Inhibition of Selenoprotein Synthesis by Selenocysteine tRNA\[^{[\text{Ser}]\text{Sec}}\] Lacking Isopentenyladenosine*

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A common posttranscriptional modification of tRNA is the isopentenylation of adenine at position 37, creating isopentenyladenosine (i\(^6\)A). The role of this modified nucleoside in protein synthesis of higher eukaryotes is not well understood. Selenocysteine (Sec) tRNA (tRNA\[^{[\text{Ser}]\text{Sec}}\]) decodes specific UGA codons and contains i\(^6\)A. To address the role of the modified nucleoside in this tRNA, we constructed a site-specific mutation, which eliminates the site of isopentenylation, in the Xenopus tRNA\[^{[\text{Ser}]\text{Sec}}\] gene. Transfection of the mutant tRNA\[^{[\text{Ser}]\text{Sec}}\] gene resulted in 80% and 95% reduction of steps in the translation process (1). A tRNA position complementary to position 37 in tRNAs reading codons beginning with uridine has important physiologic effects on translation efficiency, especially with regard to tRNA suppression of stop codons.

In E. coli, the i\(^6\)A modification is not required for attachment of an amino acid to tRNA by aminocarboxyl tRNA synthetases (10). However, this modification does appear to be important for efficient binding of aminocarboxyl-tRNA to ribosomes (11). Isopentenylation of position 37 in tRNA appears to stabilize the first position of the codon-anticodon interaction and thereby assists in preventing first position misreading (10). However, both \_in vitro\_ (12) and \_in vivo\_ (13) evidence indicate that the i\(^6\)A modification increases third position misreading due to decreased proofreading. Together these results show that, in yeast and bacteria, isopentenylation at position 37 in tRNAs reading codons beginning with uridine has important physiologic effects on translation efficiency, especially with regard to tRNA suppression of stop codons.

Mammalian selenocysteine (Sec) tRNA\[^{[\text{Ser}]\text{Sec}}\] also contains i\(^6\)A at position 37 (see Ref. 14 for review and Ref. 15 for subsequent work). Compared with other mammalian tRNAs, this tRNA is unique in that Sec biosynthesis occurs on the tRNA after it is aminoacylated with serine, it is 90 nucleotides long, which is the longest eukaryotic tRNA sequenced to date, and it contains only four other modified nucleosides in addition to i\(^6\)A whereas other tRNAs contain many more modified bases (14). Sec tRNA\[^{[\text{Ser}]\text{Sec}}\] decodes UGA and inserts Sec into nascent selenoproteins.

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1 The abbreviations used are: i\(^6\)A, isopentenyladenosine; Sec, selenocysteine; ALLIN, N-acetyl-leu-leu-norleucinal; hp, base pairs; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; mcm\(^5\)U, 5-methylcarboxymethyluridine; mcm\(^5\)Um, 5-methylcarboxymethyluridine-2'-O-methylribose;
as a stop codon requires a stem-loop structure or structures in the 3′-untranslated region of selenoprotein mRNAs as well as other translational factors specific for Sec incorporation (16).

In this paper, we examined the role of iA at position 37 of tRNA{Ser}^{Sec} on selenoprotein biosynthesis in vivo by generating Sec tRNAs lacking this modified base. Our studies indicate that structural changes at position 37 affect the decoding properties of the altered tRNA{Ser}^{Sec} and its ability to decode UGA and incorporate Sec into proteins. The effect of altered Sec tRNA{Ser}^{Sec} on selenoprotein expression is not because of differences in aminoacylation of the tRNA, biosynthesis of Sec on the tRNA, transcriptional events, protein degradation, or selenoprotein translation initiation. Most likely, the presence of iA is important for efficient translation of Sec codons by tRNA{Ser}^{Sec}.

EXPERIMENTAL PROCEDURES

Materials—Toq DNA polymerase, isopyropl-1-thio-β-n-galactopyranoside, and 5-bromo-4-chloro-3-indolyl β-n-galactopyranoside (X-gal) were obtained from Fisher. 5′-Phospho-CTP (300 μM/mmol) was obtained from NEN Life Science Products, and [3H]serine (32 Ci/mmol) from Amer sham Pharmacia Biotech, and [35S]selenous acid (180 Ci/mmol) was from the Missouri Research Reactor Facility. All restriction enzymes and the cloning vectors pGEM-T and pGEM-3zf(+) were obtained from Promega. Tri reagent® for the isolation of total RNA was obtained from Molecular Research Center. Protein A linked to Sepharose CL-4B, rabbit anti-β-galactosidase antibody, N-acetyl-leu-leu-norleucinal (ALLN), and all tissue culture reagents were obtained from Sigma. LipofectAMINE and Plus reagents were obtained from Life Technologies, Inc. Enhanced chemiluminescence reagents and GeneScreen Plus membranes were obtained from NEN Life Science Products. The plasmid pUCPST, containing a 193-bp Aval-HindIII fragment encoding the human tRNA{Ser}^{Sec} gene (17) ligated into pUC18, was kindly provided by Dr. Alan Diamond (University of Illinois-Chicago). The plasmid hD2/SeP, expressed in Xenopus oocytes, was provided by Dr. Michael W. Buettner (Harvard Medical School), the full-length rat cDNA for GAPDH used in Northern blot analyses was provided by Dr. Daniel Ortiz (Tufts Medical School), and lovastatin was from Merck.

Cell Culture—Stock cultures of CHO-K1 cells were maintained at 37 °C in a humidified chamber with a 5% CO2 atmosphere in Ham’s F-12 medium containing 5% (v/v) newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mA sodium glutamine, and 25 mA Hepes, pH 7.4 (H-NCS).

Site-directed Mutagenesis—The construct (A37C-XTSS) was created by site-directed mutagenesis using a 1.04-kilobase EcoRI/BamHI fragment of the Xenopus tRNA{Ser}^{Sec} gene (18) as a template. Site-directed mutagenesis was carried out by polymerase chain reaction amplification of the 1.04-kilobase EcoRI/BamHI fragment in 2 reaction steps. Initially, a fragment of A37C-XTSS was generated using the KpnI-tailed 29-mer (5′-CGGGTATCCACCATACTACAACAGC-3′) and the 19-mer (5′-GCTACAGGTTGGAAGCTGG-3′) to yield a 348-bp product. Subsequently, a partially overlapping fragment was generated using the 20-mer (5′-GGATGATGAGCGGATAGC-3′) and PstI-tailed 29-mer (5′-AACGCTAGCTGATGTTATGGG-3′) to yield a 365-bp product. A portion of each reaction was combined along with the KpnI-tailed 29-mer and PstI-tailed 29-mer and amplified by polymerase chain reaction using temperatures of 94°C for denaturation, 50°C for annealing, and 72°C for extension. The polymerase chain reaction product was purified on a 1% agarose gel, cut with KpnI and PstI, and cloned into pGEM-3zf (+). The plasmid was transformed into Escherichia coli (DH5α) and cloned into M13.KM101(Str+) for propagation. Probes were radiolabeled for 2 hours at 37°C with [α-32P]dCTP using DECAPrime II (Ambion Inc.). Cells were harvested at 60% confluence, and incubated at 62°C for a minimum of 16 h. Following hybridization, the membranes were washed for 2 h in 1% (w/v) SDS, 0.1× SSC at 62°C with multiple changes of the wash buffer. Dot blot analysis of RNA{Ser}^{Sec} was accomplished as described previously (15). Immunoprecipitation of total RNA for Northern blot analysis by anti-iA serum (32) was performed essentially as described (15).

Inhibition of Selenoprotein Synthesis—CHO cells were transfected with either A37A-XTSS or A37C-XTSS DNA by electroporation and allowed to grow for 4 days in H-NCS supplemented with 50 mg/ml Na2SeO3. The cells were harvested, radiolabeled for 4 h with [35S]selenious acid as described previously (24) at a concentration of 44 μCi/ml of (A37C-XTSS) or 56 μCi/ml (A37A-XTSS), washed, and then frozen until used for RNA extraction and RNA blot chromatography as described above.

[35S]Selenoprotein Synthesis—CHO cells were transfected and transcribed as described above. On day 3, the cells were radiolabeled for 3 h in H-NCS containing 2 μCi/ml of [35S]Selenious acid unless otherwise indicated. Cells were washed and harvested for protein determination (25). Laemmli (26) or Schagger (27) SDS-PAGE was performed as indicated. Polyaerylamide gels were processed for fluorography and quantification of radiolabel incorporation was accomplished using a PhosphorImager (Molecular Dynamics) and ImageQuant software (Molecular Dynamics).

Northern Hybridization—Total cellular RNA was isolated (28), electrophoresed on 1% agarose/formaldehyde gels (29), transferred to GeneScreen Plus, and probed for type I deiodinase using a 770-bp Smal/NcoI fragment of the G21-D10 plasmid (30) in a standard hybridization buffer (1× NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 25 μg/ml salmon sperm DNA). The blot was stripped of probe by incubation in 0.1 M sodium citrate, 0.1% SDS, and 1% (w/v) sodium dodecyl sulfate at 65°C for 20 min. The blot was reprobed with 0.05 μg of plasmid DNA encoding the human type I deiodinase (30) as probe. The blot was reprobed for 2 h at 37°C with [α-32P]dCTP using DECAPrime II (Ambion Inc.). Cells were harvested at a final concentration of 0.1 mA, and incubated at 62°C for a minimum of 16 h. Following hybridization, the membranes were washed for 2 h in 1% (w/v) SDS, 0.1× SSC at 62°C with multiple changes of the wash buffer. Dot blot analysis of RNA{Ser}^{Sec} was accomplished as described previously (15). Immunoprecipitation of total RNA for Northern blot analysis by anti-iA serum (32) was performed essentially as described (15).

Hepatocytes were isolated by collagenase digestion at 1:10 dilution of intensifying screens, and quantification was accomplished using a PhosphorImager (Molecular Dynamics) and ImageQuant software (Molecular Dynamics).

Western Blotting—CHO cells were seeded and transfected as described above. Cells were harvested for protein determination (25) and SDS-PAGE (26) as described. Protein gels were transferred to Protran (Schleicher and Schuell) as described previously (30). Membranes were blocked with 5% (w/v) nonfat milk in TBS-Tween (20 mm Tris, pH 7.4, 150 mm NaCl, and 0.1% Tween 20). Rabbit antisera directed against a peptide in the N-terminal region of type I deiodinase (30) was used at a 1:1000 dilution in TBS-Tween containing 5% (w/v) nonfat milk. Alternatively, rabbit antisera directed against β-galactosidase was used at a 1:1000 dilution. Goat anti-rabbit horseradish peroxidase was used as the secondary antibody at a 1:4000 dilution in TBS-Tween containing 5% (w/v) nonfat milk. Enhanced chemiluminescence was performed according to the manufacturer (NEN Life Science Products).
RESULTS

One means of defining the physiological role of \( i^\Delta A \) on tRNA\(^{Sec}\) is to eliminate the adenosine at position 37, the site of isopentenylation. As described under "Experimental Procedures," we created an A37C mutation in the Xenopus tRNA\(^{Sec}\) gene (A37C-XTSS) by site-directed mutagenesis. Next, we transfected A37C-XTSS into CHO cells, isolated total RNA, and analyzed for Sec tRNA expression by northern hybridization with tRNA\(^{Sec}\) probe (Fig. 1A, lanes 1 and 2). This experiment shows that A37C-XTSS transfected cells express a new Sec tRNA with faster mobility than the endogenous CHO Sec tRNA. Upon quantification, we observed that the expression levels of A37C-Sec tRNA exceeded the level of the endogenous CHO tRNA by almost 4-fold.

To determine if A37C-Sec tRNA was isopentenylated, we immunoprecipitated total RNA from cells transfected with either empty vector or A37C-XTSS with antisera prepared in rabbits immunized with isopentenyladenosine (32) and performed Northern blots on the immunoprecipitates (lanes 1 and 2). Previous work showed that these antibodies do not recognize unmodified adenosine (32). Whereas the endogenous, CHO Sec tRNA was precipitated by our anti-\( i^\Delta A \) antibodies in both samples, the A37C-Sec tRNA (lane 4) previously observed (lane 2) was not. These data indicated that transfection of A37C-XTTS results in overexpression of a Sec tRNA lacking isopentenyladenosine.

We also determined whether lovastatin caused reduced levels of isopentenyladenosine in Sec tRNAs (Fig. 1B). Lovastatin is a potent, specific inhibitor of mevalonic acid biosynthesis (35), the precursor for the isopentenyl moiety at position 37 of Sec tRNAs. Lovastatin treatment of A37A-XTSS transfected cells did not seem to affect either endogenous CHO or exogenous A37A-Sec tRNA expression (lanes 1 and 2). However, the amount of \( i^\Delta A \)-containing Sec tRNA was reduced ~50% in total RNA from lovastatin-treated cells (compare lanes 3 and 4). The decreased anti-\( i^\Delta A \) immunoprecipitation in lovastatin-treated cells is consistent with reduced isopentenyl modification of the tRNAs.

To address the effect that A37C-Sec tRNA has on cells, we examined the capability of A37C-XTSS transfected cells to decode UGA Sec codons. Fig. 2 depicts the synthesis of selenoproteins in CHO cells as measured by \( 75^{Se} \) incorporation. As seen in untransfected cells (lane 1), there are several endogenously expressed proteins that contain \( 75^{Se} \). This pattern is consistent with what others have found in similar labeling experiments (33, 34). Furthermore, we analyzed selenoprotein synthesis in CHO cells transfected with plasmids containing cDNAs for type I deiodinase (G21-D10) and type II deiodinase (hD2/SelP). Transfection with G21-D10 alone resulted in the expression of deiodinase (compare lanes 1 and 2), and co-transfection with the A37A-XTSS construct appeared to have little or no effect on the level of type I deiodinase expression (compare lanes 2 and 3). However, co-transfection with the mutant A37C-XTSS construct resulted in a dramatic reduction in type I deiodinase expression (lane 4). Although type II deiodinase expression was not as strongly expressed (lanes 5 and 6) as the type I form (lanes 2 and 3), its expression was affected similarly by co-transfection with the wild-type (lane 6) and the mutant.
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Fig. 2. A37C-Sec tRNA expression reduces deiodinase synthesis in CHO cells. CHO cells were transfected with vector (pGEM-T) alone, type I (G21-D10), or type II (hD2/SelP) deiodinase plasmid DNA alone or in combination with A37A-XTSS or the mutant A37C-XTSS gene as described under “Experimental Procedures.” On day 3, CHO cells were pulsed for 3 h with 2 μCi/ml of \(^{75}\)Se in H-NCS. The cells were harvested, an extract was prepared, and 100 μg of cell protein were electrophoresed on 13% SDS-PAGE gels. Gels were processed for fluorography, and quantification of \(^{75}\)Se-selenoprotein synthesis was accomplished by Phosphorimage analysis using ImageQuant software. This represents a typical fluorogram of \(^{75}\)Se incorporation into type I and type II deiodinase. Lane 1, control, 1.6 μg of pGEM-T DNA; lane 2, 0.4 μg of G21-D10 DNA + 1.2 μg of pGEM-T DNA; lane 3, 0.4 μg of G21-D10 DNA + 1.2 μg of A37A-XTSS DNA; lane 4, 0.4 μg G21-D10 DNA + 1.2 μg A37C-XTSS DNA; lane 5, 0.4 μg of hD2/SelP + 1.2 μg of pGEM-T DNA; lane 6, 0.4 μg of hD2/SelP + 1.2 μg of A37A-XTSS DNA; and lane 7, 0.4 μg of hD2/SelP + 1.2 μg of A37C-XTSS DNA.

| Transfection          | Type I deiodinase\(^a\) | Type II deiodinase\(^a\) | Thioredoxin reductase\(^b\) | 24-kDa protein\(^b\) | 18-kDa protein\(^b\) |
|-----------------------|-------------------------|-------------------------|---------------------------|-------------------|-------------------|
| Deiodinase            | 115                     | 76                      | 114                       | 120               | 103               |
| + A37A-XTSS           |                         |                         |                           |                   |                   |
| Deiodinase + A37C-XTSS| 22                      | 6                       | 91                        | 74                | 81                |

CHO cells were transfected with type I (G21-D10) or type II (hD2/SelP) deiodinase plasmid DNA alone or in combination with A37A-XTSS or the mutant A37C-XTSS gene as described under “Experimental Procedures.” On day 3, CHO cells were pulsed for 3 h with 2 μCi/ml \(^{75}\)Se in H-NCS. The cells were harvested, an extract was prepared, and 100 μg of cell protein were electrophoresed on 13% SDS-PAGE gels. Gels were processed for fluorography, and quantification of \(^{75}\)Se-selenoprotein synthesis was performed.

\(^a\) Quantitative data of transfected proteins from Fig. 2 are represented as the percentage of control in comparison to the respective deiodinase construct alone.

\(^b\) Quantitative data of endogenous selenoproteins is an average of expression levels from transfection with either G21-D10 or hD2/SelP and expressed as percentage of control relative to the average of lanes 2 and 5.

5 and 7). These data indicate that A37C-Sec tRNA does not act as a general inhibitor of protein synthesis, but inhibits selenoprotein expression (note that the immunoreactive protein seen at ~21 kDa represents nonspecific binding of our antiserum).

In the above experiments, the function of i6A was addressed by creating a mutation in the tRNA gene sequence, transfecting this mutant construct into CHO cells, and examining the ability of cells transfected with the mutant Sec tRNA gene to support selenoprotein synthesis. This method addressed the function of the intact, modified nucleoside at position 37 of Sec tRNA. As a second approach to discern the function of the intact, modified nucleoside at position 37 of Sec tRNA, we treated cells with 10 μM Lovastatin. Lovastatin is a potent, specific inhibitor of mevalonic acid biosynthesis (35), and mevalonic acid is a precursor for the iso-
Cells cultured under serum-free conditions in the presence of 10 μM lovastatin was unaffected by serum removal. CHO deiodinase (0.4 μg G21-D10) was unaffected by serum removal. CHO cells cultured for 21 h under a variety of growth conditions prevent cell cycle arrest. The magnitude of inhibition, however, was much less than that observed with lovastatin treatment. Note that 75Se incorporation into type I deiodinase mRNA when compared with cells transfected with G21-D10 alone (lane 2). Interestingly, the ratio of type I deiodinase to GAPDH in cells subject to serum deprivation (lane 6) was similar to that observed under the other conditions (lanes 2–5). These results indicate that the manipulations that cause a decreased synthesis of type I deiodinase do not alter rates of protein synthesis. Experiments were carried out in duplicate procedures, except that on day 2, cells received 10 μM lovastatin (lanes 3 and 4) or 10 μM lovastatin + 2 mM mevalonic acid (lanes 5 and 6). In addition, CHO cells were incubated in serum-free conditions (2% (w/v) bovine serum albumin in Ham’s F12 containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 25 mM Heps, pH 7.4) for 18 h in the absence (lanes 7 and 8) or presence of 10 μM lovastatin (lanes 9 and 10). CHO cells were pulsed for 3 h with 2 μCi/ml of 75Se in serum-free Ham’s F12 containing 5 mg/ml Na2SeO3 in the presence or absence of the respective drug treatments. The cells were harvested for PAGE, and 100 μg of cell protein were electrophoresed on 10% acrylamide spacer, 12% acrylamide resolving Schagger gels. Gels were processed for fluorography, and [75Se]selenoprotein synthesis direct quantification was accomplished by Phosphorimage analysis using ImageQuant software.

![Fig. 3. Effect of A37C-XTSS co-transfection on type I deiodinase and β-galactosidase protein levels.](image1)

CHO cells were transfected with either type I deiodinase (0.4 μg G21-D10) or β-galactosidase plasmid DNA (0.4 μg of pNASS) alone or in combination with A37A- or A37C-XTSS DNA as described under “Experimental Procedures.” Western blotting of type I deiodinase and β-galactosidase was performed as described under “Experimental Procedures.” Lane 1, control, 1.6 μg of pGEM-T DNA; lane 2, G21-D10 + 1.2 μg of pGEM-T DNA; lane 3, G21-D10 + 1.2 μg of A37A-XTSS DNA; lane 4, G21-D10 + 1.2 μg of A37C-XTSS DNA; lane 5, pNASS + 1.2 μg of pGEM-T DNA; lane 6, pNASS + 1.2 μg of A37A-XTSS DNA; lane 7, pNASS + 1.2 μg of A37C-XTSS DNA. Full-length type I deiodinase and β-galactosidase proteins are indicated by the arrows.

![Fig. 4. Lovastatin and serum deprivation effect on selenoprotein expression.](image2)

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pentenyl moiety at position 37 of tRNA. As observed above (Fig. 1C), lovastatin treatment of cells expressing A37A-Sec tRNA resulted in an ~50% decrease in the amount of i6A-containing Sec tRNA without affecting total Sec tRNA expression. This experimental protocol allowed us to further address the function of the isopentenyl modification on selenoprotein biosynthesis.

CHO cells were transfected with G21-D10 plasmid DNA and subsequently cultured for the final 21 h under a variety of conditions in the presence or absence of lovastatin. [75Se]Selenium acid was added during the final 3 h to monitor selenoprotein synthesis. Experiments were carried out in duplicate (Fig. 4), and the resulting data were quantified (Table II). The pattern of selenoprotein labeling in the absence of lovastatin is shown in lanes 1 and 2. In the presence of 10 μM lovastatin, selenoprotein labeling was dramatically reduced (lanes 3 and 4), whereas the addition of mevalonic acid to the culture medium reversed the effect of lovastatin (lanes 5 and 6). Quantifying this data showed that all cellular selenoproteins were reduced from 55 to 95% with the exception of an 8.5-kDa protein. The presence of the 8.5-kDa protein is enhanced, and its identity is unknown.

It has previously been shown that 10 μM lovastatin results in cell-cycle arrest at G1/G0 phase (32). To ascertain how much of the lovastatin effect was because of cell cycle arrest, we cultured cells under serum-free conditions. CHO cells cultured for 21 h under serum-free conditions (lanes 7 and 8) showed reduced 75Se incorporation into several selenoproteins compared with CHO cells cultured in H-NCS (lanes 1 and 2). The latter growth conditions prevent cell cycle arrest. The magnitude of inhibition, however, was much more than that observed with lovastatin treatment. Note that 75Se incorporation into type I deiodinase (~27 kDa) was unaffected by serum removal. CHO cells cultured under serum-free conditions in the presence of 10 μM lovastatin showed a further reduction in 75Se incorporation into all proteins, except the 8.5-kDa protein (lanes 9 and 10). Most notably affected was type I deiodinase, whose synthesis was unaffected by serum-free conditions.

Together these data point to a crucial role of the isopentenyl group at position 37 of the Sec tRNA in selenoprotein expression. However, the decreased synthesis of type I deiodinase may be explained by lower levels of deiodinase mRNA, a decrease in protein stability, or a reduction in mRNA translation.

First, we used Northern blot analysis to determine whether the type I deiodinase mRNA levels were reduced in A37C-XTSS-transfected or lovastatin-treated CHO cells. As shown in Fig. 5, type I deiodinase mRNA is undetectable in the absence of G21-D10 transfection (lane 1). When normalized for GAPDH expression, neither A37C-XTSS transfection (lane 4) nor lovastatin treatment (lane 5) affected the amount of type I deiodinase mRNA when compared with cells transfected with G21-D10 alone (lane 2). Interestingly, the ratio of type I deiodinase to GAPDH in cells subject to serum deprivation (lane 6) was similar to that observed under the other conditions (lanes 2–5). These results indicate that the manipulations that cause a decreased synthesis of type I deiodinase do not alter rates of...
TABLE II
Quantification of \(^{75}\text{Se}\) selenoprotein synthesis in CHO cells under various incubation conditions

| Protein | Incubation Conditions |
|---------|----------------------|
|         | H-NCS | H-NCS + Lovastatin | H-NCS + Lovastatin + MVA | Serum-free | Serum-free |
|         | % of control | % of control | % of control | Serum-free | Serum-free |
| kDa     |        |                  |                          |            |            |
| 54      | 100    | 45              | 105                     | 57         | 38         |
| 24      | 100    | 23              | 96                      | 93         | 21         |
| 21      | 100    | 21              | 114                     | 61         | 7          |
| 18      | 100    | 32              | 147                     | 57         | 1          |
| 15      | 100    | 4                | 106                     | 85         | 20         |
| 13      | 100    | 31              | 103                     | 97         | 31         |
| 8.5     | 100    | 214             | 105                     | 129        | 183        |

Fig. 5. Northern blot of type I deiodinase RNA. Total RNA from CHO cells transfected with vector (pGEM-T) alone (lane 1) or type I deiodinase (G21-D10) plasmid DNA alone (lane 2) or in combination with A37A-XTSS (lane 3) or A37C-XTSS (lane 4) was isolated as described under “Experimental Procedures.” In addition, total RNA was isolated from CHO cells transfected with G21-D10 and treated for 21 h with 10 μMLovastatin (lane 5) or under serum-free conditions (lane 6). Total RNA (5 μg) was resolved on a 1% agarose/formaldehyde gel and transferred to GeneScreen Plus. The membrane was first probed for type I deiodinase using a 770-bp Smal/NcoI fragment of the G21-D10 plasmid (A), stripped, and subsequently probed for GAPDH (B) as described under “Experimental Procedures.”

gene transcription or mRNA stability. This implicates a post-transcriptional event as the cause for the observed reduction in selenoprotein synthesis by the mutant A37C-Sec tRNA and the lovastatin-induced Sec tRNA lacking \(^{\text{iA}}\)A.

Next, we performed a pulse-chase experiment to determine if co-transfection with the mutant A37C-XTSS construct affected the degradation rate of type I deiodinase protein (Fig. 6). CHO cells were co-transfected with G21-D10 and either A37A-XTSS (lanes 1–4) or A37C-XTSS (lanes 5–8). After one day, the cells were pulse-labeled with \(^{75}\text{Se}\) for 1.5 h and chased for various times, and the amount of radiolabeled type I deiodinase was determined after SDS-PAGE and fluorography. Densitometric scans showed that A37C-XTSS-transfected cells synthesized ~35% less type I deiodinase during the pulse interval compared with A37A-XTSS-transfected cells. However, the rate of disappearance of type I deiodinase was similar in both sets of cells. These data indicate the expression of the mutant tRNA does not affect the stability of type I deiodinase.

Lastly, we sought to determine if A37C-XTSS inhibited type I deiodinase expression by decreasing translation. Inhibition of translation could occur at two possible sites: initiation of synthesis or, given the unique nature of selenoprotein biosynthetic decoding of the internal Sec UGA. Impaired read through of the internal UGA would cause termination of translation and release of a truncated protein.

Fig. 7 is a Western blot analysis showing the level of both the full-length and truncated type I deiodinase in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of co-transfected A37C-XTSS. We also analyzed the content in cells treated with ALLN, an inhibitor of cellular proteolysis (lanes 3 and 4). The approximate relative expression of the full-length protein in lanes 1–4 is 1.0, 0.35, 0.15, and 0.06, respectively.

If A37C-Sec tRNA caused decreased initiation of translation, we would expect to observe similar reductions in both the full-length and truncated type I deiodinase proteins. On the other hand, if the mutant Sec tRNA inhibited expression of the full-length type I deiodinase by blocking decoding of the internal UGA, we would observe a reciprocal increase in the amount of truncated protein. In comparing lanes 1 and 2, we see an ~3-fold reduction in the amount of full-length protein, yet no change in the expression of the truncated translate. This observation does not support either mechanism for A37C-Sec tRNA inhibition of translation.

We then looked at the effect of ALLN on the expression of type I deiodinase proteins (Fig. 7, lanes 3 and 4). ALLN caused a dramatic increase in the amount of the truncated protein. This observation indicates the truncated protein is subject to extremely rapid degradation, probably as a result of the proteasome-dependent protease system (36). Given the abundance of truncated versus full-length protein in the presence of ALLN, we conclude that the overwhelming majority (~95%) of type I deiodinase mRNA-directed translation is terminated at the internal UGA. Similarly, synthesis of the full-length protein is extremely inefficient (~5% of total mRNA-directed translation).

Unfortunately, this grossly disproportional pattern of expression for the two forms of type I deiodinase prevents us from determining if A37C-Sec tRNA directly inhibits decoding of the internal UGA. Co-transfection of the mutant tRNA gene reduces full-length deiodinase expression by ~60% in ALLN-treated cells. However, (as noted above) only ~5% of mRNA-directed translation produces the full-length protein. Therefore, if the mutant tRNA does inhibit decoding, we would expect to observe only an ~3% (60% of 5%) increase in the amount of truncated protein. Such a small difference would not be detected in the immunoblots from ALLN-treated cells.

If the initiation of translation is inhibited, we would predict a 60% decline in truncated deiodinase expression in the ALLN-
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extracts were prepared, and elution profiles of both A and B were compared with either A37A-XTSS (Fig. 8lanes 1–4) or A37C-XTSS (Fig. 8lanes 5–8) as described under “Experimental Procedures.” On day 2, the cells were pulse-labeled with 4 μCi/ml of [75Se]selenious acid for 1.5 h in selenium-free growth medium. Following the pulse, the monolayers were washed and chased for the indicated times in growth medium containing 50 ng/ml sodium selenite. Cells were processed for PAGE, and 100 μg of cell protein were electrophoresed on 10% acrylamide gels. Gels were processed for fluorography and 75Se type I deiodinase quantification was accomplished by Phosphorimage analysis using ImageQuant software. The position of full-length type I deiodinase is indicated by the arrow.

Another possible explanation for the reduction in 75Se incorporation into protein seen with lovastatin treatment and A37C-XTSS expression could be because of the lack of or reduced selenocysteinyl-tRNA[Ser]Sec biosynthesis because of changes at position 37. Fig. 8 shows the RPC-5 column elution profiles of [75Se]selenocysteinyl-tRNA[Ser]Sec from CHO cells transfected with either A37A-XTSS (Fig. 8A) or A37C-XTSS (Fig. 8B). The elution profiles of both in vivo [35S]labeled tRNA (Fig. 8A) and in vitro labeled [3H]seryl-tRNA (Fig. 8C) from CHO cells overexpressing A37A-XTSS show two peaks; the position 34, 5-methylcarboxymethyluridine (mcm5U, peak II) and 5-methylocarboxymethyluridine-2′-O-methyl ribose tRNAs[Ser]Sec (mcm5Um, peak III) as previously reported (37). Peak I, observed in Fig. 8B is most likely the A37C-Sec-tRNA given its relatively early elution from the RPC-5 column as was seen for tRNA[Ser]Sec lacking i6A (38). Similarly peak I, in Fig. 8D isolated from cells overexpressing A37A-XTSS and subsequently treated with 20 μM lovastatin most certainly lacks i6A. Therefore it can be concluded that i6A is not necessary for aminoclaylation with serine or in the biosynthesis of Sec.

The efficiency of aminoclaylation of tRNA[Ser]Sec and biosynthesis of Sec were still unknown. Measurement of these parameters was accomplished by quantifying the relative amount of tRNA[Ser]Sec in each of these peaks by dot blotting and the amount of 75Se associated with the purified Sec tRNA by liquid scintillation counting. The calculated specific radioactivity (Table III) is a measure of the ability of the tRNA to become aminoclaylated with Sec. Experiment 1 shows the specific activities of the two wild type forms of Sec-tRNA (derived from peaks II and III, Fig. 8A) from cells transfected with A37A-XTSS. Experiment 2 compares the purified mutant, A37C-Sec-tRNA (peak I, Fig. 8B), with the two forms of A37A-Sec-tRNA (peaks II and III, Fig. 8B). Note, the specific radioactivity is approximately the same for all three Sec-tRNA species. These data indicate that the mutant Sec tRNA is aminoclaylated with [75Se]Sec to a similar extent as A37A-Sec tRNA. Thus, a lack of or a reduction in aminoclaylation of A37C-Sec tRNA or in the biosynthesis of Sec cannot explain the observed decreases in selenoprotein synthesis seen in Figs. 2 and 3.

To determine if tRNA[Ser]Sec minus i6A may have an effect at the level of translation, we examined the codon recognition properties of the mutant isoacceptor and the i6A lacking isoacceptor induced by lovastatin inhibition. As shown in experiments 1 and 2 in Table IV, changes at position 37 from i6A to either A37C or unmodified A37A did not alter the specificity of the seryl-tRNA[Ser]Sec for recognizing UGA in a ribosome binding assay. The cysteine codons, UGU and UGC, as well as the tryptophan codon, UGG, were poorly recognized by all seryl-tRNA[Ser]Sec isoacceptors tested in this assay. However, when...
we examined specific binding as a percentage of added seryl-tRNA\[^{75}\text{Se}\] to UGA in this assay, both tRNAs exhibited reduced binding to UGA in comparison to the wild-type isoacceptor. Specific binding of the A37C- and i\(^6\)A-lacking Sec tRNA to UGA was decreased by 62 and 69%, respectively.

Although E. coli ribosomes were used in the ribosomal binding studies, it should be noted that the genetic language of mammals and bacteria were shown to be the same in this assay using E. coli ribosomes (39) and that strong responses relative to their assigned codons of mammalian Cys-, Arg-, and Trp-tRNAs to UGA were observed in this same assay using E. coli ribosomes (40). The later observation suggests that mammalian Cys-, Arg-, and Trp-tRNAs are capable of misreading UGA and, indeed, these tRNAs serve as authentic suppressors of UGA stop codons in mammals (41, 42). We conclude, therefore, that the data shown in Table IV demonstrate a defect in the decoding properties of tRNA\[^{75}\text{Se}\] lacking the i\(^6\)A modification in mammalian protein synthesis.

**DISCUSSION**

In this study we have provided evidence that changes at position 37 of Sec tRNA result in a tRNA with reduced abilities to bind to UGA in an *in vitro* ribosomal binding assay and to support selenoprotein synthesis *in vivo*. These results elucidate the role of i\(^6\)A in mammalian cells that seems to be similar to that observed in bacteria and yeast. In *E. coli* (8) and *S. cerevisiae* (9), the inability to synthesize i\(^6\)A results in a decreased efficiency to suppress stop codons. Similarly, the lack of i\(^6\)A on a nonsense serine suppressor, *sup3-i*, in an antisuppressor mutant of *S. pombe*, *sin1*, leads to its inactivation (6). In addition to the decreased stop codon suppression seen in bacteria and yeast, we have shown that removal of the i\(^6\)A from Sec tRNA results in reduced selenoprotein synthesis in mammalian cells.

Our observations of reduced selenoprotein synthesis are quite intriguing. First, the effect of A37C-XTSS transfection on type I deiodinase synthesis (Table I) does not mimic the effect on endogenous selenoprotein synthesis. Whereas a definitive effect of A37C-Sec tRNA expression on endogenous selenoproteins was observed, the reduction in synthetic rates did not approach the 80–90% decreases observed with type I and II deiodinases. Attempts to differentiate between a unique effect of A37C-sec tRNA expression on endogenous selenoprotein synthesis and a general effect on transfected selenoprotein cDNAs was attempted using HepG2 cells. This cell line reportedly expresses functional type I deiodinase activity (6). In addition to the decreased stop codon suppression seen in bacteria and yeast, we were unable to observe detectable \(^{75}\text{Se}\)-radiolabeling of type I deiodinase under our culture conditions indicating that the synthetic rate is too low for determining the effect of A37C-Sec tRNA on endogenous type I deiodinase synthesis.

**Why does expression of the mutant, A37C-tRNA\[^{75}\text{Se}\]?** A plausible explanation is that replacing the highly modified i\(^6\)A nucleoside with an unmodified base results in an isoacceptor that, by analogy to the corresponding tRNAs in bacteria and yeast,
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Table IV
Codon recognition properties of tRNA^{Ser}_{Sec} isoacceptors

| tRNA^{Ser}_{Sec} sample | Experiment | None<sup>a</sup> | Codon | UGA | UGG | UGU | UGC |
|-------------------------|------------|------------------|-------|-----|-----|-----|-----|
| A37<sup>b</sup>         | 1          | 306              | 1388  | 163  | 218 | 432 | 1168|
| iA37                    | 1          | 1148             | 4734  | 1376 | 476 | 1168|
| mcmmU34<sub>i6A</sub>   | 2          | 560              | 2918  | 596  | 582 | 626 |
| A37C<sup>c</sup>        | 3          | 472              | 3544  | 28   | 582 | 626 |
| iA37 mcmmU34<sup>d</sup>| 3          | 902              | 8140  | 735  |     |     |
| iA37                    | 3          | 926              | 8130  | 721  |     |     |

<sup>a</sup> None is the [H]serine (cpm) bound to ribosomes in the absence of trinucleoside diphosphate.

Cho cells were transfected, and the tRNA was purified as described under “Experimental Procedures.” Total tRNA was aminocylated in vitro with [H]serine and chromatographed on a RPC-5 column as described. Specific binding to ribosomes is represented by the binding in the presence of trinucleoside diphosphate minus binding in the absence of trinucleoside diphosphate, and the value is given in parentheses as a percent of the total [H]serine (cpm) added to the reaction.

decodes UGA less efficiently than the fully modified form. Our co-transfections were performed with the aid of liposomes, and the mutant tRNA and cDNA plasmids were mixed prior to the addition of the liposomes. Under these conditions, the mixture of exogenous DNA forms complexes with the liposomes. It is highly likely both types of DNA are present in every complex. Therefore, those cells that take up a liposome-DNA complex are receiving both types of plasmid, and it is unlikely a significant amount of cells take up one type of DNA without the other. Also, knowing that transfection efficiency is not 100%, some percentage of the population is not receiving any mutant tRNA. However, the entire population is exposed to a radiolabeled 75Se precursor. Consequently, if the mutant tRNA were inhibiting endogenous selenoprotein synthesis, we would only observe a decline in radiolabeled incorporation equivalent to the percentage of cells expressing the mutant tRNA. As seen in Table I, we observed a small, but significant decline (9–26%) in the incorporation of 75Se into several endogenous selenoproteins when transfected with the mutant tRNA. On the other hand, all cells on the monolayer receive lovastatin and are inhibited by the drug. This may explain why endogenous selenoproteins and transfected type I deiodinase synthases were inhibited by the drug.

Of course other possibilities exist to explain the lack of effect of A37C-Sec tRNA on endogenous selenoprotein synthesis. For example, several laboratories have provided evidence for the existence of supramolecular translation complexes containing mRNAs, ribosomes, EF-1α, tRNAs, and tRNA synthetases (44–46). According to the channelling model of Stapolonis and Deutscher (46), tRNAs are directly transferred from aminoacyl-tRNA synthetases to elongation factor to ribosomes without dissociation into the cellular fluid. Upon leaving the ribosome, uncharged tRNAs are directly transferred back to their cognate synthetases, without being released into the surrounding cytosol. There, they are recharged with amino acid and then bound by EF-1α for another elongation cycle in the same supramolecular complex. This model may explain the effect of transfected Sec-specific tRNA on transfected but not endogenous selenoprotein synthesis.

Selenoprotein mRNAs expressed from endogenous genes would be expected to be continuously transcribed and exported to the cytoplasm at a low, constitutive level. Newly synthesized selenoprotein mRNA and Sec tRNA from the transfected genes would likely undergo a sharp, steep burst of transcription and export. Thus, most of the endogenous selenoprotein mRNAs would already be in the cytoplasm assembled into translation complexes at the time of transfection. The newly transcribed mRNAs, following export, would predominantly be free to assemble together into new supramolecular complexes, producing an apparent transfection-specific effect of the introduced wild-type or mutant tRNA<sup>Sec</sup> gene.

As was shown in Fig. 5, no effect of A37C-XTSS co-expression or lovastatin treatment on type I deiodinase mRNA levels was observed. Whereas increased selenoprotein degradation could account for some of the decreased 75Se-labeled proteins in Fig. 2 (lanes 4 and 7) and Fig. 4 (lanes 3 and 4), we hypothesized that A37C-Sec tRNA and iA-lacking Sec tRNA affect translation. To address this, we examined the ability of these tRNAs to recognize UGA in a well established ribosomal binding assay (22). The results in Table IV provide evidence that changes at position 37 of the Sec tRNA affect translation, specifically the step involved in codon binding. Specific binding to UGA was reduced ~70% for both the A37C- and iA-lacking tRNA<sup>Sec</sup> populations compared with the corresponding wild-type tRNA. These results are in close agreement to those of Ohama et al. (47). They observed that an early eluting 75Se-labeled tRNA showed a 50% decrease in specific binding to UGA compared with the full modified Sec tRNA. They presumed this to be a Sec-tRNA lacking iA based on previous observations in Xenopus oocytes (38).

Our hope was to test the ability of these individual Sec tRNA populations to support UGA translation in vitro. Rabbit reticulocyte translation systems programmed with specific mRNAs are widely used for in vitro synthesis of proteins. However, to our knowledge an in vitro translation system to faithfully measure the efficiency of selenoprotein synthesis does not exist. Previously, Berry et al. (30, 48) demonstrated in vitro synthesis of type I deiodinase using a rabbit reticulocyte system. However, these investigators determined that the commercially available in vitro translation systems are, in addition to being quite inefficient, a measure of UGA suppression and not Sec incorporation into protein. Given that the A37C- and iA-lacking tRNA<sup>Sec</sup> both exhibit binding to UGA, we would expect them to show some level of UGA translation. However, without a reliable system to accurately measure translation efficiency, we are unable to definitively demonstrate a reduced translational ability of either the A37C- or iA-lacking tRNA<sup>Sec</sup> gene.
its function, e.g., inhibiting other modifications, affecting tRNA stability, altering tertiary structure, and inhibiting general protein synthesis. However, we show the lack of adenosine at position 37 does not affect the ability of A37C-Sec tRNA to bind Sec, as judged by normal amounts of $^{75}$Se incorporation into the mutant tRNA (Fig. 8B and Table III). This observation indicates adenosine 37 is not required for serine-specific aminoacylation and tRNA-dependent conversion to Sec, mediated by aminoacyl-tRNA synthetase and selenocysteine synthase, respectively. Furthermore exchanging guanosine for adenosine at position 37 of Sec tRNA does not affect the tertiary interaction of d-T-arms in the tRNA. Lastly, the expression of transfected b-galactosidase is not reduced by co-transfection with the A37C-Sec tRNA gene (Fig. 3), showing lack of adenosine at position 37 does not grossly affect general protein synthesis.

These data provide evidence of a crucial role for the iA modification on tRNA$^{Sec}_{Sec}$ for decoding UGA Sec codons. Future directions are aimed at determining the precise mechanism by which changes at position 37 of Sec tRNA result in decreased selenoprotein synthesis.

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REFERENCES

1. Hagervall, T. G., Ericson, J. U., Edberg, K. B., Ji-nong, L., and Bjork, G. R. (1990) Biochim. Biophys. Acta 1050, 263–266
2. Bjork, G. R., Ericson, J. U., Gustafsson, C. E. D., Hagervall, T. G., Jonasson, Y. H., and Wikstrom, P. M. (1987) Annu. Rev. Biochem. 56, 263–287
3. Bartz, J. K., Kline, L. K., and Soll, D. (1970) J. Mol. Biol. 39, 414–415
4. Caillet, J., and Droogmans, L. (1988) J. Bacteriol. 170, 4147–4152
5. Dihanich, M. E., Najarian, D., Clark, R., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1987) Mol. Cell. Biol. 7, 177–184
6. Janner, F., Vogeli, G., and Fluri, R. (1980) J. Mol. Biol. 135, 111–126
7. Denich, M. E., Najarian, D., Clark, R., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1987) Mol. Cell. Biol. 7, 177–184
8. Janner, F., Vogeli, G., and Fluri, R. (1980) J. Mol. Biol. 135, 111–126
9. Pétrole, A., Laga, A., and Elgers, D. (1983) Mol. Gen. Genet. 190, 289–294
10. Janner, F., Vogeli, G., and Fluri, R. (1980) J. Mol. Biol. 135, 111–126
11. Denich, M. E., Najarian, D., Clark, R., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1987) Mol. Cell. Biol. 7, 177–184
12. Janner, F., Vogeli, G., and Fluri, R. (1980) J. Mol. Biol. 135, 111–126
13. Denich, M. E., Najarian, D., Clark, R., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1987) Mol. Cell. Biol. 7, 177–184
14. Hatfield, D. L., Gladyshev, V. N., Park, S. I., Chittum, H. S., Carlson, B. A., et al. (1998) Biochemistry 37, 3753–3759
15. Hatfield, D. L., Gladyshev, V. N., Park, S. I., Chittum, H. S., Carlson, B. A., et al. (1998) Biochemistry 37, 3753–3759
16. Hatfield, D. L. (1994) Biochemistry 33, 4248–4250
17. Hatfield, D. L. (1994) Biochemistry 33, 4248–4250
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