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Methylation of the ribosyl moiety at position 34 of selenocysteine tRNA$^{[Ser]Sec}$ is governed by both primary and tertiary structure

LARK KYUN KIM,[1] TAMIKO MATSUFUJI,[2] SENYA MATSUFUJI,[3] BRADLEY A. CARLSON,[4] SUKWON S. KIM,[1] DOLPH L. HATFIELD,[4] and BYEONG JAE LEE[1]

[1]Laboratory of Molecular Genetics, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea
[2]Department of Internal Medicine (Daisan Hospital), Jikei University School of Medicine, Tokyo 105-8461, Japan
[3]Department of Biochemistry II, Jikei University School of Medicine, Tokyo 105-8461, Japan
[4]Basic Research Laboratory, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

ABSTRACT

The selenocysteine (Sec) tRNA$^{[Ser]Sec}$ population in higher vertebrates consists of two major isoacceptors that differ from each other by a single nucleoside modification in the wobble position of the anticodon (position 34). One isoacceptor contains 5-methylcarboxymethyluridine (mcmU) in this position, whereas the other contains 5-methylcarboxymethyluridine-2′-*O*-methylribose (mcmUm). The other modifications in these tRNAs are N^6-isopentenyladenosine (i^6A), pseudouridine (ψ), and 1-methyladenosine (m^1A) at positions 37, 55, and 58, respectively. As methylation of the ribose at position 34 is influenced by the intracellular selenium status and the presence of this methyl group dramatically alters tertiary structure, we investigated the effect of the modifications at other positions as well as tertiary structure on its formation. Mutations were introduced within a synthetic gene encoded in an expression vector, transcripts generated and microinjected into *Xenopus* oocytes, and the resulting tRNA products analyzed for the presence of modified bases. The results suggest that efficient methylation of mcmU to yield mcmUm requires the prior formation of each modified base and an intact tertiary structure, whereas formation of modified bases at other positions, including mcmU, is not as stringently connected to precise primary and tertiary structure. These results, along with the observations that methylation of mcmU is enhanced in the presence of selenium and that this methyl group affects tertiary structure, further suggest that the mcmUm isoacceptor must have a role in selenoprotein synthesis different from that of the mcmU isoacceptor.

Keywords: 1-methyladenine; 2′-*O*-ribose methylation; 5-methylcarboxymethyluridine; 5-methylcarboxymethyluridine-2′-*O*-methylribose; N^6-isopentenyladenosine; pseudouridine; selenocysteine; tRNA modification

INTRODUCTION

The selenocysteine (Sec) tRNA$^{[Ser]Sec}$ population in higher vertebrates consists of two major isoacceptors that differ from each other by a single 2′-*O*-methyl group attached to the ribose at position 34 (reviewed in Hatfield et al., 1999). One isoacceptor contains 5-methylcarboxymethyluridine (mcmU) at this position, whereas the other contains 5-methylcarboxymethyluridine 2′-*O*-methylribose (mcmUm). The intracellular level of the methylated form increases from as little as 2.5-fold to as much as 8-fold more than the unmethylated form depending on the cell (Hatfield et al., 1991; Choi et al., 1994) or tissue type (Diamond et al., 1993; Chittum et al., 1997) in response to selenium. In addition, the tertiary structures of these two forms are dramatically affected by the presence or absence of this methyl group (Diamond et al., 1993). The biological significance of the methylated form is not known, but the influence of selenium on its biosynthesis and the consequence of its presence on tertiary structure suggest that it must have a specific role in selenoprotein biosynthesis (Jung et al., 1994).

Reprint requests to: Byeong Jae Lee, Laboratory of Molecular Genetics, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea; e-mail: imbglmg@plaza.snu.ac.kr.

In addition to the presence of mcmU or mcmUm at position 34, Sec tRNA[Ser]Sec contains only three other modifications compared to the 15–17 modified bases that occur in most other tRNAs (reviewed in Hatfield et al., 1999). Both Sec isoacceptors contain N^6-isopentenyladenosine (i^6A), pseudouridine (ψ), and 1-methyladenosine (m^1A) at positions 37, 55, and 58, respectively.

Grosjean and collaborators have examined the formation of modified nucleosides in tRNA by microinjecting wild-type transcripts and site-specific mutant transcripts into *Xenopus* oocytes and observing the resulting modifications (see Grosjean et al., 1996; Tewari et al., 1996; Morin et al., 1998 and references therein). These investigators proposed that a host of maturation enzymes fell into one of two major groups; those that were extremely sensitive to changes in the three-dimensional structure of tRNA (designated tRNA architecture by Grosjean et al., 1996) and those that were insensitive to such changes. With respect to the common base modifications shared by the tRNAs analyzed by Grosjean and collaborators and those in tRNA[Ser]Sec, ψ formation at position 55 and m^1A formation at position 58 were insensitive to changes in tRNA architecture (Grosjean et al., 1996). i^6A formation at position 37 was relatively slower than that of m^1A and it was not clear into which class the corresponding modifying enzyme fits (Tewari et al., 1996). Although m^1A and ψ have been reported to have a role in governing tRNA architecture (Kim et al., 1974; Arnez & Steitz, 1994), the role of these modified positions in tRNA[Ser]Sec structure and function is still unresolved.

Because methylation of the ribose at position 34 in tRNA[Ser]Sec is influenced by selenium status (Hatfield et al., 1991; Diamond et al., 1993; Choi et al., 1994; Chittum et al., 1997) and because the presence of this methyl group dramatically affects the tertiary structure of this tRNA (Diamond et al., 1993), it was of considerable interest to determine the role of the other modified positions and tRNA architecture in tRNA[Ser]Sec that influence the 2′-*O*-methylation of the ribosyl moiety. In the present study, we examined the effects of mutations at each position normally containing a modified base and at positions elsewhere in tRNA[Ser]Sec that effect tertiary structure on the 2′-*O*-methylation step, as well as on the formation of modified bases at the other positions. The data show that 2′-*O*-methylation of the ribosyl moiety at the wobble position is retarded by mutating position 58 and completely abolished by mutating other modified sites. In addition, mutations that are known to influence tertiary structure (i.e., at positions 16–19) also abolish methylation of the ribose at position 34. As described herein, it is likely that ψ at position 55 also influences tertiary structure of tRNA[Ser]Sec, but m^1A at position 58 does not. Therefore, both primary structure and tRNA architecture apparently play a role in ribose methylation at position 34. Mutation of the A at position 58 (A58U) also inhibited ψ formation at position 55, suggesting that m^1A, or at least an A, is required at position 58 for ψ synthesis. Mutation of the wobble base (U34G) did not affect modification at positions 37, 55, or 58.

RESULTS

As a means of assessing the roles of the modified nucleosides in tRNA[Ser]Sec structure and function and, in particular, the methylated ribosyl moiety at position 34, several mutations were introduced into the gene of this tRNA that was cloned into a pBluescript expression vector. Mutations and mutation positions in tRNA[Ser]Sec are summarized in Figure 1. Single mutations were generated at positions 37 (A37G), 55 (U55G), and 58 (A58U) where i^6A, ψ, and m^1A, respectively, occur and at position 34 (U34G), where mcmU or mcmUm occurs. A double mutation was introduced at positions 37 and 55 (A37G:U55G) and multiple mutations in the D-loop at positions 16–19 (U16A:G17C:G18C:U19A). Mutations in the D-loop were prepared to assess the role of tertiary structure in modified nucleoside formation, as the D-loop is known to influence tertiary structure (Kim et al., 1974).

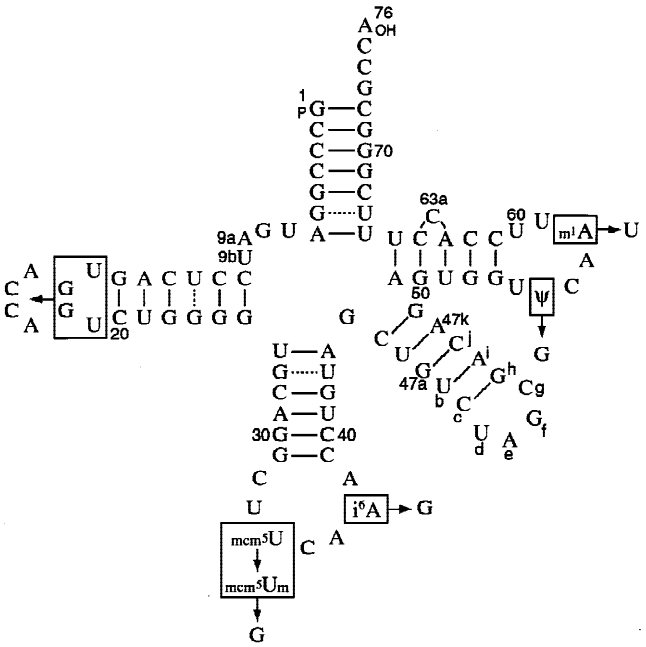

FIGURE 1. Positions of Sec tRNA[Ser]Sec gene mutations. Sec tRNA[Ser]Sec is shown in a cloverleaf form with the modified nucleosides. The site of single mutations at positions 34 (U34G), 37 (A37G), 55 (U55G), and 58 (A58U), of a double mutation at positions 37 and 55 (A37G:U55G), and of multiple mutations in the D-loop at positions 16–19 (U16A:G17C:G18C:U19A) can be deduced from the boxed bases shown. Although the cloverleaf form shown in the figure is as a 7/5 base-paired structure [i.e., 7 bp in the acceptor stem and 5 bp in the T-stem; see Ioudovitch & Steinberg (1999) and references therein], it should be noted that evidence also exists for a 9/4 base-paired structure [see Hubert et al. (1998) and references therein].

Tertiary structure and serylation of tRNA[Ser]Sec mutants

Two important aspects of tRNA function that can be readily examined in mutant tRNAs are to assess the role of the base change(s) on tertiary structure and on aminoacylation. We determined whether the primary transcripts of the tRNA[Ser]Sec mutants maintained tertiary structure by immunoprecipitation with an antibody that recognizes tertiary interaction of the D- and T-arms of tRNA[Ser]Sec (see Table 1). An autoantibody (NM) that recognizes the tertiary interaction of the D- and T-arms of unmodified tRNA[Ser]Sec (see Materials and Methods) precipitated wild-type Sec tRNA, A58U, A37G, and U34G mutants, but did not precipitate U55G, A37G:U55G, and the D-loop mutants as well as wild-type serine tRNA. Another autoantibody (EW) that recognizes the tetranucleotide-loop of the D-arm (see Materials and Methods) precipitated all Sec tRNA mutants except the D-loop mutant. These results strongly suggest that the immunoprecipitation with the NM antibody can be employed to determine the tertiary structure of Sec tRNA. It should be noted, however, that to obtain direct evidence for tertiary structure, more accurate methods (e.g., NMR or X-ray crystallography) should be used. Because A58U was readily precipitated with the NM antibody, this mutation presumably had no effect on tRNA[Ser]Sec tertiary structure. However, U55G appeared to affect tertiary structure, as demonstrated by its inability to bind the antibody. In addition, the chromatographic properties of U55G were affected, but those of A58U were not affected (see below), providing further evidence that the tertiary structure of the former, but not the latter mutant, was altered. The tertiary structure of the double mutant, A37G:U55G, was affected. As expected, the mutations of 4 nt in the D-loop disrupted tertiary structure. The tertiary structure-recognizing antibody (NM) did not bind the primary transcript of this mutant. Furthermore, as shown below, the chromatographic properties of this mutant, after incubation in oocytes, were severely affected (as compared to that of wild-type tRNA[Ser]Sec), suggesting that the D-loop mutations rendered the tRNA unstable.

Sec tRNA[Ser]Sec is initially aminoacylated with serine and Sec is then biosynthesized on its tRNA (Hatfield et al., 1999). The major identity elements within tRNA[Ser]Sec for aminoacylation with serine by seryl-tRNA synthetase are the discriminator base at position 73 and the long extra arm (Wu & Gross, 1993; Ohama et al., 1994). The acceptor stem, D-, and T-stems also play a role (Amberg et al., 1996). The ability, therefore, of each single mutant or of the multiple mutants to be aminoacylated with serine was examined (Table 1). A58U and U55G were almost as efficiently aminoacylated as the corresponding wild-type transcript, whereas the serylation activity of A37G was reduced compared to that of the wild-type, unmodified transcript. It is of interest to note that U55G is aminoacylated as efficiently as A58U, even though the tertiary structure of the former is affected whereas that of the latter is not affected (see Table 1 and below). Thus, the tertiary structure of tRNA[Ser]Sec as influenced by ψ at position 55 appears to affect aminoacylation only slightly. U34G was almost as efficiently aminoacylated as the wild-type, unmodified tRNA[Ser]Sec, whereas A37G was less efficiently aminoacylated. Aminoacylation of the double mutant, A37G:U55G, and the four-base D-loop mutant was very inefficient as compared to the corresponding wild-type tRNA.

TABLE 1. Serylation and tertiary structure of mutants.

	Immunoprecipitation[a]		
tRNA	NM	EW	Serylation[b]
Sec-WT	+	+	++++
Ser-WT	−	−	+++
A58U	+	+	+++
U55G	−	+	+++
A37G	+	+	++
U34G	+	+	++++
A37G:U55G	−	+	+
D-loop mutant	−	±[c]	+

[a]NM and EW denote results of immunoprecipitation with the sera from these patients. NM antibody recognizes the tertiary interaction of the D- and T-arm of tRNA[Ser]Sec and EW antibody only the tetra-loop at the D-arm (see Materials and methods).

[b]The amounts of serylation relative to the wild-type were expressed as the number of + symbols. ++++: 80–100% of wild-type; +++: 60–80% of wild-type; ++: 40–60% of wild-type; and +: 20–40% of wild-type. Wild-type tRNA[Ser]Sec yielded 35,970 dpm after the blank value (3,220 dpm) was subtracted.

[c]The value of ± was about 30% of Sec-WT.

Modified nucleoside analyses of Sec tRNAs[Ser]Sec

Transcripts were generated from wild-type and mutant tRNA[Ser]Sec genes that were labeled specifically with either ^{32}P-UTP or ^{32}P-ATP. Labeled transcripts were microinjected into the cytoplasm of *Xenopus* oocytes. Following overnight incubation, the resulting tRNA[Ser]Sec products were extracted from oocytes and chromatographed on an RPC-5 column. Individual peaks that eluted from the column were isolated, the peaks digested with nuclease P1, and digests analyzed by two-dimensional thin-layer chromatography (TLC) for the presence of U and modified U-containing nucleotides and A and modified A-containing nucleotides. Elution profiles from the column for each tRNA[Ser]Sec are shown in Figure 2 and nucleoside analyses are shown in Figure 3 and are summarized in Table 2. In Figure 3, the results of wild-type and only five mutants were shown, as the positions of nucleotides from U55G mutant are similar to those given. Although the profiles shown in Figure 2 were obtained with ^{32}P-labeled U tRNAs, similar elution profiles were obtained in each case with the

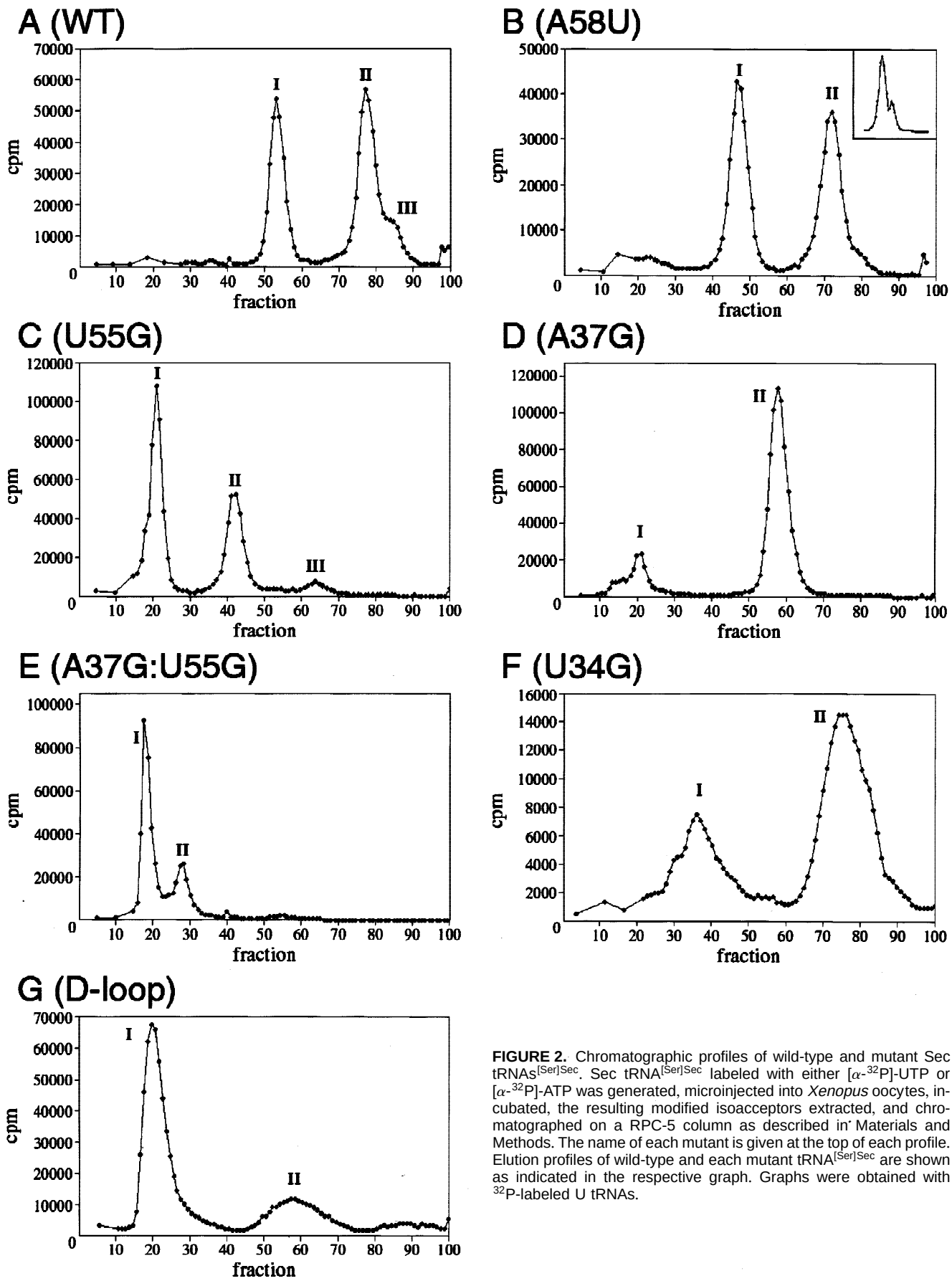

FIGURE 2. Chromatographic profiles of wild-type and mutant Sec tRNAs[Ser]Sec. Sec tRNA[Ser]Sec labeled with either [α-^{32}P]-UTP or [α-^{32}P]-ATP was generated, microinjected into *Xenopus* oocytes, incubated, the resulting modified isoacceptors extracted, and chromatographed on a RPC-5 column as described in Materials and Methods. The name of each mutant is given at the top of each profile. Elution profiles of wild-type and each mutant tRNA[Ser]Sec are shown as indicated in the respective graph. Graphs were obtained with ^{32}P-labeled U tRNAs.

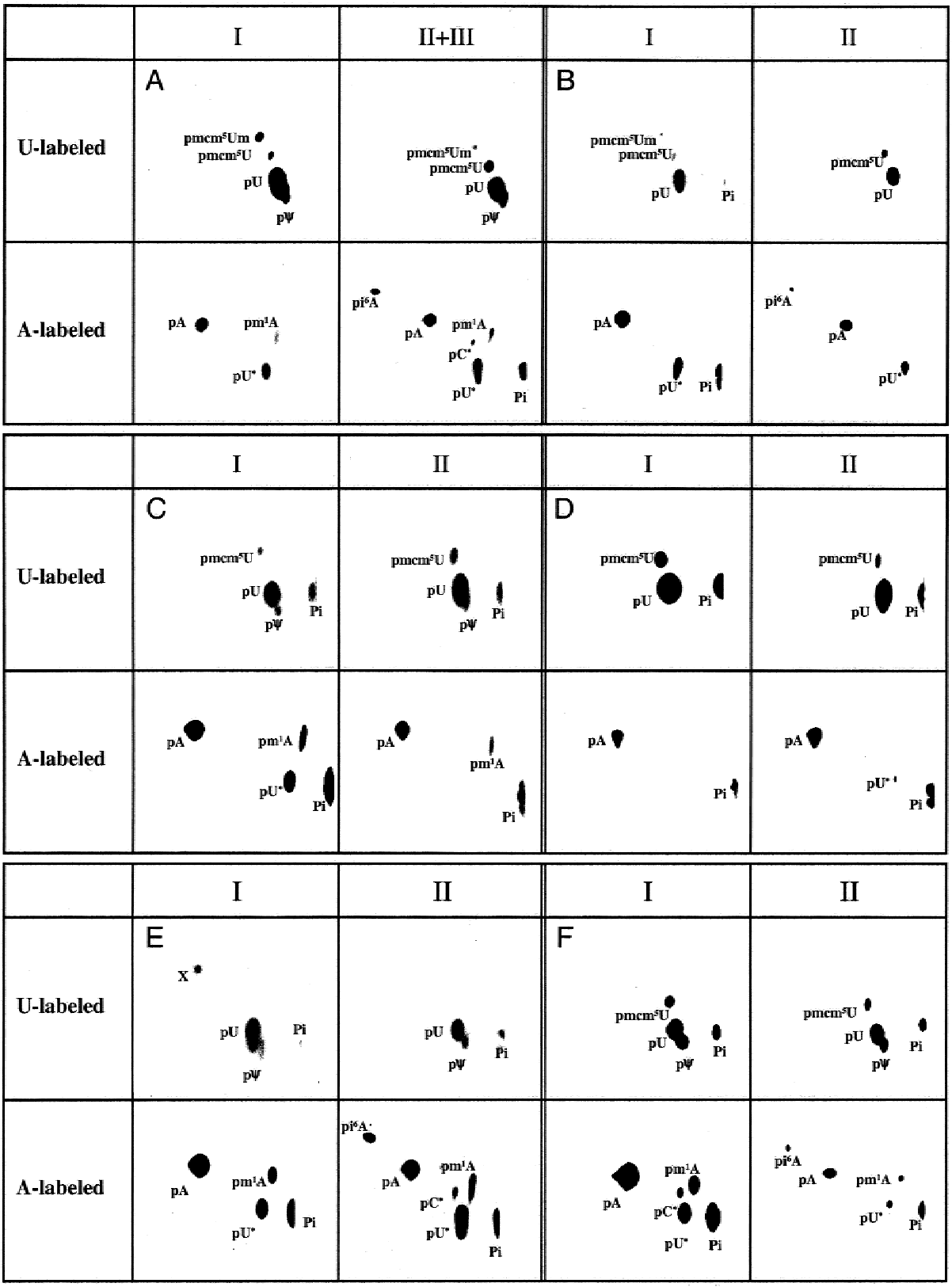

FIGURE 3. Identification of modified nucleotides in isoacceptors of wild-type and mutant Sec tRNAs$^{[Ser]Sec}$. Individual peaks of wild-type and mutant tRNAs that were resolved by RPC-5 chromatography (see Fig. 2) were isolated, digested with nuclease, and individual nucleotides resolved by two-dimensional TLC as described in Materials and Methods. Numbers on the top of each column designate the peak number isolated by RPC-5 chromatography (see Fig. 2). **A**: wild-type; **B**: A58U; **C**: A37G; **D**: double (A37G:U55G); **E**: U34G; and **F**: D-loop mutants. Cryptic U and cryptic C (see text) are designated as pU* and pC*, respectively. In **C**, the unidentified spot on the A-labeled U34G mutant peak I is marked as X. Pi that appears in some of the graphs is inorganic phosphate generated in the digests.

TABLE 2. Summary of modified nucleotides in wild-type and mutant Sec tRNAs[Ser]Sec [a].

	U-Labeled			A-Labeled	
	$_{p}$mcm^{5}U	$_{p}$mcm^{5}Um	$_{p}\psi$	$_{p}$m^{1}A	$_{p}$i^{6}A
Sec tRNA I	+ [b]	+	+	+	− [c]
Sec tRNA II	+	+	+	+	−
A58U I	+	+	−	−	−
A58U II	+	± [d]	−	−	+
U55G I	+	−	−	+	−
U55G II	+	−	−	+	+
U55G III	+	−	−	−	+
A37G I	+	−	+	+	−
A37G II	+	−	+	+	−
A37G:U55G I	+	−	−	−	−
A37G:U55G II	+	−	−	−	−
U34G I	−	−	+	+	−
U34G II	−	−	+	+	+
D Loop I	+	−	+	+	−
D Loop II	+	−	+	+	+

[a] Nucleotides generated from nuclease digests of individual peaks that had been labeled with [α-^{32}P]-UTP or [α-^{32}P]-ATP (see Fig. 2) were identified as given in Materials and Methods and the legend of Figure 2.

[b] +: the amount of each modified nucleotide detected is about one molecule in each tRNA and the relative amount of each modified nucleotide was calculated based on the percentage of pU or pA.

[c] −: not detected.

[d] Although pmcmUm was not detected in this sample after overnight incubation in *Xenopus* oocytes (see Figs. 2B and 3B), its formation could be detected by reinjection of Peak II for an additional overnight incubation (see inset to Fig. 2B and text), suggesting that formation of mcmUm in this mutant was retarded.

corresponding ^{32}P-labeled A tRNAs. The results are discussed below.

Wild-type Sec tRNA[Ser]Sec

The elution profile of wild-type tRNA[Ser]Sec from the RPC-5 column consisted of two major peaks and a trailing shoulder, designated Peaks I–III, respectively, as shown in Figure 2A. Because it is known that Peak II contains mcmU and Peak III contains mcmUm (Choi et al., 1994), these two peaks were pooled from this chromatographic run and digested with nuclease. Nuclease digests of Peak I and Peaks II–III were analyzed by two-dimensional TLC (see Fig. 3A). The various spots on the chromatograms were identified (Choi et al., 1994) and the data are summarized in Table 2. Interestingly, the initial eluting peak, Peak I, contained all modified bases, mcmU, mcmUm, ψ, and m^{1}A, with the exception of i^{6}A. Peaks II–III contained all nucleoside modifications, but, as expected, the relative intensity of the new spot containing mcmUm was much less than that of mcmU (see Fig. 3). The fact that Peak II elutes later from the column than Peak I, and thus is more hydrophobic, is likely due to the presence of i^{6}A, whereas the methyl group on the ribose in Peak III likely accounts for it being more hydrophobic than Peak II (Choi et al., 1994).

When tRNAs were labeled with [α-^{32}P]-ATP, a spot with similar intensity as pA that migrated at the position of pU was detected (see Fig. 3). It is not clear how this spot arises in ATP-labeled experiments, but its presence has been observed previously in similar *Xenopus* oocytes studies and is not due to contamination by [α-^{32}P]-UTP (Choi et al., 1994). The cryptic pU spot occurred in all mutants examined in this study with the exception of Peak II from A37G and Peak I from A37G:U55G (see Fig. 3C and D, respectively, and below).

m^{1}A mutant (A58U)

Following the incubation period of A58U in *Xenopus* oocytes, the resulting tRNA[Ser]Sec species were chromatographed on the RPC-5 column (see Fig. 2B). The elution profile of this tRNA mutant was quite similar to that of wild-type tRNA[Ser]Sec (Fig. 2, compare A and B). Peak I contained mcmU and mcmUm, but no ψ (see Fig. 3B and Table 2). Peak II of U58G contained mcmU and i^{6}A, but no detectable mcmUm (see Fig. 3B and Table 2). There was a very slight late eluting shoulder on Peak II that we suspected might be the mcmUm-containing isoacceptor (i.e., Peak III). This trailing shoulder was present in amounts too low to detect mcmUm and was present in much lower amounts than the corresponding Peak III formed in wild-type tRNA[Ser]Sec (Fig. 2, compare A and B). We, therefore, reinjected the U58G mutant Peak II into oocytes and, after overnight incubation, rechromatographed this material. Peak III was synthesized as shown in the inset in Figure 2B and this peak contained mcmUm and i^{6}A (data not shown). The fact that formation of Peak III was slower in this mutant compared to the wild type also suggested that the mutation at position 58 retarded methylation at position 34.

ψ mutant (U55G)

Column chromatography of U55G following its incubation in oocytes yielded three peaks (Fig. 2C). Peak I eluted much earlier from the column than the corresponding Peak I in the wild-type profile. The alteration in chromatographic properties of U55G Peak I is likely due to disruption of the tertiary structure of tRNA[Ser]Sec. This peak contained mcmU and m^{1}A (Table 2). Peak II contained i^{6}A in addition to mcmU and m^{1}A, and the greater hydrophobicity of this peak is likely due to the presence of i^{6}A. U55G Peak II elutes earlier from the column than the corresponding wild-type Peak II, suggesting that its tertiary structure is likely disrupted. Peak III, interestingly, lacked m^{1}A, but contained mcmU and i^{6}A (Table 2). It is more hydrophobic than Peaks I and II, which is likely due to the lack of m^{1}A. It is not clear how the absence of this modification can make this species more hydrophobic.

i^{6}A mutant (A37G)

A37G generated two peaks by column chromatography (Fig. 2D). The earlier eluting, minor peak also contained a front-running shoulder. Both early eluting components were pooled, analyzed, and found to contain mcmU, ψ, and m^{1}A (Fig. 3C and Table 2). Peak II contained the same base modifications as Peak I and neither peak contained mcmUm. Thus, a mutation at the i^{6}A position does not affect mcmU, ψ, or m^{1}A formation. Although the tertiary structure of A37G is not distorted (even though the presence of i^{6}A renders tRNA$^{[Ser]Sec}$ more hydrophobic), this mutant does not form mcmUm. However, i^{6}A formation is not a prerequisite for ribose methylation, as the wild-type species appears to form mcmUm without prior i^{6}A synthesis (see Peak I in Fig. 2A and Table 2). It would seem, therefore, that the presence of at least an A in position 37 is required for methylation at position 34.

The cryptic pU that was observed in most other tRNAs labeled with [α-^{32}P]-ATP was not detected in Peak II (Fig. 3C and Table 2). Because Peak I does contain the cryptic pU spot, then A at position 37 is not essential for its formation. However, there is a correlation with the occurrence of this cryptic base and an A at position 37 (see A37G:U55G mutant below). At present, the identity of this spot is not known and it is possible that it may be a derivative of A.

ψ/i^{6}A double mutant (A37G:U55G)

A37G:U55G produced two peaks by column chromatography (Fig. 2E). Peak I has chromatographic properties similar to the corresponding peaks in U55G (Fig. 2, compare C and E). The relative amount of Peak III in U55G was quite small compared to Peaks I and II, whereas this peak was not observed in the double mutant. A37G:U55G Peak II elutes earlier from the column than U55G Peak II. The double mutant did not form mcmUm or m^{1}A (see Fig. 3D and Table 2). Both U55G and A37G, however, formed m^{1}A (see above) and thus it is not clear why the double mutant affected base modification at position 58. Peak I did not contain the cryptic pU spot and the amount of this spot in Peak II relative to pA was much less than in the wild type and other mutants (Table 2). This observation also demonstrates a correlation in mutation at position 37 and the formation of the cryptic pU.

Wobble position mutant (U34G)

The wobble position of the anticodon, position 34, is where mcmU and mcmUm occur. U34G produced two major peaks by column chromatography (see Fig. 2F). Peak I contained ψ and m^{1}A and Peak II contained ψ, m^{1}A, and i^{6}A (Fig. 3E and Table 2). Thus, mutation at position 34 did not affect formation of the other modified bases. Peak I contained, in addition, an unidentified spot (designated X) that migrated near that of i^{6}A when this mutant was labeled with [α-^{32}P]-UTP (see Fig. 3E).

D-loop mutant (U16A:G17C:G18C:U19A)

The D-loop is known to have an important role in tRNA tertiary structure by interacting with the TψC-arm (Kim et al., 1974). Thus, U16A:G17C:G18C:U19A was introduced, as it clearly would affect tertiary structure, and the effect of tRNA$^{[Ser]Sec}$ architecture on modified nucleoside formation could readily be assessed. This mutant produced two peaks by column chromatography (Fig. 2G). Peak I elutes early from the column indicating that the tertiary structure is affected by this mutant. This peak contains mcmU, ψ, and m^{1}A, but not mcmUm (Fig. 3F and Table 2). In addition, this mutant contained the cryptic pU spot and an unidentified spot that migrates like pC (see Fig. 3F) that were observed above in wild-type Sec tRNA$^{[Ser]Sec}$. Peak II also contains each modified base observed in Peak I and, in addition, contained i^{6}A.

DISCUSSION

As noted in the Introduction, the intracellular level of the mcmUm isoacceptor may increase several-fold more than the unmethylated form in response to selenium in different cells (Hatfield et al., 1991; Choi et al., 1994) or specific tissues (Diamond et al., 1993; Chittum et al., 1997). Furthermore, the tertiary structure of Sec tRNA$^{[Ser]Sec}$ is altered dramatically following 2′-*O*-ribose methylation at position 34 (Diamond et al., 1993). In the present study, we observed that efficient 2′-*O*-ribose methylation of tRNA$^{[Ser]Sec}$ requires both the naturally occurring primary and tertiary structures. Formation of the other two modifications in the anticodon loop of tRNA$^{[Ser]Sec}$, i^{6}A and mcmU, do not have such stringent requirements. It is, therefore, tempting to speculate that the mcmUm isoacceptor might be preferentially or selectively used in selenoprotein synthesis (Jung et al., 1994). The large increase in the amount of this species in response to selenium would suggest it plays a specific role, as this element also increases the level of selenoprotein synthesis (Bermano et al., 1995; Diamond et al., 1996; Berggren et al., 1997; Yeh et al., 1997). It is of interest to note that Berry and collaborators have reported that the stem-loop structure(s) located in the 3′-untranslated region of selenoprotein mRNAs, designated the SECIS (Sec insertion sequence) element, which is (are) responsible for dictating UGA as a Sec codon, fall into two classes (Low & Berry, 1996; Grundner-Culemann et al., 1999). Another tempting speculation is that the two classes of SECIS elements may complement the two Sec isoacceptors where one isoacceptor form and one SECIS element

form are utilized in concert for insertion of Sec into specific nascent selenopolypeptides, whereas the other two components are utilized together in the biosynthesis of other selenoproteins. This would of course mean that there are also two classes of UGA Sec codons. In any case, it is important to elucidate the formation of these two isoacceptors and in particular the mcmUm isoacceptor.

Both m^1A and ψ formation at positions 58 and 55, respectively, occur in $tRNA^{Tyr}$ at an early stage in *Xenopus* oocytes, as they are formed in the nucleus prior to the time the primary transcript is fully processed (Melton et al., 1980). m^1A formation is an efficient process in *Xenopus* oocytes (Morin et al., 1998). It has previously been shown that these two modifications also occur early in $tRNA^{[Ser]Sec}$ maturation within the nucleus of *Xenopus* oocytes (Choi et al., 1994; Sturchler et al., 1994). In the present study, the ψ mutant, U55G, did not form mcmUm, but did form the base modifications at positions 34, 37, and 58. The chromatographic properties of this mutant were altered dramatically, providing further evidence that the base in this position plays an important role in tertiary structure (Kim et al., 1974; Arnez & Steitz, 1994). The m^1A mutant, A58U, did not form ψ at position 55 and thus an A is required in position 58 for ψ synthesis. mcmUm was formed in A58U, albeit at a much slower rate than in wild-type $tRNA^{[Ser]Sec}$. As the chromatographic properties of this mutant were similar to those of wild-type $tRNA^{[Ser]Sec}$, we conclude that the tertiary structure of this mutant is not affected by the absence of an A at position 58. We can also conclude that a U at position 55 is sufficient to maintain the tertiary structure of this tRNA, as ψ was not formed in A58U.

We also observed in the present study the occurrence of cryptic pU and pC when the tRNA was labeled with [α-^{32}P]-ATP, as was previously found in another study (Choi et al., 1994). These labeled nucleotides are not contaminants of UTP and CTP, as they were not generated in the A37G mutant and as was previously reported by Choi et al. (1994). They might be an intermediate in the biosynthesis of a modified base, a degradation product of a modified base, or possibly the result of editing (see Hatfield et al., 1999 for review). However, their presence in high levels in some tRNA digests would suggest that these possibilities are unlikely. Although the origin of these nucleotides are of considerable interest, their identity must await a future study.

The formation of the 2′-*O*-methylribose at position 34 was quite sensitive to all base mutations examined in this study, whereas mcmU at this position was fairly insensitive. The data suggest that this methylation step requires the prior formation of each modified base and, in addition, the overall tertiary structure is important in its synthesis. Furthermore, the data provide strong evidence that this step occurs late in the maturation process. Grosjean and collaborators (Droogmans et al., 1986) have examined the 2′-*O*-ribose methylation at position 34 in $tRNA^{Phe}$ in *Xenopus* oocytes and have observed that guanosine, cytosine, or uridine in this position was efficiently methylated. These investigators were not sure if the methylation occurred as the result of a single enzyme or more than one, but it did occur in the cytoplasm, indicating that it was a late reaction. Whether the enzyme that methylates the ribosyl moiety at position 34 in $tRNA^{[Ser]Sec}$ is the same protein (or one of the proteins) as that examined by Grosjean and collaborators remains to be established. However, it is clear from the studies described herein, as well as those demonstrating a role of selenium in enriching the levels of this isoacceptor (Hatfield et al., 1991; Diamond et al., 1993; Choi et al., 1994; Chittum et al., 1997) and as a result of the presence of the methyl group having a pronounced effect on tertiary structure (Diamond et al., 1993), that the mcmUm form must play some unique role in selenoprotein biosynthesis that is different than that of the mcmU form.

MATERIALS AND METHODS

Materials

Xenopus laevis females were purchased from Xenopus I, restriction enzymes, Klenow enzyme, and T7 RNA polymerase from New England Biolabs, plasmid pSP65, transcription reagents, human placental RNase inhibitor, and RQ1 DNase from Promega, oligonucleotides, nucleases P1, [α-^{32}P]-ATP, and [α-^{32}P]-UTP (specific activities ~3,000 Ci/mmol), L-[^{3}H]serine (33 Ci/mmol), and Protein A-Sepharose CL4B from Amersham Pharmacia Biotech, [α-^{32}P]-CTP (~3,000 Ci/mmol) from NEN, Taq polymerase from Takara, and Cellulose TLC plates (nonfluorescent) from Sigma. Adogen and Plaskons used in tRNA column chromatography were kindly provided by K. Bruce Jacobson.

Plasmid construction

Human genes for $tRNA^{[Ser]Sec}$ and $tRNA_{IGA}^{Ser}$ (Ohama et al., 1994) were cloned in pSP65 (Promega) at the *Pst*I and *Eco*RI sites. Mutations were introduced to the $tRNA^{[Ser]Sec}$ gene with the overlapping PCR method described by Higuchi et al. (1988). For preparation of the A37G:U55G double mutant, the A37G mutation was introduced to the U55G mutant. The primers used are shown in Table 3. The constructs were verified by Dye Terminator sequencing with an ABI PRISM 373 sequencer.

In vitro transcription

Each tRNA gene was amplified by PCR with an upstream primer containing the T7 promoter (SECT7FP: ggctgcag taatacgactcact) and one of the downstream primers (SERRPL: tggcgtagtcggcaggatt for $tRNA_{IGA}^{Ser}$; SECRPS: tggcgccc gaaaggtggaa for $tRNA^{[Ser]Sec}$) on the pSP65 plasmid containing wild-type human $tRNA_{IGA}^{Ser}$ (Ohama et al., 1994), human $tRNA^{[Ser]Sec}$, or its mutants. The PCR products were

TABLE 3. Primers used for mutant construction.

Outside primers (on pSP65)		PSPSQ1: tgagcggataacaatttcac AZSP6S: gatttaggtgacactataga
Mutagenesis primers	For A58U	TM031: tagcgacagagtggttcatttccacctttcgg TM032: ccgaaaggtggaaatgaaccactctgtcgcta
	For U55G	TM059: tagcgacagagtggtgcaattccacctttcgg TM060: ccgaaaggtggaattgcaccactctgtcgcta
	For A37G	TM065: ggtctggggtgcaggcttcagacctgtagctgtcta TM066: tagacagctacaggtctgaagcctgcaccccagacc
	For U34G	TM063: ggtctggggtgcaggctgcaaacctgtagctgtcta TM064: tagacagctacaggtttgcagcctgcaccccagacc
	For U16A:G17C:G18C:U19A	TM015: atgatcctcagaccactggggtgcaggctt TM016: aagcctgcaccccagtggtctgaggatcat

extracted with phenol/chloroform/isoamyl alcohol (25:24:1, PCI) and precipitated with ethanol. The products were blunt-ended with Klenow enzyme in the presence of deoxynucleoside triphosphates, extracted with PCI, precipitated with ethanol, and used as transcription templates. Transcription of the tRNA genes was carried out in a 25-μL mixture containing 0.2 μg of template, 15 U of T7 RNA polymerase (Promega), 40 U of human placental RNase inhibitor (Promega), 10 μCi of [α-^{33}P]-CTP (NEN), 0.4 mM each of ribonucleoside triphosphates, 10 mM dithiothreitol, 6 mM $MgCl_2$, 2 mM spermidine, and 40 mM Tris-HCl, pH 7.9. For the preparation of nonlabeled transcripts, [α-^{33}P]-CTP was omitted. The mixtures were incubated at 37 °C for 1 h, then DNase I (RNase-free, 1 U, Promega) was added, and the mixtures incubated for a further 30 min. The transcripts were loaded on a 10% polyacrylamide gel containing 8 M urea. The gel was run at a constant 300 V for 2 h, and then autoradiographed. The band corresponding to the full-length tRNA was excised and RNAs eluted using the elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% SDS) (Sambrook et al., 1989). Gel slices were incubated for 8 h at 37 °C and the eluents were extracted with phenol and precipitated with ethanol.

Immunoprecipitation

Identification and characterization of human sera containing anti-tRNA$^{[Ser]Sec}$ autoantibodies will be reported elsewhere (T. Matsufuji, D. Hatfield, T. Ohama, J.F. Atkins, R.F. Gesteland, & S. Matsufuji, in prep.). ^{33}P-labeled transcript (10 pmol) was mixed with 50 μg/mL yeast tRNA (Sigma), 1 mg/mL BSA, 1 mM DTT and 1 U/μL RNase inhibitor in 1× NET buffer (50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 150 mM M NaCl, 0.05% Nonidet P-40) in a total volume of 0.2 mL. Human serum (10 μL) was added to the mixture and incubated at room temperature for 1 h by mixing with a rotary mixer. Protein A-Sepharose CL4B (80 μL of 50% [v/v] suspension in 1× NET containing 1 mg/mL BSA) was then added to the mixture and mixing was continued for an additional 15 min. Protein A-Sepharose was collected by centrifugation (8,000 rpm for 2 min) and washed three times with 0.5 mL of 1× NET. Yeast tRNA (20 μg) was added to the pellet and the total volume was increased to 0.4 mL with TE buffer (pH 7.6). The mixture was extracted with PCI, the aqueous phase was collected, and RNA precipitated with ethanol. The pellet was dissolved in 10 μL of TE buffer (pH 7.6), mixed with 15 μL of denaturation buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue), warmed at 37 °C for 10 min, and subjected to polyacrylamide gel electrophoresis on an 8% acrylamide-8 M urea gel [130 mm (W) × 120 mm (H) × 0.75 mm] in Tris-borate buffer. After electrophoresis, the gel was fixed with 10% acetic acid for 30 min and vacuum dried. The tRNA bands were visualized and quantitated with a FLA2000 image analyzer (Fuji).

Aminoacylation of Sec tRNA$^{[Ser]Sec}$

Serylation reaction was carried out in a 25-μL mixture containing 10 pmol of T7-transcribed tRNA, 1.5 μL of aminoacyl-tRNA synthetase partially purified from rabbit reticulocytes, 2.5 μCi of L-[^{3}H]-serine, 2 mM each of the other 19 amino acids, 50 mM Tris-HCl, pH 7.4, 6 mM ATP, and 20 mM $MgCl_2$ at 37 °C for 20 min. After incubation the mixture was diluted with 0.5 mL of water and then 8 mL of 5% TCA. The precipitate was collected on a GF-C filter (Whatman) set on a filtration funnel with a vacuum pump. The filter was washed with 5 mL of 5% TCA twice and 10 mL of ethanol, transferred to scintillation fluid in a vial, and counted with a scintillation counter. The assay was performed in duplicate. The number of counts obtained with each mutant was compared to that of wild-type Sec tRNA$^{[Ser]Sec}$ after the blank value was subtracted.

Microinjection into *Xenopus* oocytes and RPC-5 column chromatography

Oocytes were obtained from mature *Xenopus* females. Stage VI oocytes were selected and maintained in OR-2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM HEPES, pH 7.8) at room temperature. Forty nanoliters of the mixture containing 2–3 × 10^5 cpm of ^{32}P-labeled tRNAs (prepared as described above) were injected into the cytoplasm of oocytes and the oocytes incubated at 20 °C for 16 h.

After incubation, tRNAs were extracted as described (Tobian et al., 1985; Lee et al., 1987), loaded on a RPC-5 column (Kelmers & Heatherly, 1971), and the column developed in a 0.525–0.70 M NaCl linear gradient as described (Diamond et al., 1993; Choi et al., 1994). Column fractions were counted in a liquid scintillation counter (Pharmacia), the peaks representing isoacceptors pooled, precipitated in ethanol, collected, and prepared for nuclease digestion as described (Choi et al., 1994).

Minor-base analysis

Samples isolated from RPC-5 columns (see above) were completely digested with nuclease P1 by incubating in 50 μL containing 50 mM ammonium acetate, pH 5.3, at 37 °C for 16 h. Three microliters of each digest were applied to a cellulose TLC plate (20 × 20 cm) and the resulting 5′-monophosphate nucleosides resolved by two-dimensional chromatography for 8 h in each direction using solvent A and C of Silberklang et al. (1979). Autoradiographs were prepared from developed plates by exposing for about 12 h.

Modified bases were identified by cochromatographing the standards, pA, pC, pU, and pG (Sigma). Standards were detected by UV irradiation and the relative mobility rates (R*f*) of each modified base were calculated and compared with those of known modified bases (Silberklang et al., 1979; Diamond et al., 1993; Choi et al., 1994).

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