Effects of Release Factor 1 on in Vitro Protein Translation and the Elaboration of Proteins Containing Unnatural Amino Acids†

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ABSTRACT: An in vitro protein synthesizing system was modified to facilitate the improved, site-specific incorporation of unnatural amino acids into proteins via readthrough of mRNA nonsense (UAG) codons by chemically misacylated suppressor tRNAs. The modified system included an S-30 extract derived from *Escherichia coli* that expresses a temperature-sensitive variant of *E. coli* release factor 1 (RF1). Mild heat treatment of the S-30 extract partially deactivated RF1 and improved UAG codon readthrough by as much as 11-fold, as demonstrated by the incorporation of unnatural amino acids into positions 25 and 125 of HIV-1 protease and positions 10 and 22 of *E. coli* dihydrofolate reductase. The increases in yields were the greatest for those amino acids normally incorporated poorly in the in vitro protein synthesizing system, thus significantly enhancing the repertoire of modified amino acids that can be incorporated into the proteins of interest. The substantial increase in mutant protein yields over those obtained with an S-30 extract derived from an RF1 proficient *E. coli* strain is proposed to result from a relaxed stringency of termination by RF1 at the stop codon (UAG). When RF1 levels were depleted further, the intrinsic rate of DHFR synthesis increased, consistent with the possibility that RF1 competes not only at stop codons but also at other mRNA codons during peptide elongation. It thus seems possible that in addition to its currently accepted role as a protein factor involved in peptide termination, RF1 is also involved in functions that control the rate at which protein synthesis proceeds.

The suppression of a nonsense codon using a chemically misacylated suppressor tRNA during mRNA translation (*1*−*8*) constitutes a powerful strategy for preparing proteins containing unnatural amino acids at predetermined sites (*9*−*19*). This strategy has been implemented in a number of laboratories, generating numerous modified proteins and polypeptides of utility for the study of protein structure and function (*9*−*29*). Other than studies involving the incorporation of unnatural amino acids into membrane bound proteins by micro-injection into *Xenopus* oocytes, (*30*, *31*) the preparation of mutant proteins containing unnatural amino acids has relied mainly on the techniques of in vitro protein synthesis mediated by rabbit reticulocyte lysate (*16*), wheat germ extract (*32*), or bacterial S-30 extract (*10*).

In vitro protein synthesis, while applicable to a wide variety of proteins, presently has the disadvantage of producing only limited quantities of protein (*27*). Efforts to increase yields have focused on sustaining protein synthesis for longer periods of time (*33*), or saturating the translation reaction with mRNA to enhance protein synthesis as demonstrated in our earlier (gene amplification with transcription/translation) methodology (*34*). Readthrough of nonsense codons with misacylated suppressor tRNAs imposes further constraints on protein production. Even with recent improvements in protein synthesis technology, it is still difficult to predict the facility of incorporating a specific unnatural amino acid in vitro at a given site prior to performing the experiment. The only general predictor of efficiency of incorporation of an amino acid is the structure and chemical nature of the particular amino acid per se. While hydrophobic amino acids tend to be incorporated with high efficiency, charged or polar amino acids are particularly troublesome, typically resulting in low suppression efficiencies (*14*, *16*, *27*).

One factor which influences the prospects for suppression of a nonsense codon by a suppressor tRNA is the competition with intrinsic release factors, which detect in-frame stop codons along the mRNA and mediate termination of protein synthesis. Release factor 1 (RF1) is responsible for the termination at the stop codons UAA and UAG, whereas release factor 2 (RF2) recognizes UAA and UGA stop codons (*35*). Due to the redundant recognition of the UAA stop codon by both RF1 and RF2, it should be possible to selectively delete one release factor and rely on the other to mediate termination of protein synthesis at the UAA stop codon. Because the UAG stop codon has been the nonsense codon of choice by which unnatural amino acids have been introduced into proteins (*9*−*29*), diminution of RF1 should lessen competition with the misacylated suppressor tRNA and thus promote the incorporation into protein of the unnatural amino acid.

On the basis of earlier studies which found that a thermosensitive mutation in *Escherichia coli* release factor 1 increased the readthrough levels of several suppressor tRNAs in vivo at 37 °C (*36*, *37*), we sought to prepare a coupled S-30 transcription/translation system for the elaboration of proteins in vitro. Concurrent with our investigation,

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Steward et al. (*29*) reported without experimental detail that a heat-shocked S-30 translation system containing a temperature sensitive RF1 gave increased yields of incorporation of three fluorescent amino acids, although neither the coparative yields nor accuracy of translation were discussed.

Presently, we describe the preparation and analysis of an optimized S-30 coupled transcription/translation system having a thermosensitive RF1. Partial deactivation of RF1 by mild heat shock confirmed our predictions, demonstrating an increase in the yield of full-length protein in vitro containing unnatural amino acids, as demonstrated using DHFR and HIV-1 protease. The increase was found to correlate with a decrease in the concentration of active RF1. Addition of active RF1 to the heat-shocked S-30 once again reduced the incorporation of the unnatural amino acid, and also decreased the initial rate of DHFR synthesis. These changes in the rate of DHFR synthesis are proposed to result from an alteration in the competition between RF1, suppressor tRNAs, and cognate tRNAs for codons translated within the ribosome during peptide elongation. Together, these results suggest that RF1 may participate during both elongation and termination phases of protein synthesis.

EXPERIMENTAL PROCEDURES

General Methods and Materials. Amino acid mixtures utilized during translation experiments, as well as endonucleases *Pst*I, *Sac*I, *Bcl*I, *Bam*HI, *Hin*dIII, *Nde*I, and *Nco*I, were purchased from Promega Corporation (Madison, WI). *Pfu*I DNA polymerase and *E. coli* competent cells were from Stratagene Cloning Systems (La Jolla, CA). T4 RNA ligase, T4 DNA ligase, T4 polynucleotide kinase, and endonuclease *Fok*I were obtained from New England Biolabs (Beverly, MA). Kits for plasmid isolation were purchased either from Qiagen (Chatsworth, CA) or PGC Scientific (Gaithersburg, MD). Synthetic oligonucleotides were obtained from Cruachem, Inc. (Dulles, VA) or Midland Certified Reagent Co. (Midland, TX). While isopropyl β-D-thiogalactopyranoside was from Boehringer Mannheim (Indianapolis, IN), all other biochemicals/enzymes including NTP's, phopho(enol)pyruvate, pyruvate kinase, cAMP, GMP, amino acids, and folinic acid were obtained from Sigma Chemicals (St. Louis, MO). AmpliScribe transcription kits and T7 RNA polymerase were purchased from Epicentre Technologies (Madison, WI); [^{35}S]methionine (1000 Ci/mmol, 10 μCi/μL) was from Amersham Corporation. Plasmids pET14 b(+) and pET28 b(+) were obtained from Novagen (Madison, WI); plasmid pTrc99A was from Pharmacia Biotech (Piscataway, NJ). The HIV-1 protease substrate, anthranilyl-Lys-Ala-Arg-Val-Nle-(*p*-NO_2)-Phe-Glu-Ala-Nle-NH_2 was purchased from Bachem (King of Prussia, PA). Nitroveratryloxycarbonyl (NVOC)-protected pdCpA derivatives of aspartic acid, cysteic acid, β,β-dimethylaspartic acid, *threo*-β-methylaspartic acid, and *erythro*-β-methylaspartic acid were prepared as described previously (*16*), as were pentenoyl-protected pdCpA derivatives of valine and phenylalanine (*38*). The synthesis of *N*-NVOC- or *N*-pentenoyl-protected pdCpA derivatives of *N*-methyltryptophan, naphthylalanine, and naphthylglycine will be described elsewhere (*39*).

Fluorescence spectral measurements were made using a Hitachi F2000 fluorescence spectrophotometer. Phosphorimager analysis was performed using a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 3.2 software. Radioactivity measurements were made using a calibrated phosphorimager screen, such that the pixel density of the image could be related directly to the amount of radioactivity present in the sample.

Construction of Expression Plasmids Having the Gene for Dihydrofolate Reductase. Plasmid pTZRKE, a derivative of pTZ19R encoding wild-type *E. coli* DHFR (*40*), was modified for this study. The *Hind*III-*Bcl*I fragment from pTZRKE containing a 5′-noncoding region was replaced by a synthetic oligonucleotide duplex carrying a *trc* promoter, Shine-Dalgarno site, and a sequence encoding a hexahistidine fusion peptide, affording plasmid pTHD. The same oligonucleotide duplex, containing the bacterial promoter elements and hexahistidine fusion peptide, was also used to modify pTZN2 (*16*) to yield plasmid pTHD10. Construction of pTHD22 was carried out in a fashion identical to pTHD except for oligonucleotide-directed replacement of the TGG codon for Trp-22 by the amber stop codon TAG. Restriction analysis and dideoxy DNA sequencing verified the nucleotide sequences in all three plasmids (*41*).

Construction of Expression Plasmids Having the Gene for HIV-1 Protease. Expression vectors pTHPR (wild-type HIV-1 protease) and pTHPR25 (amber stop codon at position 25), were constructed de novo by PCR-mediated gene synthesis (*42*, *43*). Briefly, a PR gene sequence (*44*), optimized for prokaryotic expression, was assembled from two gene cassettes and cloned into an appropriately designed synthetic polylinker within pTrc99A. The gene cassettes were prepared by specifically arranging synthetic oligonucleotides (70−100 bases in length) such that one entire DNA strand of each gene cassette was contiguous. This was accomplished by annealing three 5′-phosphorylated oligonucleotides in an end-to-end fashion using complementary linking oligonucleotide splints (24-mer) at each 3′-5′ junction to ensure proper assembly. The gaps present at each junction were ligated together using T4 DNA ligase to afford one complete strand of the gene cassette. The complementary strand was synthesized during the course of a PCR in the presence of appropriate primers. The amplified gene cassettes were subsequently digested with either *Sac*I/*Bcl*I or *Bcl*I/*Pst*I and cloned stepwise within a synthetic polylinker region of pGem 3zf(+). The resulting gene was altered at both the 5′- and 3′-termini using mutagenic primers and PCR to allow cloning between *Nde*I and *Bam*HI sites. The hexahistidine fusion peptide, derived from pET 14b, and the PR genes were then cloned into pTrc99A between *Nco*I and *Bam*HI sites to afford plasmids pTHPR and pTHPR25.

The tethered dimer of HIV-1 protease was constructed, as described previously (*45*), using a PCR-mediated technique to link two wild-type PR genes in tandem. The tethered dimer gene containing a stop codon at position 125, pPRPR125, was constructed in similar fashion except that the C-terminal monomer was derived from the PR gene in plasmid pTHPR25. Because of toxicity associated with the PRPR gene, production of a stable *E. coli* cell line necessitated the cloning of PRPR under a silent promoter. In this case, PRPR was cloned into pET28b(+) containing a T7 promoter, ribosomal binding site, and N-terminal hexahistidine fusion peptide to yield plasmid pPRPR. The nucleotide sequences of all plasmids were verified by restriction analysis and dideoxy DNA sequencing (*41*).

In Vitro Transcription of 5′-Monophosphate-Enriched Suppressor tRNA (46). Plasmid pYRNA8, encoding the yeast suppressor $tRNA^{Phe}_{CUA}$ (*5*), was linearized with *Fok*I and then transcribed using an AmpliScribe T7 RNA polymerase transcription kit. Preparative *Fok*I digests typically contained 100 μg of plasmid DNA, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, and 40 units of *Fok*I in a total volume of 100 μL. Reactions were incubated at 37 °C for 4 h. A solution containing the digested plasmid DNA was then extracted with phenol−chloroform, precipitated with ethanol, and then transcribed using an AmpliScribe T7 transcription kit in a buffered reaction mixture (1 mL total volume) containing 7.5 mM of ATP, CTP, and UTP, 5 mM GTP, 20 mM GMP, 10 mM dithiothreitol, 20 nM template DNA, and 100 μL of the T7 RNA polymerase preparation for 12 h at 42 °C. The transcribed tRNA was precipitated from the reaction mixture by the addition of 4 volumes of ethanol and then collected by centrifugation and dried under vacuum. The crude tRNA was dissolved in 80% formamide containing 100 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol, then applied to an 8% denaturing polyacrylamide gel (40 cm × 22 cm × 2 mm), and subjected to electrophoresis at 600 V for 4.5 h. The RNA was visualized by UV shadowing (*47*), excised from the gel, and recovered by crush and soak (*48*) with 100 mM sodium acetate, pH 4.5, 1 mM EDTA and 0.01% SDS at 23 °C for 16 h. The tRNA was recovered by ethanol precipitation, dried, and then redissolved in RNase-free water prior to use.

Chemical Misacylation of Suppressor tRNA-C_{OH}. Chemical misacylation reactions were carried out in 100 μL (total volume) of 50 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 50 μg of suppressor tRNA-C_{OH}, 1.0 A_{260} unit protected aminoacyl-pdCpA (5−10-fold molar excess), 20% dimethyl sulfoxide, and 200 units of T4 RNA ligase. After incubation at 37 °C for 25 min, the reaction was quenched by the addition of 0.1 volume of 3 M sodium acetate, pH 4.5, and the tRNA was precipitated with 2.5 volumes of ethanol. The efficiency of ligation was estimated by gel electrophoresis (pH 5.0) (*49*). Deprotection of NVOC-containing aminoacyl-tRNAs was carried out at a tRNA concentration of 1 mg/mL in 1 mM potassium acetate, pH 4.5. The aminoacyl-tRNAs were cooled to 2 °C and irradiated with a 500 W mercury−xenon lamp using both Pyrex and water filters. Typically, monosubstituted NVOC aminoacyl-tRNAs were irradiated for 2.5 min, while disubstituted NVOC aminoacyl tRNAs were deblocked for 5 min. After irradiation, deblocked acylated suppressor tRNAs were ethanol precipitated from aqueous solution, dried under diminished pressure, and then used in in vitro suppression experiments.

Pentenoyl-protected aminoacyl-tRNAs were deprotected at room temperature for 20 min in 5 mM I_2 from a stock solution containing 25 mM I_2 dissolved in 1:1 tetrahydrofuran:H_2O (*8*). Following deblocking, the solution was centrifuged, and the cleared supernatant was adjusted to 0.3 M sodium acetate and treated with 4 volumes of ethanol to precipitate the acylated tRNA and remove trace amounts of I_2. After the tRNA pellet was washed with 70% ethanol, the deprotected aminoacyl-tRNA was used immediately in in vitro suppression experiments.

S-30 Preparation and Reactions. The preparation of the S-30 extracts from *E. coli* XAC [*zce*, *ara*, *D(lacpro)*, *gyr A*, *arg E-am*, *rpo B*, *thi*] (*37*) and XAC−RF [*zce*, *ara*, *D(lacpro)*, *gyr A*, *arg E-am*, *rpo B*, *thi*, *uar*] (*37*) was carried out according to a modified version of the Pratt procedure (*49*). *E. coli* XAC and XAC−RF were grown at room temperature (23 °C) in a 10-L fermentor until the optical density at 450 nm was 1.7. The cells were collected by centrifugation, washed, and pressed at 8400 psi in a French cell press, such that the only deviation from the S-30 protocol (*50*) was the preincubation temperature and duration. For both XAC and XAC−RF strains, the S-30 extract preincubation mixture was incubated at 30 °C for 150 min followed by an increase in temperature to 37 °C and further incubation at this elevated temperature for 15 min. Finally, the S-30 extracts were dialyzed, centrifuged, frozen in liquid N_2, and stored in 200-μL aliquots at −80 °C until use (total yield 15 mL).

Translation reactions carried out in the presence of S-30 extract were performed essentially as described by Lesley et al. (*51*) with minor modifications. In a typical experiment, proteins were synthesized in a reaction mixture (100 μL) that contained 10 μg of plasmid DNA dissolved in diethyl pyrocarbonate-treated water, 40 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM dithiothreitol, 11 mM magnesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM isopropyl β-D-thiogalactopyranoside, 20 mM ATP and GTP, 5 mM CTP and UTP, and 10 mM cAMP) (*50*), 100 μM of amino acids minus methionine, 50 μM methionine, 40 μCi of [^{35}S]-*S*-methionine, and 30 μL of S-30 extract. For translation of pPRPR, the in vitro translation reaction was supplemented with 1200 units of T7 RNA polymerase (200 units/μL). Suppression reactions (100 μL) contained 25 μg of deblocked misacylated suppressor tRNA dissolved in 10 μL of diethylpyrocarbonate-treated water and were incubated at 37 °C for 45 min. For readthrough studies of HIV-1 protease and DHFR, the XAC−RF S-30 extract was heat shocked at 42 °C for 6 min (or as otherwise indicated) prior to the assembly of the translation reaction. Protein was precipitated from the reaction mixtures by the addition of 5 volumes of acetone followed by centrifugation. The protein pellets were dried and resuspended in sodium dodecyl sulfate loading buffer prior to polyacrylamide gel electrophoresis (*52*). Dried gels were visualized and analyzed using a Molecular Dynamics PhosphorImager followed by exposure to Kodak X-OMAT film.

HIV-1 Protease Assay. HIV-1 protease activity in crude S-30 extract translation reactions was measured quantifying the fluorescence increase of the nonapeptide HIV-1 protease substrate anthranilyl-Lys-Ala-Arg-Val-Nle-(*p*-NO_2)-Phe-Glu-Ala-Nle-NH_2. A typical protease assay contained 9.1 μM anthranilyl-Lys-Ala-Arg-Val-Nle-(*p*-NO_2)-Phe-Glu-Ala-Nle-NH_2 and 50−100 ng (40−50 μL of translation reaction) of crude HIV-1 protease tethered dimer in 900 μL of 50 mM Mes, pH 6.0, containing 1 mM EDTA, 0.9 M NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, and 5% dimethyl sulfoxide (*53*). All reaction components were mixed together, except the substrate, and incubated at 23 °C for 10 min. The substrate was then added to the reaction, and the rate of fluorescence increase was measured using an Hitachi

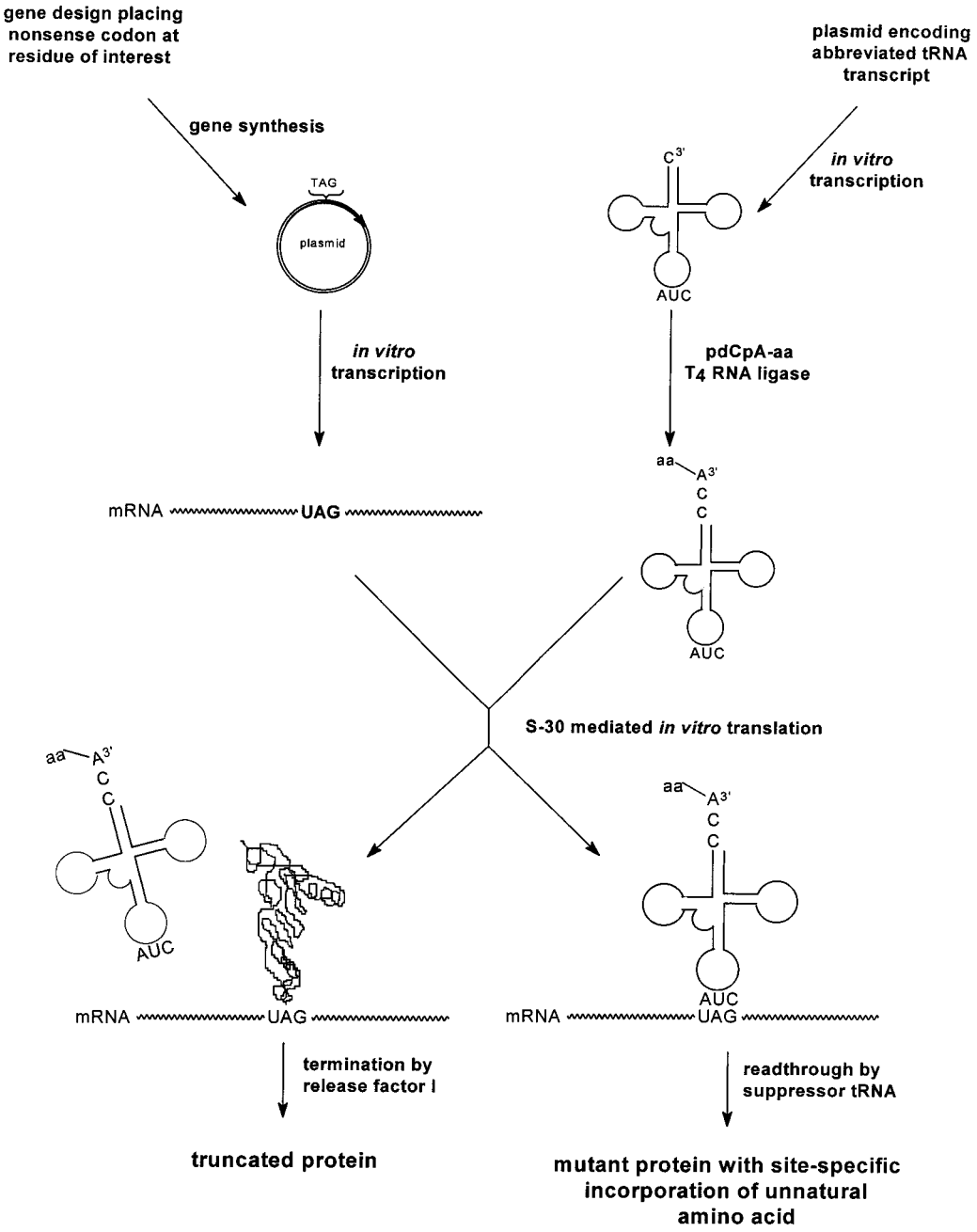

FIGURE 1: Strategy employed for studying the site-specific incorporation of unnatural amino acids into DHFR and HIV-1 protease. The scheme demonstrates a competition between *E. coli* release factor 1 and acylated suppressor tRNA, which results either in a release factor 1-dependent termination of protein synthesis or readthrough of the UAG stop codon by the suppressor tRNA. By using an *E. coli* S-30 extract depleted in release factor 1, readthrough of the UAG stop codon is favored.

F2000 fluorescence spectrometer (excitation, 337 nm; emission, 420 nm) at 23 °C.

RESULTS

The general strategy presented in Figure 1 has been utilized successfully in studying the extent to which *E. coli* RF1 interferes with the incorporation of unnatural amino acids into proteins during in vitro translation. As indicated, this includes the use of a misacylated suppressor tRNA to effect readthrough of a UAG (stop) codon in the mRNA of interest. The scheme also notes the probable competition between *E. coli* RF1 and the misacylated suppressor tRNA, ultimately leading to increased premature termination of protein synthesis and decreased nonsense codon suppression, such that the yield of the desired protein is diminished.

Construction of Expression Plasmids. The extent to which an S-30 extract depleted of RF1 could promote protein synthesis was studied by observing the extent of protein expression from genes encoding *E. coli* dihydrofolate reductase (DHFR) and HIV-1 protease (PR), as well as from the same genes bearing amber (UAG) stop codons at the desired sites of insertion of unnatural amino acids. Amber stop codons were introduced at positions 10 and 22 of *fol* and positions 25 and 125 in the genes of PR (PR25) and the tethered dimer of PR (PRPR125), respectively (Figure 2). While the plasmid encoding DHFR was a modified version of an earlier plasmid that employed a T7 promoter (*17*), the sequences encoding PR and PRPR were synthesized de novo. Preparation of the PRPR gene construct involved the use of PCR to alter the encoding DNA sequence of the 3′-terminus

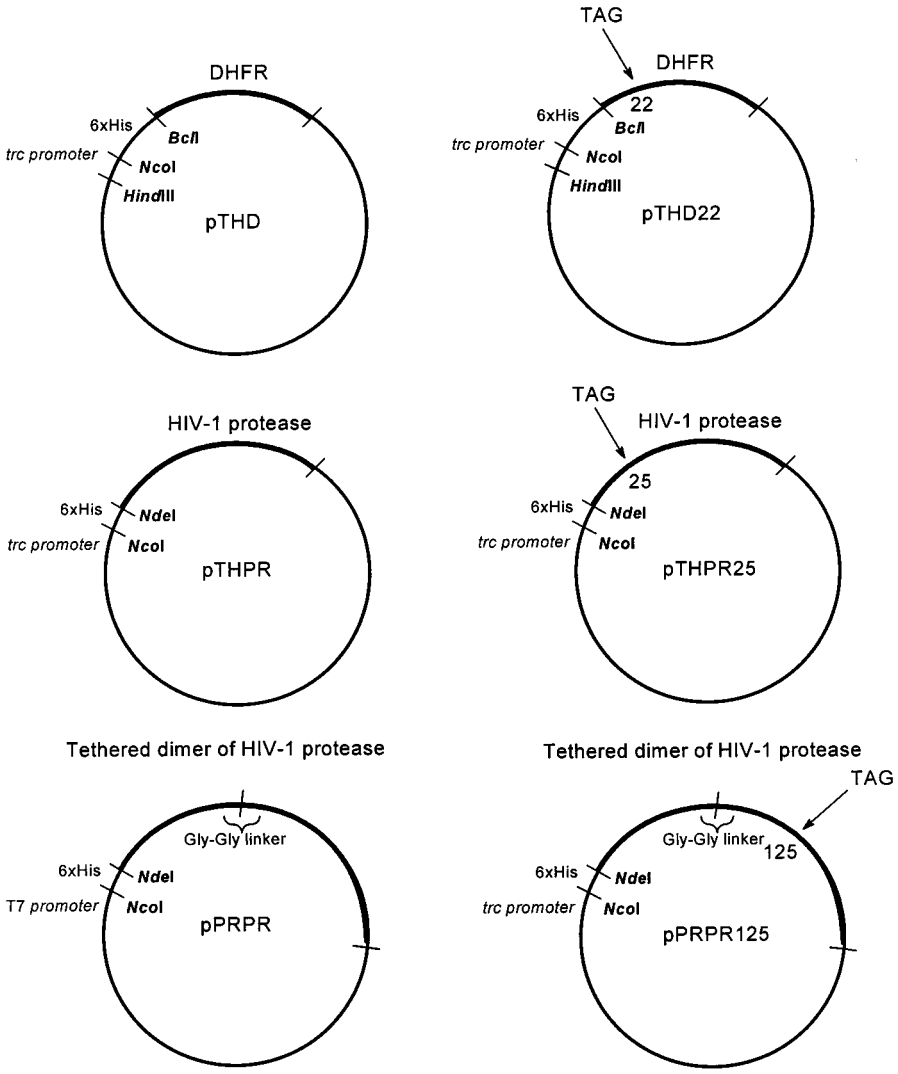

FIGURE 2: Plasmid constructs used in coupled S-30 transcription/translation systems for the synthesis of wild-type DHFR (pTHD), PR (pTHPR) and the tethered dimer of HIV-1 protease (pPRPR), as well as mutants at position 22 in DHFR (pTHD22), 25 in PR (pTHPR25), and 125 in the tethered protease dimer (pPRPR125). The plasmid construct used to elaborate DHFR containing TAG at codon position 10 was identical to pTHD22 except for the position of the nonsense codon.

of PR, as well as the 5′-terminus of the corresponding wild-type gene, such that the two peptide monomers were linked in tandem by a Gly-Gly dipeptide (*54*). A DNA sequence encoding an N-terminal hexahistidine fusion peptide was present within all plasmids, enabling the derived DHFR and PR proteins to be purified by Ni^{2+}-based affinity chromatography (*54*). Typical levels of wild-type protein production in the prepared S-30 extracts were 30−50 μg/mL for all gene constructs.

Comparison of S-30 Translation Systems Produced from E. coli Strains XAC−RF and XAC. To facilitate a better understanding of the nature of suppressor tRNA-RF1 competition, in vitro transcription/translation systems were prepared using S-30 extracts from two strains of *E. coli*. The first, XAC−RF, contains a thermosensitive RF1 and is almost completely deficient in *E. coli* RF1 activity at 37 °C, yet retains the majority of RF1 activity at 25 °C (*37*). The second, XAC (*37*), is the parent strain of XAC−RF and served as the wild-type control. To maximize translation activity of XAC−RF, an optimized procedure involved permissive growth at 23 °C during bacterial culture followed by incubation at 30 °C for 150 min and then a mild heat shock at the nonpermissive temperature of 37 °C for 15 min during a preincubation step in the preparation (*50*). To permit assessment of the effects of diminishing *E. coli* RF1 activity in the S-30 extract, preparations of S-30 extract derived from XAC−RF and XAC were made under identical conditions.

In vitro suppression reactions were carried out using DHFR mRNA having UAG at codon position 22 in the presence of XAC−RF- and XAC-derived S-30 extracts and [^{35}S]methionine, so that the amount of DHFR formed as a function of time could be determined. At each time point, an aliquot of the reaction was quenched, analyzed by SDS-PAGE, and the radiolabeled band corresponding to DHFR was then quantified by phosphorimager analysis. *N*-Methyltryptophan was incorporated into position 22 of DHFR with 5-fold greater suppression efficiency in the XAC−RF strain (Table 1). The extent of DHFR synthesis in XAC−RF was also increased relative to that obtained using an S-30 extract prepared from MRE600, a strain employed extensively for in vitro protein synthesis. Moreover, on the assumption that the initial rate of DHFR synthesis was limited by the efficiency of UAG codon suppression, the initial rate of suppression was almost seven times greater: 60.6 pmol of DHFR was synthesized per min using the XAC−RF system

Table 1: Synthesis of DHFR from mRNAs Containing a UAG Codon at Position 10 or 22

	suppression efficiency (%)[a]					
	position 10			position 22		
amino acid[b]	XAC-RF system	XAC system	MRE600	XAC-RF system	XAC system	MRE600
c	1.3	0.2	1.6		1.0	1.3
d	1.2	1.4	1.0	0.5	1.3	4.3
N-methyltryptophan	24	9	13.2	45	8.5	2.2
naphthylalanine	29	19	14	13	15	12
naphthylglycine	68	22	22	23	12	35
valine	47	35	44	50	36	35
phenylalanine	46	31	33	28	27	24

[a] 5′-Monophosphorylated yeast $tRNA^{Phe}_{CUA}$ was employed for all experiments. [b] Introduced as an activated ester of the suppressor tRNA. [c] No suppressor tRNA. [d] Unactivated full length suppressor tRNA.

Table 2: Initial Rates[a] of DHFR Synthesis from mRNA Having a UAG Codon at Position 22 in S-30 Extracts Containing *N*-Methyltryptophanyl-$tRNA^{Phe}_{CUA}$ and Varying Ratios of XAC and XAC−RF S-30[b]

ratio of XAC:XAC−RF S-30 extract	rate of DHFR synthesis (pmol/min)
0:1	60.6 ± 0.1
1:1	42.6 ± 2.1
2:1	18.9 ± 0.9
1:0	9.3 ± 0.7

[a] Calculations of the initial rates of synthesis were based on the linear increase in the amount of full length DHFR over the first 10 min of translation, as judged by phosphorimager analysis of a 17.5% SDS polyacrylamide gel. [b] Wild-type DHFR mRNA was translated at a rate of 85.3 ± 1.7 pmol/min in XAC−RF S-30 extract and 55.0 ± 3.6 pmol/min in XAC S-30 extract.

as compared to 9.3 pmol/min in the XAC system (Table 2). The difference in the rates of DHFR synthesis is proposed to result from the difference in the concentrations of functional *E. coli* RF1 able to compete with the misacylated suppressor tRNA via UAG codon recognition.

Because *E. coli* strain XAC is the parent strain of XAC−RF, the two strains are genetically identical except for the thermostability mutation in RF1. If both S-30 extracts demonstrate similar translation activity of wild-type DHFR, any observed differences noted in UAG codon readthrough should be a direct consequence of the relative concentration of RF1 present during DHFR synthesis. To ascertain whether the observed increase in UAG codon readthrough was attributable to a decrease in the concentration of functional RF1, XAC S-30 and XAC−RF S-30 were mixed in varying ratios and in vitro translation of DHFR was performed. In vitro translation of wild-type DHFR in XAC−RF and XAC S-30 afforded similar protein yields, namely, 1671 and 1964 pmol/mL respectively, suggesting that both S-30 extracts had similar translation activities (Figure 3). However, when increasing amounts of XAC S-30 were added to XAC−RF, the level of UAG readthrough decreased. At 1:1 XAC:XAC−RF, the extent of DHFR synthesis decreased 2-fold, presumably reflecting decreased *N*-methyltryptophanyl-$tRNA_{CUA}$ decoding of a UAG codon at position 22 in DHFR, relative to XAC−RF readthrough. Likewise, when the proportion of XAC S-30 was increased to 2:1 XAC:XAC−RF, the amount of DHFR synthesis decreased 2-fold further to a level approximating that obtained in the presence of XAC S-30 alone.

Corresponding decreases in the initial rate of DHFR synthesis were observed with increasing amounts of active RF1. The presence of competent RF1 was found to slow DHFR production in both the readthrough and wild-type studies (Table 2). In the case of UAG readthrough at position 22 in DHFR with *N*-methyltryptophanyl-$tRNA_{CUA}$, 1:1 XAC:XAC−RF slowed DHFR production from 61 to 43 pmol/min, whereas 2:1 XAC:XAC−RF slowed DHFR synthesis more than 3-fold. Not only was the initial rate of DHFR synthesis slower in the case involving UAG readthrough but also for wild-type DHFR. While not as dramatic as in the readthrough cases, wild-type DHFR expressed in XAC S-30 was translated at a rate of 55 pmol/min compared to 85 pmol/min in XAC−RF, thereby suggesting that RF1 might also compete with cognate tRNAs for the ribosomal A-site during peptide elongation.

Translation Accuracy During the In Vitro Synthesis of a Tethered Dimer of HIV-1 Protease in a Heat-Shocked XAC−RF S-30 System. To investigate the accuracy of UAG decoding in the XAC−RF S-30 system, a gene encoding the tethered dimer of HIV-1 protease was constructed. The UAG stop codon was placed at position 125 within the active site of the enzyme to facilitate the visualization of the 16 kDa truncated peptide resulting from termination of protein synthesis in the absence of aminoacylated suppressor tRNA. Moreover, extensive site-directed mutagenesis studies have demonstrated that within the active site of HIV-1 protease only aspartic acid supports protease function (*45*, *55*, *56*), such that any proteolytic activity associated with the enzyme must have resulted from the incorporation of aspartic acid at position 125.

Readthrough was studied as a function of increasing duration of XAC−RF S-30 heat shock at the nonpermissive temperature of 42 °C. The XAC−RF S-30 extract, prior to translation, was heat shocked for 0, 10, 20, and 30 min. In vitro translation reactions were then performed in the presence of mRNA encoding the tethered dimer of HIV-1 protease (PRPR125), and misacylated suppressor tRNA activated with aspartic acid. With increasing duration of heat shock, the level of readthrough increased: 15% at 0 min, 32% at 10 min, 52% at 20 min, and 65% at 30 min (Figure 4A). An identical experiment performed with XAC S-30 extract failed to show any increase in the level of readthrough product; the level of readthrough was maintained at 4% (Figure 4B).

The HIV-1 protease activity associated with the heat-shocked XAC−RF S-30 translation reactions was assayed to ensure that translation accuracy was not sacrificed for an increase in protein yield. While the specific activity of HIV-1 protease arising from wild-type mRNA (PRPR) was un-

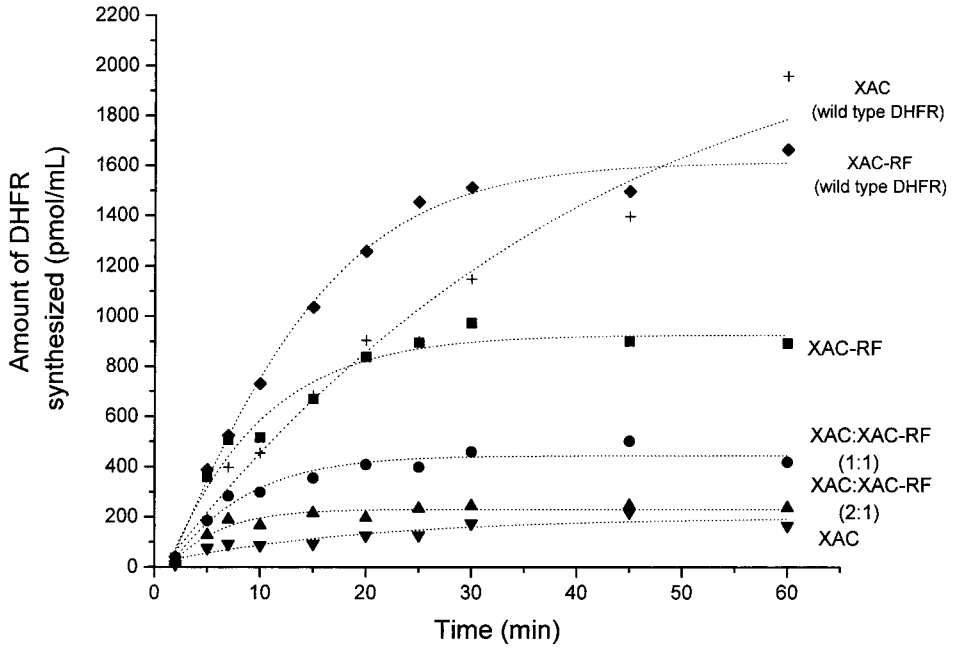

FIGURE 3: Time course of DHFR synthesis, as judged by phosphorimager analysis of a 17.5% SDS polyacrylamide gel. The DHFR was elaborated either in XAC−RF S-30 (■), XAC S-30 (▼), 2:1 XAC:XAC−RF S-30 (▲), or 1:1 XAC:XAC−RF S-30 (●) extract using plasmid pTHD22 in the presence of 5′-monophosphorylated *N*-methyltryptophanyl-tRNA$^{Phe}_{CUA}$ and [^{35}S]methionine. Wild-type DHFR was also synthesized in XAC−RF S-30 (◆) and XAC S-30 (+) extracts to provide positive controls for the respective S-30 translation systems. All extracts were heat shocked at 42 °C for 6 min. Statistical analysis indicated the error for individual points to be no greater than ± 6%.

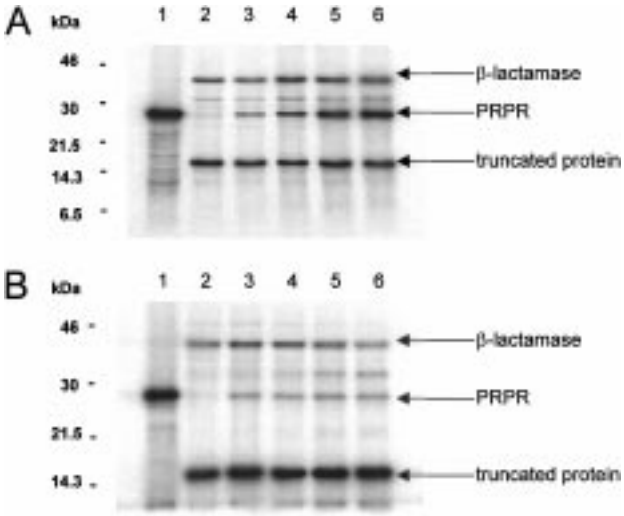

FIGURE 4: Autoradiograms of 15% SDS polyacrylamide gels illustrating the effects of heat shocking XAC−RF (A) and XAC (B) extracts on subsequent in vitro protein synthesis. The tethered dimer of HIV-1 protease was elaborated using plasmid pPRPR125 in the presence of 5′-monophosphorylated aspartyl-tRNA$^{Phe}_{CUA}$ and [^{35}S]methionine. Lane 1: tethered dimer of HIV-1 protease elaborated from wild-type mRNA. Lane 2: mRNA from plasmid pPRPR125 + full length unacylated tRNA$^{Phe}_{CUA}$. Lanes 3−6: pPRPR125 expressed in either XAC−RF (A) or XAC (B) which had been heat shocked at 42 °C for lane 3, 0 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min.

changed, the specific activity of HIV-1 protease (PRPR125) synthesized from UAG codon readthrough by an aspartylated suppressor tRNA$^{Phe}_{CUA}$ decreased with increasing duration of heat shock. Heat shock incubations of 0, 10, and 20 min afforded HIV-1 protease whose specific activity was much higher compared to that obtained following a 30-min heat shock (Figure 5). For example, after a 20-min heat shock, the specific activity of the protease dimer was found to be 1.4×10^5 units min^{-1} mg^{-1} versus only 0.8×10^5 units min^{-1} mg^{-1} after a 30-min heat shock. This drop in activity paralleled an increased level of readthrough at 30 min (Figure 4A, lane 6) and suggests a decrease in the accuracy of UAG suppression.

To understand the process by which translation accuracy may decrease while readthrough increases, the level of readthrough was titrated versus heat shock duration in the presence of wild-type mRNA encoding the tethered dimer of HIV-1 protease, and in the presence of the tethered dimer mRNA containing UAG at position 125. In the presence of wild-type mRNA, the specific activity associated with HIV-1 protease did not change with increasing heat shock duration suggesting that translation is still accurate even though the amount of active RF1 decreases (Figure 5). However in the absence of suppressor tRNA, increasing heat shock duration led to an increase in the level of UAG readthrough at position 125 (Figure 6). Heat shock incubations between 0 and 6 min

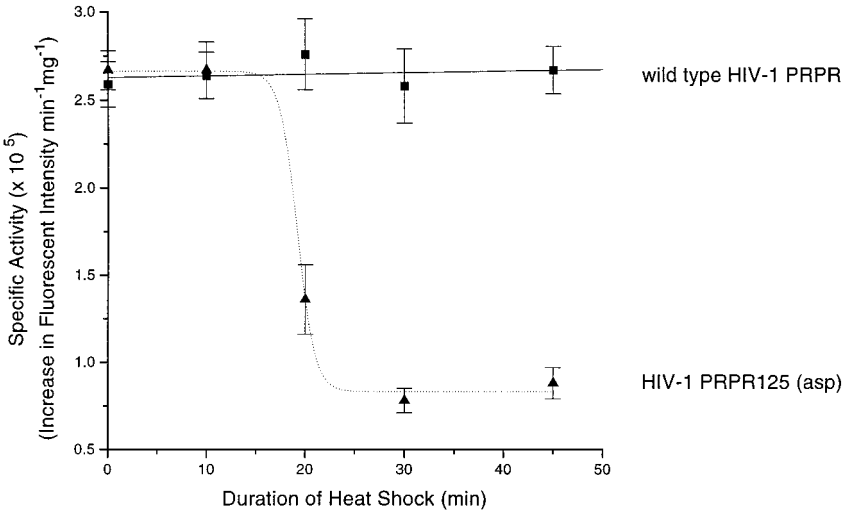

FIGURE 5: Effects of increasing heat shock duration of XAC−RF S-30 extract on the specific activity of HIV-1 protease (PRPR) arising from wild type mRNA (■) and of HIV-1 protease (PRPR125) synthesized from UAG codon readthrough at position 125 by a chemically acylated aspartyl-tRNA$^{Phe}_{CUA}$ (▲).

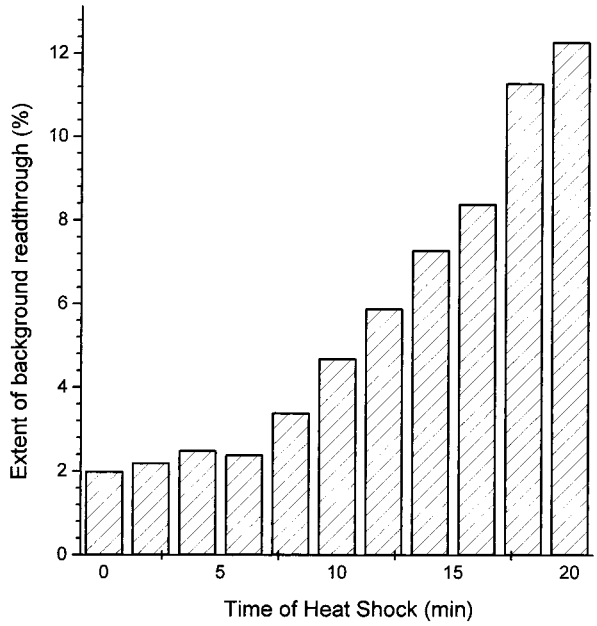

FIGURE 6: The bar graph illustrates an increase in background readthrough when XAC−RF S-30 extract is heat shocked for various lengths of time at 42 °C in the absence of suppressor tRNA$^{Phe}_{CUA}$. With increasing heat shock time, the extent of UAG readthrough at position 125 in the tethered dimer of HIV-1 protease increases as much as 6-fold over 20 min relative to wild-type HIV-1 protease (PRPR). Heat shocking the XAC−RF for 6−8 min had little effect on the level of background readthrough.

were found to have little effect on subsequent readthrough. Yet heat shocking XAC−RF S-30 for more than 6 min was found to increase the level of background readthrough linearly to 12% at 20 min. This increase in readthrough is presumably due to a depletion of functional RF1 such that endogenous tRNAs not normally capable of reading through UAG may begin to compete, albeit with lesser efficiency than suppressor tRNA.

Incorporation of Unnatural Amino Acids into DHFR and HIV-1 Protease is Facilitated by Heat Treatment of XAC−RF S-30. XAC−RF S-30 extract was able to promote suppression at codon 25 in HIV-1 protease mRNA, as well as positions 10 and 22 in DHFR mRNA. Shown in Table 3 are the results for HIV-1 protease synthesis using misacylated

Table 3: Synthesis of HIV-1 Protease from a mRNA Containing a UAG Codon at Position 25

	suppression efficiency (%)[b]		
amino acid[a]	XAC-RF S-30 system[c]	XAC S-30 system	MRE600 S-30 system
d	1.2	0.3	1.1
e	2.3	1.2	2.2
aspartic acid	22	3.8	0.8
cysteic acid	17	2.1	n.d.[f]
β,*β*-dimethylaspartic acid	17	2.0	n.d.[f]
threo-*β*-methylaspartic acid	17	2.6	n.d.[f]
erythro-*β*-methylaspartic acid	33	7.2	n.d.[f]
N-methyltryptophan	45	4.2	2.2
naphthylalanine	57	24	26
naphthylglycine	75	22	28

[a] Introduced as an activated ester of the suppressor tRNA. [b] Relative to the synthesis of HIV-1 protease from wild-type mRNA in the absence of any suppressor tRNA. 5′-Monophosphorylated yeast tRNA$^{Phe}_{CUA}$ was employed for all experiments. [c] Heat shocked for 6 min. [d] No suppressor tRNA. [e] Unactivated full length suppressor tRNA. [f] n.d.: not detected.

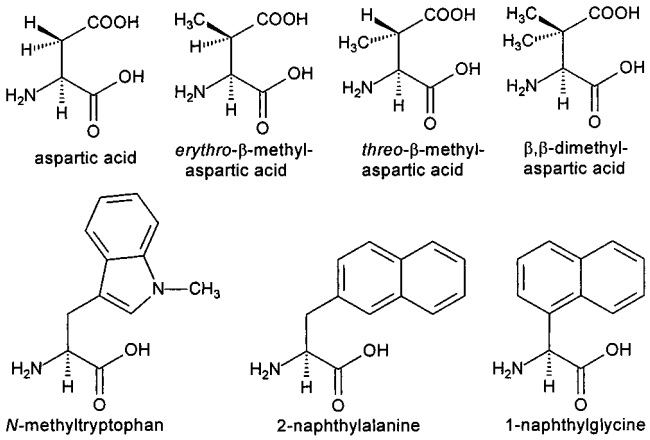

FIGURE 7: Amino acid analogues attached to suppressor tRNA$^{Phe}_{CUA}$ for the study of UAG codon readthrough.

suppressor tRNAs activated with charged and uncharged amino acids (Figure 7). Given the relatively poor interaction of the charged species with elongation factor-Tu·GTP (EF-Tu·GTP) (*57*), suppression with the analogous suppressor tRNAs may be difficult as well. Indeed, attempts at

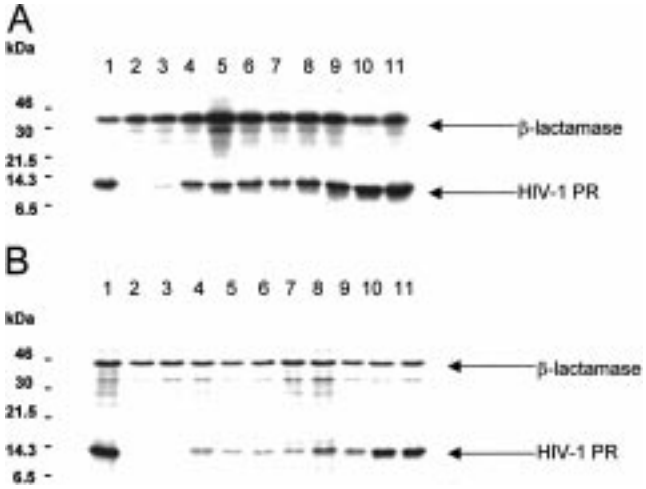

FIGURE 8: Autoradiogram of a 17.5% SDS-polyacrylamide gel illustrating the in vitro synthesis of [^{35}S]methionine-labeled HIV-1 protease using an S-30 extract prepared either from XAC−RF (A) or XAC (B) as described in the Experimental Section. Lane 1: HIV-1 protease elaborated from wild-type mRNA. Lane 2: mRNA from plasmid pTHPR25, no suppressor tRNA. Lane 3: mRNA from plasmid pTHPR25 + full length $tRNA^{Phe}_{CUA}$. Lanes 4−11: protease expressed from pTHPR25 mRNA in the presence of suppressor $tRNA^{Phe}_{CUA}$ activated with lane 4, aspartic acid; lane 5, cysteic acid; lane 6, *β*,*β*-dimethylaspartic acid; lane 7, *threo*-*β*-methylaspartic acid; lane 8, *erythro*-*β*-methylaspartic acid; lane 9, *N*-methyltryptophan; lane 10, naphthylalanine; lane 11, naphthylglycine.

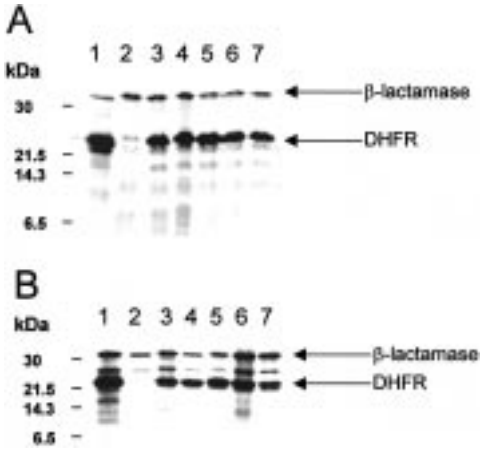

FIGURE 9: Autoradiogram analysis of a 15% SDS-polyacrylamide gel illustrating the in vitro synthesis of [^{35}S]methionine-labeled DHFR in an S-30 extract prepared either from *E. coli* XAC−RF (A) or *E. coli* XAC (B). Lane 1: DHFR expressed from wild-type mRNA. Lane 2: mRNA from plasmid pTHD10 + full length unacylated $tRNA^{Phe}_{CAU}$. Lanes 3−7: DHFR elaborated from pTHD10 mRNA in the presence of suppressor $tRNA^{Phe}_{CAU}$ activated with lane 3, phenylalanine; lane 4, valine; lane 5, naphthylalanine; lane 6, naphthylglycine; lane 7, *N*-methyltryptophan. The smaller bands associated with the full length DHFR product are a consequence of initiation of translation at an internal methionine codon (*17*).

incorporating aspartic acid and several of its analogues into HIV-1 protease using XAC S-30 extracts typically resulted in very poor incorporation (Table 3). On the other hand, readthrough levels in heat-shocked XAC−RF S-30 extract were higher, permitting the incorporation of charged amino acids and facilitating the insertion of several uncharged amino acids. Phosphorimager analysis following 17.5% SDS-PAGE (Figure 8) demonstrated the successful incorporation of charged aspartic acid analogues into protease with suppression efficiencies ranging from 17% to 33%, while three amino acids lacking any charge were incorporated with efficiencies of 45−75% (Table 3). Again, the increase in yields using a S-30 extract from strain XAC−RF afforded DHFR yields much greater than what could be realized in a system prepared from MRE600.

To ensure that the increases in unnatural amino acid incorporation mediated by heat shocked XAC−RF extract were not protein specific, several natural and unnatural amino acids were incorporated into positions 10 and 22 of DHFR. On the basis of phosphorimager analysis following 15% SDS-PAGE (Figure 9), the XAC−RF S-30 extract was found to promote the readthrough of the UAG stop codon at both positions 10 and 22. While the increases in DHFR yields were not as great as in the case of charged amino acid insertion within HIV-1 protease, readthrough was 2−5-fold greater for some uncharged species in heat-shocked XAC−RF S-30 relative to that obtained with XAC S-30 (Table 1). Thus, regardless of the protein sequence or varying context effects which may influence codon recognition, the XAC−RF S-30 extract facilitates unnatural amino acid incorporation.

DISCUSSION

The use of chemically misacylated suppressor tRNAs for the readthrough of a nonsense codon placed at predetermined positions in mRNAs has extended site-directed mutagenesis technology to allow the elaboration of proteins with amino acids not normally incorporated. While simple site-directed mutagenesis allows ample production of mutant proteins from recombinant sources, the incorporation of unnatural amino acids by the use of chemically misacylated suppressor tRNAs is generally performed in vitro. Not only does the cell free approach allow the direct and facile introduction of the misacylated suppressor tRNA into the translation system but it also enables rapid synthesis and monitoring of the desired protein. Unfortunately, most cell free translation systems produce significantly less protein than is obtained in vivo, with typical levels of expression less than 100 μg of protein per mL of incubation mixture. To ensure that the amount of mutant protein is as great as possible, the efficiency of suppression of the nonsense codon must be as high as possible. Some amino acids, especially charged species, have been shown to be incorporated with much lower efficiencies from suppressor tRNAs than hydrophobic amino acids (*14*, *16*), possibly due to less efficient interaction with elongation factor Tu (*55*, *56*). Reasoning that the low suppression efficiency must be due to some extent to competition with *E. coli* RF1 for the UAG codon, we sought to eliminate RF1 from the S-30 extract and delineate the effects of RF1 depletion.

On the basis of earlier observations that resulted in an increase in nonsense suppression in vivo (*36*, *37*), we carried out experiments using *E. coli* strain XAC−RF which is known to have a thermosensitive RF1 (*37*). Deactivation of RF1 within the XAC−RF S-30 extract was accomplished by heat shocking the extract after preparation at a nonpermissive temperature of 42 °C. The heat-shocked XAC−RF S-30 extract was found to increase the level of incorporation of several amino acids into DHFR and HIV-1 protease (Tables 1 and 3). While the increase in readthrough of the UAG codon was presumably due to a decrease in the

competition between RF1 and *N*-methyltryptophanyl-tRNA$^{Phe}_{CUA}$, the heat-shocked S-30 system was replenished with varying amounts of active RF1 to verify that RF1 was actually responsible for limiting UAG codon readthrough. Upon the addition of increasing amounts of XAC S-30 extract, a source of active RF1, the level of readthrough by *N*-methyltryptophanyl-tRNA$^{Phe}_{CUA}$ diminished, suggesting the competitive role that RF1 plays in blocking the access of suppressor tRNA to the UAG stop codon.

While other explanations for the apparent increase in the rate of protein synthesis (Figure 3) can be envisioned, one possible consequence of depleting RF1 may be greater access of tRNAs to their respective codons during protein synthesis, thereby altering the rate of protein synthesis. As shown in Table 2, decreasing the concentration of RF1 present within the translation reaction increased the apparent initial rate at which translation proceeded. Compared to the standard *E. coli* XAC S-30 extract, the rate of DHFR synthesis using the XAC−RF S-30 extract increased almost 7-fold in the presence of *N*-methyltryptophanyl-tRNA$^{Phe}_{CUA}$. Likewise, the rate of wild-type DHFR synthesis also increased by more than 50% with the XAC−RF S-30 extract. Since this mRNA for DHFR does not utilize UAG as an internal stop codon, the observed change in the rate of DHFR synthesis from wild-type mRNA demonstrates unequivocally that the effects of RF1 are not limited to those mediated at the stop codon UAG. Moreover, the observed rate increases could be readily reversed upon the addition of XAC S-30 containing active RF1 (Table 2). Because XAC−RF and XAC are isogenic in every regard other than the thermal sensitivity of RF1, the variation in the rates of DHFR synthesis must be a consequence of the varying levels of active RF1 present during translation.

The extent to which the XAC−RF S-30 extract was heat shocked was found to be crucial not only for ensuring the homogeneity of in vitro translated proteins but also for minimizing background readthrough. As shown in Figure 6, heat shocking the XAC−RF S-30 extract at the nonpermissive temperature of 42 °C for 10 min resulted in increased levels of aspartyl-tRNA$^{Phe}_{CUA}$ readthrough of UAG at position 125 in the tethered dimer of HIV-1 protease. While further heat shocking afforded higher yields of full length product, the specific activity of the protease product decreased (Figure 5). Because aspartic acid is the only amino acid presently known to support protease function at position 125 (*52*, *54*), the decrease in specific activity, which accompanied an increase in full length protein product, must be attributed to the improper delivery of amino acids other than aspartic acid into position 125. The specific activity loss does not appear to be a consequence of limiting amounts of acylated suppressor tRNA, since this tRNA species was present in at least 6-fold molar excess compared to the protein elaborated in any experiment. Similarly, heat shock incubation times greater than 6 min resulted in increased levels of readthrough when the resulting S-30 extract was employed in the absence of suppressor tRNA (Figure 6). While the nature of this readthrough is uncertain, similar observations have been noted in vivo using an *E. coli* strain having a similar thermosensitive RF1 mutation (*36*). As in the present cases, readthrough in the absence of suppressor tRNA is presumed to have resulted from a reduction in the concentration of active RF1, allowing endogenous tRNAs to act as suppressors albeit with low efficiency. This evidence is consistent with the interpretation that while a moderate reduction in the concentration of active RF1 may be tolerated without serious loss of fidelity, complete deactivation of RF1 can result in nonsense codon readthrough by normal cellular tRNAs and ultimately produce a modified protein containing microheterogeneity at the site intended for introduction of the unnatural amino acid.

By thermally depleting the RF1 in XAC−RF S-30 by heat shock of limited duration, we have been able to realize an increase in the yields of proteins containing unnatural amino acids at predetermined positions. In comparison to the isogenic *E. coli* XAC S-30 extract, the XAC−RF S-30 extract not only promoted the incorporation of large, reasonably hydrophobic amino acids such as *N*-methyltryptophan and naphthylalanine, but also facilitated nonsense codon readthrough by misacylated tRNAs activated with charged amino acids that have previously been noted to be troublesome (*14*, *16*). In the case of aspartic acid analogues, we have found the enhanced efficiency of nonsense codon readthrough to be particularly important since such species are utilized poorly during protein synthesis, due either to their charged nature or an increased lability in the translation milieu. While such species typically require side chain protection for efficient incorporation (*16*), an increase in the efficiency of nonsense codon readthrough can also afford greater yields of full length protein. Because the S-30 extract is not able to sustain protein synthesis for longer than 30−40 min under the conditions typically employed for in vitro protein synthesis (Figure 3), the initial rate at which the nonsense codon is utilized by the corresponding suppressor tRNA is critical for accumulation of the desired protein product. For aspartic acid and its analogues, the observed increases in the expression of various analogues of HIV-1 protease have been as high as eleven-fold (Table 3). Because the absolute yields of proteins produced in a given experiment are a significant function of the age and condition of the S-30 extract employed, the absolute yields obtained in comparisons such as those in Tables 1 and 3 will vary. However, in replicate experiments the rank order of effects from one amino acid to another did not vary and the magnitude of the increases due to the deletion of RF1 was always comparable. We estimate the intrinsic error due to experimental manipulations to be ±3%.

It is interesting to consider the foregoing results in the context of an established competition between suppressor tRNAs, cognate tRNAs, and RF1 at the ribosomal level. With any decrease in the concentration of active RF1 within the translation system, an associated increase was noted in the apparent rate of protein synthesis. An ambiguity in the interpretation of these data is that we measured the rate of full length DHFR production, rather than the actual rate of peptide bond formation (Table 3). Nonetheless, the apparent increase in the rate of protein synthesis even in the presence of wild-type mRNA suggests that RF1 may compete with both cognate and suppressor tRNAs at the ribosomal A-site while scanning the translating mRNA for stop codons (*58*). In the absence of such putative competition, tRNAs may gain access to their appropriate codons more easily, thereby increasing the rate of protein synthesis. This phenomenon may help explain the mechanism by which *E. coli*, for example, can maintain high rates of protein synthesis in

vigorously growing cultures. Adamski et al. noted that in rapidly doubling *E. coli* cultures, the expression of RF1 increased 4-fold while the number of ribosomes increased 11-fold (*59*). This disproportionate increase in ribosomes relative to RF1 was found to increase with higher growth rates. The growing *E. coli* may decrease the ratio of RF1 to ribosomes to facilitate an increase in the rate of protein synthesis necessitated by growth demands. This is not dissimilar to the present case where RF1 deactivation within the XAC−RF S-30 leads to the same alteration of the ratio between RF1 and *E. coli* ribosomes. Modulation of the concentration of RF1 may ultimately prove important in controlling the rate of protein synthesis *in vivo*, as was demonstrated here in vitro.

We have demonstrated the preparation and advantageous use of an S-30 extract which is generally applicable to the in vitro synthesis of a wide variety of elaborated proteins containing unnatural amino acid substitutions. We suggest that the XAC−RF S-30 translation system may be of particular utility when incorporating labile or charged amino acid species not feasible using standard S-30 systems. This is illustrated in the present study for analogues of aspartic acid, which could not be incorporated into proteins readily absent this technique. This translation system may also prove useful for the in vitro study of RF1 dependent translation events such as frameshifting, termination context effects (*60*), and other stop codon bypassing mechanisms (*61*).

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