CH_3CN (1:1). Gradient elution with 0.1% TFA in CH_3CN (35–50% CH_3CN in 50 min) resolved the 8- and 16-mer peaks eluting at 40–45% CH_3CN.

two fractions were isolated that contained significant amounts of the radiolabel. The slower eluting peak (the suspected suppression product) was isolated, followed by alkaline hydrolysis (Fahnestock et al., 1970; Fahnestock & Rich, 1971) and fractionation by reverse-phase HPLC. Scintillation counting of the resulting fraction revealed that all the ^{35}S radiolabel now coeluted with a polypeptide whose sequence was that of the expected N-terminal cleavage product.

Comparison of Amber, Opal, and Ochre Suppressors. All of the studies thus far carried out with nonsense suppressors to incorporate nonnatural residues into proteins, whether conducted by us (Bain et al., 1989, 1990a,b) or the Schultz group (Noren et al., 1989, 1990), have utilized an amber (UAG) suppression site and the corresponding nonsense amber suppressor tRNA. Minor modification of the assay components (the mRNA and suppressor tRNA) enabled the efficiencies of other suppressors (UGA = opal and UAA = ochre) to be determined. Interestingly, the opal suppressor was found to give the highest level of suppression for incorporation of L-3-iodotyrosine (81%) while the ochre suppressor gave the lowest level (48%), compared with 63% for the amber suppressor determined in side-by-side rapid assays. It was also verified that suppression of each nonsense site is specific for the corresponding aminoacyl-tRNA; e.g., an amber site with an opal suppressor gives no incorporation.

Reducing the Effectiveness of the Normal Termination Process as a Potential Means of Increasing Suppression Efficiency. Suppression efficiency is a vital issue since low levels of suppression would lead to only small amounts of the desired product. Since the observed suppression efficiency is a function not only of the rate of suppression itself but also of the effectiveness of normal termination, one way to increase overall efficiency would be to disrupt termination at the nonsense site. The rabbit reticulocyte lysate system was chosen in part because it offers a potential means of doing so. Releasing factors for termination in this translation system seem to recognize a codon tetramer instead of the normal triplet codon associated with termination in prokaryotic systems (Caskey, 1980; Freifelder, 1987). Substitution of different bases in the 3′-position of the tetramer had no adverse effect on recognition but did affect the efficiency of the termination process. Thus, it appears that the 3′-terminal base is not part of the recognition process but confers an element of stability

to the mRNA/releasing factor complex that results in efficient termination. The nonsense suppression site in our initial experiments contained the tetramer UAGG (Figure 1), which is one of the four (UAGG, UAGA, UAAA, UGAA) effective termination tetramers (Caskey, 1980; Freifelder, 1987). The UAGG tetramer was employed so that in the absence of suppression translation would result in efficient termination at position 9 rather than reading through the nonsense site to produce a 16-mer lacking the nonnatural amino acid. However, by preparing a mRNA in which the codons correlating to positions 10 and 11 of the polypeptide are reversed, a tetramer containing the sequence UAGC (a sequence not normally associated with efficient termination for in vitro translations) is the result. As predicted, this change resulted in an increase in suppression efficiency, although not a dramatic one, from 63 to 75%. It is unclear whether the results of this experiment can be attributed directly to the change in the tetramer or are due, all or in part, to longer range context effects. Further investigation is warranted to more clearly understand and apply this method of termination attenuation.

CONCLUSIONS

Several important and novel facets of site-specific incorporation of nonnatural residues into proteins during in vitro translation are presented here. It is known that during in vitro translation "read-through" (i.e., suppression by endogenous aminoacyl-tRNAs during in vitro translation) does occur and can account for almost half of the total suppression during protein synthesis when suppression levels fall below 10–15%. However, by maintaining an excess of acylated suppressor tRNA during the translation reactions, not only are lower suppression levels (5–10%) avoided but, more importantly, read-through does not contribute significantly to total suppression. Additionally, we have introduced a rapid and efficient method—along with a validation of this assay system—for examining the process of site-specific incorporation of nonnatural residues during protein biosynthesis. The suppression efficiencies of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA, *N*-methyl-L-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, D-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, and L-phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA were all evaluated in cell-free translation reactions with varying results (suppression efficiency varied from 72% for *N*-methyl-L-phenylalanine to 0% for D-phenylalanine). Further validation of the rapid assay method for analysis of suppression levels was conducted with these four acylated suppressors by direct analysis of HPLC-purified 8- and 16-mer translation products. Suppression levels derived by direct analysis of the purified polypeptides were indistinguishable from values obtained indirectly by the rapid assay method. Additional translation experiments were conducted with amber (UAG), opal (UGA), and ochre (UAA) suppressors acylated with L-3-iodotyrosine. All three acylated suppressor gave reasonably good suppression levels, ranging from 82% for the opal suppressor to 48% for the ochre suppressor. And finally, a translation experiment with L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA was conducted with the less efficient termination tetramer UAGC. Comparison between the suppression levels of an mRNA containing UAGC and the tetramer employed in the initial experiments (UAGG) demonstrated the ability to increase suppression efficiency by termination attenuation.

We have recently applied this rapid assay method to a systematic survey of the structural requirements for biosynthetic incorporation of nonnatural residues into our model polypeptide system for a series of 12 semisynthetic acylated suppressor tRNAs (Bain et al., 1991a). In addition, this method has recently been employed to compare several new methods for the preparation of semisynthetic suppressor tRNAs (Bain et al., 1991b). However, broad applicability of these procedures will require advances in two important areas. First, a simplified in vitro method needs to be developed to reduce the number of laborious chemical or enzymatic steps. Second, the desire to produce larger amounts of translation product will ultimately require in vivo protein expression. In an ongoing effort to expand the versatility of this process, we are currently expanding our research to address these two issues.

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REFERENCES

Atherton, E., Brown, E., & Sheppard, R. C. (1981) *J. Chem. Soc., Chem. Commun. 21*, 1151–1152.

Bain, J. D., Diala, E. S., Glabe, C. G., Dix, T. A., & Chamberlin, A. R. (1989) *J. Am. Chem. Soc. 111*, 8013–8014.

Bain, J. D., Wacker, D. A., Kuo, E. E., & Chamberlin, A. R. (1991a) *Tetrahedron 47*, 2389–2400.

Bain, J. D., Wacker, D. A., Kuo, E. E., Lyttle, M. H., & Chamberlin, A. R. (1991b) *J. Org. Chem.* (in press).

Baldini, G., Martoglio, B., Schachenmann, A., Zugliani, C., & Brunner, J. (1988) *Biochemistry 27*, 7951–7959.

Carpino, L. A., & Han, G. Y. (1972) *J. Org. Chem. 37*, 3404–3409.

Caskey, C. T. (1980) *Trends Biochem. Sci. 5*, 234–237.

Chang, C.-D., & Meienhofer, J. (1978) *Int. J. Pept. Protein Res. 11*, 246–249.

Chapeville, R., Lipmann, F., von Ehrenstein, G., Weisblum, B., Ray, W. J., Jr., & Benzer, S. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1086–1092.

Chládek, S., & Sprinzl, M. (1985) *Angew. Chem., Int. Ed. Engl. 24*, 371–391.

Contreras, R., Cheroutre, H., Degrave, W., & Fiers, W. (1982) *Nucleic Acids Res. 10(20)*, 6353–6362.

Crick, F. H. C. (1958) *Symp. Soc. Exp. Biol. 12*, 138–163.

Dryland, A., & Sheppard, R. C. (1986) *J. Chem. Soc., Perkin Trans. 1*, 125–137.

Fahnestock, S., & Rich, A. (1971) *Science 173*, 340–343.

Fahnestock, S., Neuman, H., Sashova, V., & Rich, A. (1970) *Biochemistry 9*, 2477–2483.

Francklyn, C., & Schimmel, P. R. (1989) *Nature 337*, 478–481.

Freifelder, D. (1987) in *Molecular Biology* (Davern, C. I., Ed.) Jones and Bartlett Publishers, Inc., Boston.

Hadi, U. A. H., Malcolme-Lawes, D. J., & Oldham, G. (1977) *Int. J. Appl. Radiat. Isot. 28*, 747–749.

Hagen, M. D., Scalfi-Happ, C., Happ, E., & Chládek, S. (1988) *J. Org. Chem. 53*, 5040–5045.

Hall, K. B., Sampson, J. R., Uhlenbeck, O. C., & Redfield, A. G. (1989) *Biochemistry 28*, 5794–5801.

Happ, E., Scalfi-Happ, C., & Chládek, S. (1987) *J. Org. Chem. 52*, 5387–5391.

Hecht, S. M., Alford, B. L., Kuroda, Y., & Kitano, S. (1978) *J. Biol. Chem. 253*, 4517–4520.

Heckler, T. G., Zama, Y., Naka, T., & Hecht, S. M. (1983) *J. Biol. Chem. 258*, 4492–4495.

Heckler, T. G., Chang, L.-H., Zama, Y., Naka, T., Chorghade, M. S., & Hecht, S. M. (1984a) *Biochemistry 23*, 1468–1473.

Heckler, T. G., Chang, L.-H., Zama, Y., Naka, T., & Hecht, S. M. (1984b) *Tetrahedron 40*, 87-94.
Heckler, T. G., Roesser, J. R., Xu, C., Chang, P. I., & Hecht, S. M. (1988) *Biochemistry 27*, 7254-7262.
Heinrikson, R. L., Mora, R., & Maraganore, J. M. (1987) in *Modern Methods in Protein Chemistry* (L'Italien, J., Ed.) Pergamon Press, New York.
Hilvert, D., Hatanaka, Y., & Kaiser, E. T. (1988) *J. Am. Chem. Soc. 110*, 682-689.
Hudson, D. (1988) *J. Org. Chem. 53*, 617-624.
Khan, A. S., & Roe, B. A. (1988) *Science 241*, 74-79.
Konarska, M. M., Padgett, R. A., & Sharp, P. A. (1984) *Cell 38*, 731-736.
Kozak, M. (1984) *Nature 308*, 241-246.
Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M.-A., & Bock, A. (1988) *Nature 331*, 723-725.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783-8798.
Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., & Schultz, P. G. (1989) *Science 244*, 182-188.
Noren, C. J., Anthony-Cahill, S. J., Suich, D. J., Noren, K. A., Griffith, M. C., & Schultz, P. G. (1990) *Nucleic Acids Res. 18*, 83-88.
Payne, R. C., Nichols, B. P., & Hecht, S. M. (1987) *Biochemistry 26*, 3197-3205.
Pezzuto, J. M., & Hecht, S. M. (1980) *J. Biol. Chem. 255*, 865-869.
Roberts, J. W., & Carbon, J. (1975) *J. Biol. Chem. 250*, 5530-5541.
Roesser, J. R., Chorghade, M. S., & Hecht, S. M. (1986) *Biochemistry 25*, 6361-6365.
Roesser, J. R., Xu, C., Payne, R. C., Surratt, C. K., & Hecht, S. M. (1989) *Biochemistry 28*, 5185-5195.
Sampson, J. R., & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033-1037.
Scalfi-Happ, C., Happ, E., Ghag, S., & Chládek, S. (1987) *Biochemistry 26*, 4682-4688.
Scherberg, N. H., Seo, H., & Hynes, R. (1978) *J. Biol. Chem. 253*, 1773-1779.
Schimmel, P. R. (1989) *Acc. Chem. Res. 22*, 232-233.
Schimmel, P. (1990) *Biochemistry 29*, 9495-9502.
Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601-648.
Shih, L. B., & Bayley, H. (1985) *Anal. Biochem. 144*, 132-141.
Snopek, T. J., Sugino, A., Agarwal, K. L., & Cozzarelli, N. R. (1976) *Biochem. Biophys. Res. Commun. 68*, 417-424.
Söll, D. (1988) *Nature 331*, 662-661.
Sugino, A., Snopek, T. J., & Cozzarelli, N. R. (1977) *J. Biol. Chem. 252*, 1732-1738.
Tanimura, H., Fukazawa, T., Sekine, M., Hata, T., Efcavitch, J., & Zon, J. (1988) *Tetrahedron Lett. 29*, 577-578.
Usman, N., Ogilvie, K. K., Jiang, M.-Y., & Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109*, 7845-7854.

A Photolabile Oligodeoxyribonucleotide Probe of the Peptidyltransferase Center: Identification of Neighboring Ribosomal Components†

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ABSTRACT: In this work we report the synthesis of a radioactive, photolabile oligodeoxyribonucleotide probe and its exploitation in identifying 50S ribosomal subunit components neighboring its target site in 23S rRNA. The probe is complementary to 23S rRNA nucleotides 2497-2505, a single-stranded sequence that has been shown to fall within the peptidyltransferase center of *Escherichia coli* ribosomes [Cooperman, B. S., Weitzmann, C. J., & Fernandez, C. L. (1990) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D., & Warner, J. R., Eds.) pp 491-501, American Society of Microbiology, Washington]. On photolysis in the presence of 50S ribosomes, it site-specifically incorporates into protein L3 (identified by both SDS-PAGE and immunological methods) and into three separate 23S rRNA regions: specifically, nucleotides 2454; 2501, 2502, 2505, 2506; and 2583, 2584. These results provide clear evidence that G-2505 in 23S rRNA is within 24 Å (the distance between G-2505 and the photogenerated nitrene) of protein L3 and of each of the nucleotides mentioned above and are of obvious importance in the construction of detailed three-dimensional models of ribosomal structure. The approach we present is general and can be applied to determining ribosomal components neighboring regions of rRNA that are susceptible to binding by complementary oligodeoxyribonucleotides, both in intact 30S and 50S subunits and in subunits at various stages of reconstitution.

Bogdanov and his co-workers (Skripkin et al., 1979; Mankin et al., 1981) first introduced the notion of using oligodeoxyribonucleotides that are complementary to rRNA sequences to probe the structure of rRNA, both of the native molecule and within ribosomal subunits. More recently, Hill and his co-workers (1990) have demonstrated that single-stranded regions of rRNA can form stable complexes with their complementary oligodeoxyribonucleotides, as evidenced both by filter-binding assays using a ^{32}P-labeled probe and by the demonstration that treatment of the complex with RNase H cleaves rRNA at the appropriate position. They have exploited

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