ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 315, No. 2, December, pp. 293–301, 1994

Selenocysteine tRNA and Serine tRNA Are Aminoacylated by the Same Synthetase, but May Manifest Different Identities with Respect to the Long Extra Arm

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Received June 30, 1994, and in revised form August 15, 1994

Selenocysteine (Sec) tRNA$^{[Ser]Sec}$ donates Sec to protein, but interestingly, this amino acid is synthesized on tRNA which is first aminoacylated with serine. Thus, the identity elements in tRNA$^{[Ser]Sec}$ for aminoacylation correspond to elements for seryl-tRNA synthetase recognition. As tRNA$^{[Ser]Sec}$ has low homology to the tRNA^{Ser} isoacceptors, it would seem then that the identity elements in tRNA$^{[Ser]Sec}$ involve (1) very specific sequences, (2) conformational features, and/or (3) different points or domains for tRNA$^{[Ser]Sec}$:synthetase and tRNA^{Ser}:synthetase recognition. Initially, we confirmed that the same synthetase aminoacylates both tRNAs by showing that a mutant tRNA$^{[Ser]Sec}$ which has a blocked 3′-terminus is a competitive inhibitor of tRNA^{Ser} aminoacylation with a partially purified and a highly purified seryl-tRNA synthetase preparation. The discriminator base (base G73) is essential for aminoacylation of tRNA$^{[Ser]Sec}$ and tRNA^{Ser}, while the long extra arm plays an important role which seems to be orientation- and length-specific in tRNA^{Ser} and, in addition, may manifest sequence specificity in tRNA$^{[Ser]Sec}$. This difference in the tRNA recognition specificity is discussed. The acceptor stem, DHU stem, and TψC stem contribute to the recogntion process, but to a lesser extent than the discriminator base and the long extra arm. © 1994 Academic Press, Inc.

Key Words: **selenocysteine; serine; aminoacylation; discriminator base; mutant tRNAs; RNA base mutations.**

The flow of information through the genetic code requires two precise steps to insure the fidelity of expression. The first step requires that an amino acid be attached to its cognate tRNA, which is catalyzed by the appropriate aminoacyl-tRNA synthetase to form aminoacyl-tRNA. The second step requires that an aminoacyl-tRNA anticodon recognize the corresponding codeword(s) for insertion of the correct amino acid into the growing polypeptide chain. The precise accuracy of the first step depends on the information encoded in the synthetase for recognition of the corresponding information (designated identity elements) within tRNA. Since the attachment of the correct amino acid to tRNA is such a crucial step in translating the genetic code, the identity of tRNA by its cognate synthetase has received considerable attention in the last few years (see 1–9 for review). The discriminator base which is located at position 73 (immediately 5′ to the CCA terminus), the anticodon, bases located within the helical stems, tertiary structure, the long extra arm (characteristic of class II type tRNAs), and modified nucleotides have all been shown to play a role in the identity process in different tRNAs (see 1–9 for review).

The identity elements within selenocysteine (Sec)[2] tRNA$^{[Ser]Sec}$ for seryl-tRNA synthetase are of considerable interest for several reasons. For example, this tRNA is only one of two known tRNAs that is aminoacylated (with serine) and the amino acid is then altered on tRNA (to Sec) before its insertion into protein (see 10, 11 for glutamine synthesized on tRNA). Thus, the identity elements that reside in tRNA$^{[Ser]Sec}$ for its aminoacylation correspond to those for serine and not to those for Sec. In addition, there is little sequence homology between

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[2] Abbreviations used: Sec, selenocysteine; tRNA$^{[Ser]Sec}$, selenocysteine tRNA first aminoacylated with serine; DHU stem, dihydrouracil or D stem in tRNA; TψC stem, thymidine, pseudouridine, and cytidine or T stem in tRNA; TCA, trichloroacetic acid; DTT, dithiothreitol.

0003-9861/94 $6.00

$tRNA^{[Ser]Sec}$ and the canonical serine isoacceptors (see 12 for review and references therein). Thus, the identity elements in these tRNAs must involve: (1) very sequence-specific sites, (2) conformational features (e.g., tertiary structure), and/or (3) different sites or regions in $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ for synthetase interaction. One unusual feature of $tRNA^{[Ser]Sec}$ is that it belongs to class II tRNAs which are characterized by having a long extra arm. The only members in this class are $tRNA^{[Ser]Sec}$, $tRNA^{Ser}$, $tRNA^{Leu}$, and prokaryotic $tRNA^{Tyr}$. In light of the fact that the long extra arm has been shown to be a critical determinant in *Escherichia coli* $tRNA^{Ser}$ for synthetase recognition (13–18) and has been implicated in yeast $tRNA^{Ser}$ (18), it might be anticipated that this domain of mammalian $tRNA^{Ser}$ and $tRNA^{[Ser]Sec}$ may be critical for synthetase recognition. Indeed, the long extra arm in human $tRNA^{Ser}$ has been shown to be a critical element in seryl-tRNA synthetase recognition (19).

An additional reason that the identity elements in $tRNA^{[Ser]Sec}$ are of considerable interest to us is that we have had a long-standing interest in many different parameters of the biology of $tRNA^{[Ser]Sec}$. These include its coding properties, its role in protein synthesis, its structure, its gene and the chromosomal localization of its gene, its transcription and the biosynthesis of its modified nucleotides, its distribution in nature, and the effects of selenium on the $tRNA^{[Ser]Sec}$ population (reviewed recently in 12; also see 20, 21 for additional studies). As an extension of these studies and due to the interesting features that must govern $tRNA^{[Ser]Sec}$ identity, we undertook a study of the identity elements in this tRNA. In the course of this work, a study on the identity elements in $tRNA^{[Ser]Sec}$ was published by Wu and Gross (22). The long extra stem–loop and the discriminator base were reported to be important elements in $tRNA^{[Ser]Sec}$:seryl-tRNA synthetase interaction (22). Our approach, however, is different than the recently published work as our emphasis has focused more toward the contribution of nucleotide positions that are common to both $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ in the identity process. Further, contrary to the previous work on the identity of $tRNA^{[Ser]Sec}$ (22), we find that its long extra arm appears to manifest sequence and length specificity, as well as orientation specificity.

MATERIALS AND METHODS

Materials. L-[^{3}H]Serine (sp act 29 Ci/mmol) and γ-[^{32}P]ATP (sp act >5000 Ci/mmol) were purchased from Amersham, dsDNA cycle sequencing system and the large fragment of DNA polymerase I from Gibco BRL, Muta-Gene M13 *in vitro* mutagenesis kit from Bio-Rad, T7 RNA polymerase (50,000 U/ml) and *Nsi*I from BioLabs, RNasin from Promega, GeneAmp PCR core reagents from Perkin-Elmer Cetus, and *E. coli* $tRNA^{Tyr}$ from Boehringer Mannheim.

Methods. Seryl-tRNA synthetase was purified extensively from rabbit reticulocytes as follows: Rabbit reticulocytes were obtained from anemic rabbits by cardiac puncture and lysed (23), and the aminoacyl-tRNA synthetases were prepared according to Muench and Berg (24). Seryl-tRNA synthetase was then purified from the unfractionated synthetases by a three-step purification procedure according to Fahoum (25). Unfractionated synthetases were loaded onto a column of DEAE-Sephadex and eluted with a linear gradient of KCl. The active fractions were loaded directly onto a hydroxyapatite column and seryl-tRNA synthetase eluted with a linear gradient of potassium phosphate. Pooled active fractions were then dialyzed and loaded onto a column of *E. coli* tRNA-Sepharose, and seryl-tRNA synthetase was eluted with a linear gradient of KCl. Purified seryl-tRNA synthetase showed a single protein band with a subunit molecular weight of 60,000 as analyzed by SDS-polyacrylamide gel electrophoresis. This is in agreement with that obtained by Dang and Traugh (26) and Miseta *et al.* (27). Purified seryl-tRNA synthetase had a specific activity of 200 nmol mg^{-1} min^{-1} under standard assay conditions of serylation of tRNA.

Synthesized DNA fragments (constructs are shown in Fig. 1) containing the promoter for T7 RNA polymerase, the human tRNA gene sequence ($tRNA^{[Ser]Sec}$ or $tRNA^{Ser}$), and *Pst*I, *Nsi*I, and *Eco*RI restriction sites were cloned into the *Pst*I–*Eco*RI mutiple cloning site of M13mp19. Purified M13 ssDNAs encoding this fragment were used as template DNA for mutagenesis. Mutagenesis was carried out following the method of Kunkel (28) using the Muta-Gene M13 *in vitro* mutagenesis kit. After mutagenesis, the sequence of each mutated clone (including sequences spanning the T7 promoter and *Nsi*I restriction site) were confirmed using the cycle sequencing system. After sequencing, the wild-type and mutant clones were amplified by PCR, the amplified products were digested with *Nsi*I and then blunt-ended with Klenow fragment, and the resulting product was used as template to generate transcripts with T7 RNA polymerase. The tRNA transcripts were purified by electrophoresis on a 12% polyacrylamide–7M urea gel using purified $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ from rat liver (29) as markers. Bands were detected with ethidium bromide, cut out, and eluted from the gel strip with 200 mM NaCl for 24 h at 4°C. Traces of polyacrylamide were removed by repeated phenol–chloroform extractions and ethanol precipitations and ethidium bromide by repeated treatment with 1-butanol until the A_{260}/A_{280} ratio reached 1.9–2.0. Transcripts used in competition studies were prepared as follows: the $tRNA^{[Ser]Sec}$ gene was digested with *Eco*RI and then transcribed and purified as above. This transcript has an extra 10 nucleotides at the 3′-terminus (---CCAUCGAUGAAUU) and is designated competitor $tRNA^{[Ser]Sec}$.

Aminoacylation of 5 μg of the tRNA gene transcript was carried out in a total volume of 50 μl containing 25 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 2.5 mM ATP, and 10 mM KCl. Aliquots (4.5 μl) were removed at 1-min intervals and spotted onto 1-cm^2 Whatman 3MM filter discs that had been prewashed in 10% trichloroacetic acid (TCA) and dried. Filters were washed 3× in cold 10% TCA for 5 min, rinsed in 99% ethanol, dried, and counted in a liquid scintillation counter. For the serine acceptor activity of wild-type and mutant tRNAs, a limiting concentration of tRNA was used in the presence of an excess amount of unfractionated reticulocyte aminoacyl-tRNA synthetases. For the comparisons of relative rates of serylation of wild-type and mutant tRNA transcripts, a limiting concentration of enzyme was used to ensure the linearity of serylation and thus the initial rates of serylation. In competition studies, the aminoacylation of $tRNA^{Ser}$ in the presence or absence of the competitor $tRNA^{[Ser]Sec}$ or of *E. coli* $tRNA^{Tyr}$ was examined. The appropriate amount of seryl-tRNA synthetase to use in aminoacylation experiments was established by varying the amounts of enzyme over a wide range in reactions carried out in the initial studies.

RESULTS AND DISCUSSION

Human $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ were generated from the corresponding genes cloned into an expression vector (see Materials and Methods). The constructs are shown in Fig. 1. Following purification of the transcripts by polyacrylamide gel electrophoresis, they were serylated with a partially purified or a highly purified preparation of seryl-tRNA synthetase. Mizutani *et al.* (30) have pre-

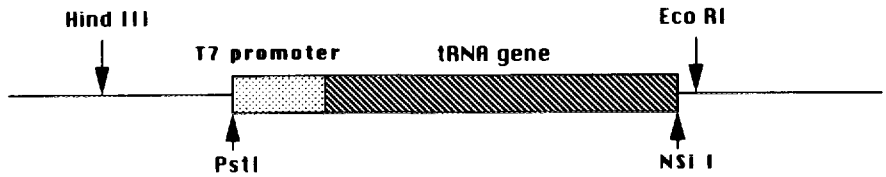

FIG. 1. Construction of the plasmid DNA containing the human $tRNA^{[Ser]Sec}$ or $tRNA^{Ser}$ gene. Synthesized ds DNA fragments were cloned into the *PstI–EcoRI* multiple cloning site of M13mp19. Transcripts generated from fragments digested with *NsiI* produced a 3'-CCA-terminus, while those digested with *EcoRI* contain a 3'-terminus with 10 additional nucleotides.

viously shown that $tRNA^{Ser}$ and $tRNA^{[Ser]Sec}$ are serylated by a highly purified preparation of seryl-tRNA synthetase from bovine liver. These investigators reported that the K_m for the naturally occurring $tRNA^{[Ser]Sec}$ is 1.25 μM and that for the naturally occurring $tRNA^{Ser}$ is 1.40 μM (30). The K_m for the $tRNA^{Ser}$ transcript is 2.50 μM (data not shown) and we did not determine the K_m for the $tRNA^{[Ser]Sec}$ transcript. To establish that both tRNAs are serylated by the same enzyme, we examined the ability of a mutant $tRNA^{[Ser]Sec}$ in which the 3'-terminus is blocked (designated competitor $tRNA^{[Ser]Sec}$) to inhibit the aminoacylation of $tRNA^{Ser}$ with both enzyme preparations (see below).

$tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ are aminoacylated by the same synthetase. The rates of aminoacylation of $tRNA^{Ser}$ in the presence or absence of $tRNA^{Tyr}$ ($tRNA^{Tyr}$ was added as a control tRNA which should not affect $tRNA^{Ser}$ serylation) and in the presence or absence of competitor $tRNA^{[Ser]Sec}$ were examined with the partially purified (Fig. 2A) or highly purified preparation of seryl-tRNA synthetase (Fig. 2B). As expected, $tRNA^{Tyr}$ did not affect the rate of aminoacylation of $tRNA^{Ser}$ (Fig. 2A). An equal amount of competitor $tRNA^{[Ser]Sec}$ as $tRNA^{Ser}$ in reactions inhibited the rate of aminoacylation about 50% with the partially purified enzyme preparation. The observation that the serylation of $tRNA^{Ser}$ was specifically inhibited by the competitor $tRNA^{[Ser]Sec}$ and not by noncognate $tRNA^{Tyr}$, when preparation of unfractionated synthetases was used in serylation, suggests that $tRNA^{[Ser]Sec}$, but not $tRNA^{Tyr}$, was recognized by seryl-tRNA synthetase among all other synthetases. In order to eliminate the possibility that the inhibition was due to the interaction of compet-

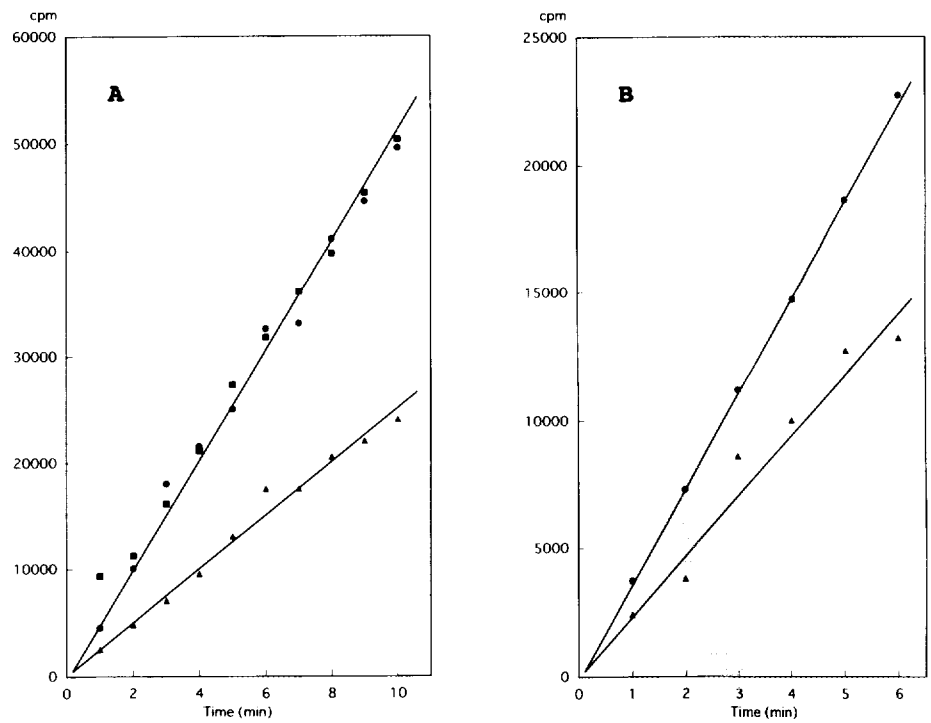

FIG. 2. Inhibition of $tRNA^{Ser}$ aminoacylation with competitor $tRNA^{[Ser]Sec}$. Aminoacylation of $tRNA^{Ser}$ by unfractionated (A) and a highly purified preparation of seryl-tRNA synthetase (B) was examined. Reactions contained 5 μg of $tRNA^{Ser}$ transcript and: ●, no addition; ■, 5 μg of *E. coli* $tRNA^{Tyr}$; ▲, 5 μg of competitor $tRNA^{[Ser]Sec}$.

itor tRNA$^{[Ser]Sec}$ with other protein factors, such as pyrophosphatase, that may result in direct inhibition of seryl-tRNA synthetase, highly purified seryl-tRNA synthetase from reticulocytes was then used. Similar inhibition (about 40%) with the highly purified enzyme preparation was observed. The difference in the levels of inhibition with the two enzyme preparations probably is due to the presence of other factors in the unfractionated synthetases, such as kinase, pyrophosphatase, and/or elongation factors that affect synthetase activities. Nonetheless, competitor tRNA$^{[Ser]Sec}$ evidently competes tRNA^{Ser} serylation in both synthetase preparations. Also, the fact that competitor tRNA$^{[Ser]Sec}$ inhibits serylation of tRNA^{Ser} with both enzyme preparations, and in particular with the highly purified enzyme, demonstrates that these tRNAs are recognized and aminoacylated by seryl-tRNA synthetase.

Identity elements in tRNA$^{[Ser]Sec}$ and tRNA^{Ser}. The structures of tRNA$^{[Ser]Sec}$ and tRNA^{Ser} are shown in their respective cloverleaf forms in Fig. 3. The secondary structure of tRNA$^{[Ser]Sec}$ is taken from that of Böck *et al.* (31) and Sturchler *et al.* (32). It should be noted that other cloverleaf forms of tRNA$^{[Ser]Sec}$ are possible (12). The bases in each tRNA that were changed are shown in bold letters in the figure and the precise, individual changes are listed in Table I. The rates of aminoacylation were examined at 1-min intervals over a 10-min period during which time the rates were linear. The rates of aminoacylation of wild-type and several representative mutant tRNAs are shown in Figs. 4A (tRNA$^{[Ser]Sec}$) and 4B (tRNA^{Ser}). All aminoacylation data with mutant tRNAs were summarized and presented in Table I. The concentration of tRNA used in the assay mixtures was the same for wild-type tRNA as that for all mutant tRNAs. The amount was 0.1 μg/μl (about 3.3 μM), which is about 1.3 to 2.5 times the K_m. The concentration of seryl-tRNA synthetase was sufficiently low such that the time course of aminoacylation remained linear for at least 10 min.

Roles of the long extra arm and loop. As noted in the Introduction the long extra arm is a likely critical domain of tRNA$^{[Ser]Sec}$ identity (see also 22). We, therefore, examined the effects of numerous point mutations within the stem and loop of the extra arm on aminoacylation. The base pairings within the stem are characterized in tRNA$^{[Ser]Sec}$ and tRNA^{Ser} by being rich in G and C. There are four G:C pairings in the stem of tRNA^{Ser}, while there are two C:G and one G:C pairings in the stem of tRNA$^{[Ser]Sec}$ (see Fig. 3). The orientation of at least two of these G and C pairings are not critical to tRNA^{Ser} identity as changing them from G:C to C:G at positions 46 and 47g (Fig. 4B) and 47 and 47f, respectively, does not impair aminoacylation (Table I). Interestingly, changing

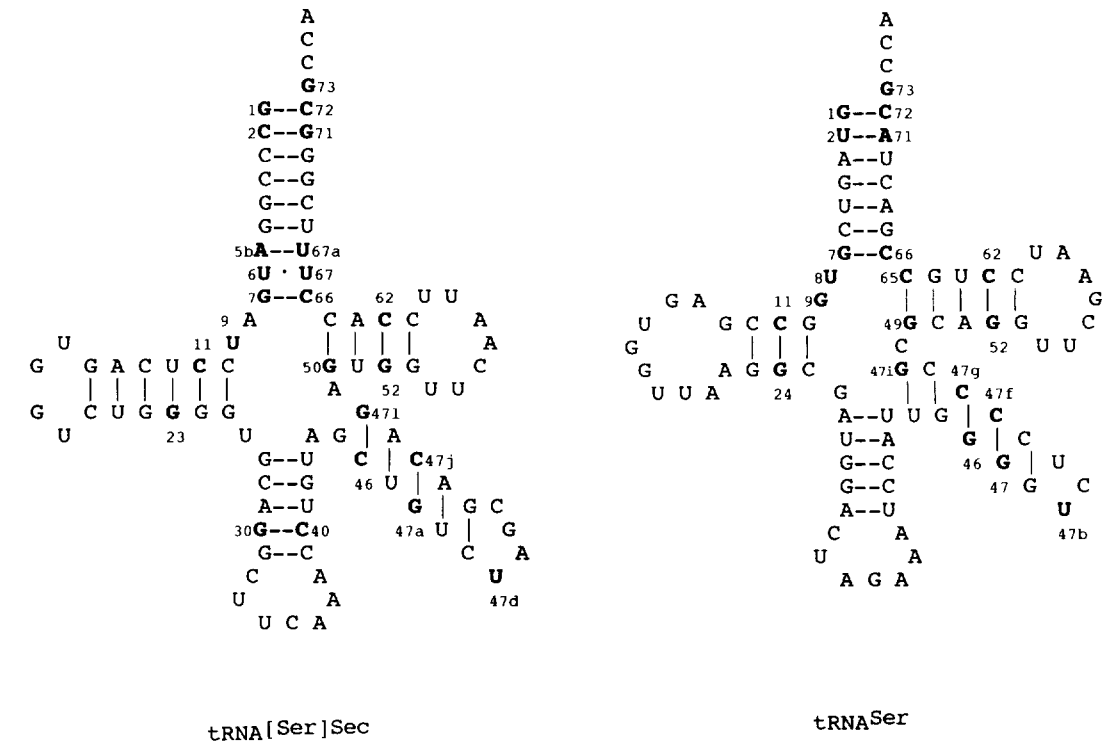

FIG. 3. Cloverleaf models of tRNA$^{[Ser]Sec}$ and tRNA^{Ser}. The bases in tRNA$^{[Ser]Sec}$ are numbered according to Sturchler *et al.* (32) and that in tRNA^{Ser} according to Sprinzl *et al.* (40). Mutated bases are shown in bold and are numbered. The individual mutated base or bases are given in Table 1.

TABLE I

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

Location	tRNA	Mutations	Initial rate of serylation[a] (wild type = 100%)
Extra arm stem	$tRNA^{Sec}$	C46:G47l → G:C	36
	$tRNA^{Ser}$	G46:C47g → C:G	100
	$tRNA^{Sec}$	G47:C47j deletion	7.2
	$tRNA^{Ser}$	G46:C47g deletion	3.9
	$tRNA^{Sec}$	G47a:C47j → C:G	51
	$tRNA^{Ser}$	G47:C47f → C:G	100
	$tRNA^{Ser}$	G47i → C	82
Extra loop	$tRNA^{Ser}$	U47b → C	111
	$tRNA^{Ser}$	U47b → G	100
	$tRNA^{Sec}$	U47d → G	36
	$tRNA^{Sec}$	U47d deletion	100
Discriminator base	$tRNA^{Sec}$	G73 → A	0.9
	$tRNA^{Ser}$	G73 → A	1.6
Acceptor stem	$tRNA^{Sec}$	G1:C72 → A:U	37
	$tRNA^{Ser}$	G1:C72 → A:U	61
	$tRNA^{Sec}$	C2:G71 → A:U	100
	$tRNA^{Ser}$	U2:A71 → A:U	138
	$tRNA^{Sec}$	U6 → A	100
	$tRNA^{Sec}$	G7 → C	71
	$tRNA^{Sec}$	A5b:U67a → C:G	100
	$tRNA^{Ser}$	G7:C66 → C:G	48
Between acceptor and D stems	$tRNA^{Ser}$	U8 → A	100
	$tRNA^{Sec}$	U9 → C	75
	$tRNA^{Ser}$	G9 → C	100
D stem	$tRNA^{Sec}$	C11&G23 → G&C[b]	107
	$tRNA^{Ser}$	C11:G24 → G:C	78
Anticodon stem	$tRNA^{Sec}$	G30:C40 → C:G	87
TΨC stem	$tRNA^{Sec}$	G50&C66 → C&G[b]	39
	$tRNA^{Ser}$	G49:C65 → C:G	100
	$tRNA^{Sec}$	G52:C62 → C:G	51
	$tRNA^{Ser}$	G52:C62 → C:G	81

[a] Transcripts were aminoacylated using the same amount of unfractionated synthetases under the standard assay conditions, and the initial rates of serylation were compared. The initial rate of aminoacylation was determined at the same tRNA transcript concentration for all constructs, which was slightly lower than the K_m for the wild-type $tRNA^{Ser}$. Under these conditions, the relative initial rates should be close to the ratio of V_{max}/K_m of the mutant relative to the V_{max}/K_m of the wild type, since $v = V_{max} \cdot [S]/([S] + K_m)$ and changes in the initial rates are small. In case of $[S] \ll K_m$, the initial rate will be more closely proportional to V_{max}/K_m.

[b] Nonpairing bases.

the orientation of the G:C pairings at positions 46 and 47l (Fig. 4A) and 47a and 47j in $tRNA^{[Ser]Sec}$ has a pronounced affect on aminoacylation, reducing the level about 2/3 in the former alteration and about 1/2 in the latter as shown in Table I. Thus, the precise sequences of the G and C base pairings appear to be more critical to the identity of $tRNA^{[Ser]Sec}$ than to that of $tRNA^{Ser}$ in the long extra arm. Deletion of the G:C base pair which is the third base pair within the stem of the extra arm of $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ (i.e., G47a:C47j and G46:C47g, respectively) reduces aminoacylation dramatically (under 10% of that of wild type) in both tRNAs (see Fig. 4 and Table I).

The first base within the loop of the extra arm is a U in both tRNAs (position 47d in Sec and 47b in Ser). The presence of this U is not a factor in identity as aminoacylation is not affected by its deletion from $tRNA^{[Ser]Sec}$ (Table I). Similarly, aminoacylation of $tRNA^{Ser}$ is not affected by alteration of this base to C or to G. However, very interestingly, changing this U to a G in $tRNA^{[Ser]Sec}$ reduces aminoacylation by about 2/3 (Table I). As deletion of this base does not affect $tRNA^{[Ser]Sec}$ aminoacylation, but changing it to G has a pronounced affect, it would seem most likely that the presence of a G lengthens the stem by an additional G:C base pair (i.e., the G at position 47d pairs with the C at position 47g) which affects the orientation of the extra stem–loop in $tRNA^{[Ser]Sec}$ and thus inhibits aminoacylation. This mutant and the deletion mutant (G47a:C47j) in $tRNA^{[Ser]Sec}$ (Table I) which has a pronounced effect on serylation provide strong evidence that there is length specificity with respect to the stem of the long extra arm. C47c in human, rabbit, and bovine $tRNA^{[Ser]Sec}$ (12) manifests a pyrimidine transition to U47c in the mouse (33) and the rat tRNAs (29). Thus, this alteration presumably has no effect on the level of aminoacylation of this tRNA with rabbit seryl-tRNA synthetase. It should be noted that the rabbit $tRNA^{[Ser]Sec}$ gene is identical in structure to that in humans (12).

The mutations in the stem of the long extra arm of $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ provide strong evidence that this domain is a critical element in their identity for synthetase recognition (Table I). The recognition of human $tRNA^{Ser}$ in this region does not appear to be base (sequence) specific, as in the case of the discriminator base described above, since reciprocal G and C base pairings and mutations within the loop do not impair aminoacylation. Thus, the stem–loop structure appears to have more of a global effect in $tRNA^{Ser}$ identity. The sequences of the base pairings in this domain of $tRNA^{[Ser]Sec}$, however, are more sensitive to synthetase interaction than are those of $tRNA^{Ser}$. This suggests that the G and C base pairings in $tRNA^{[Ser]Sec}$ play a sequence-specific role as well as an orientation-specific role in the identity process. These observations are in agreement with those of Achsel and Gross (19) and Wu and Gross (22) with the exception that these investigators did not observe any enhanced sensitivity of the sequence specificity within the stem of the $tRNA^{[Ser]Sec}$ extra arm, nor the length specificity of the stem, in the identity process. In fact, none of the point mutations examined by Wu and Gross (22) occurred within the stem or loop of the long extra arm. They were generated in the hinge bases between the stems of the anticodon and long extra arm and the stems of the long extra arm and TΨC of $tRNA^{[Ser]Sec}$. These mutations only demonstrated the orientation specificity of the long extra arm in the recognition process (22).

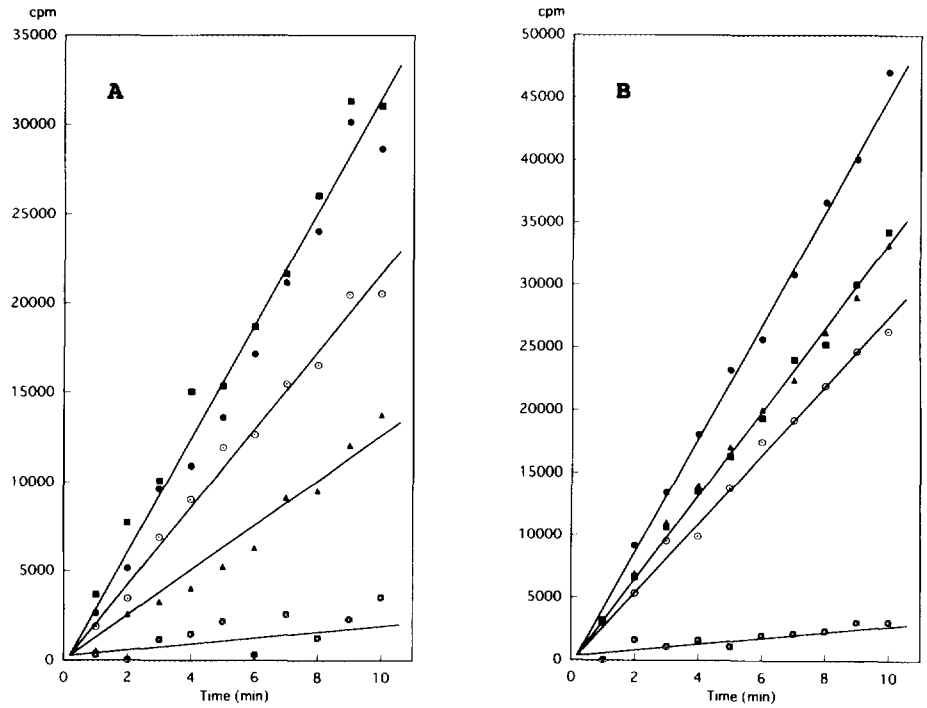

FIG. 4. Representative aminoacylation profiles of transcripts generated from the wild-type tRNA genes and from several mutant tRNA genes. Aminoacylation of $tRNA^{[Ser]Sec}$ is shown in (A) and $tRNA^{Ser}$ in (B). Symbols represent the following tRNA mutants: In A, ■, wild type; ●, 2 & 71 (C-G → A-U); ▲, 46 & 47l (C-G → G-C); ⊙, 9 (U → C); and ⊠, 47a & 47j (G-C → DEL). In B, ■, wild type; ●, 2 & 71 (U-A → A-U); ▲, 46 & 47g (G-C → C-G); ⊙, 11 & 24 (C-G → G-C); and ⊠, 46 & 47g (G-C → DEL). The data are also summarized in Table I.

Roles of position 73, the acceptor stem, DHU stem, and the bases between these two stems. Position 73 is the discriminator base and it is a critical site in the aminoacylation of many tRNAs (see 1–9 for review). This base was altered from G73 to A73 in $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$, respectively. These alterations completely inhibited the aminoacylation of both tRNAs (Table I). Thus, the discriminator base has an absolute requirement for the attachment of serine to the respective tRNA (see also 22). In *E. coli*, however, the discriminator base does not appear to be critical in the aminoacylation process (see 17 and references therein).

The acceptor stem has a role in the identity of a number of tRNAs (see 1–9 for review) including that of bacterial $tRNA^{Ser}$ (14, 34). We mutated a number of base pairs in the acceptor stem of human $tRNA^{[Ser]Sec}$. G1:C72 was changed to A1:U72 in both tRNAs. This alteration has a moderate affect on $tRNA^{Ser}$ aminoacylation (about 40% reduction), but a more pronounced effect on $tRNA^{[Ser]Sec}$ aminoacylation [about 65% reduction (Table I)]. Mutation of C2:G71 to A2:U71 in $tRNA^{[Ser]Sec}$ had no affect, while alteration of the bases in this position of $tRNA^{Ser}$ from U:A to A:U enhanced aminoacylation about 1.4 times (Fig. 4 and Table I). Mutation of the seventh base pair in the acceptor stem of both tRNAs (i.e., A5b:U67a in $tRNA^{[Ser]Sec}$ and G7:C66 in $tRNA^{Ser}$) had no affect on $tRNA^{[Ser]Sec}$ aminoacylation, but reduced that of $tRNA^{Ser}$ by about 1/2 (Table I). Mutation of U6 to A6 in $tRNA^{[Ser]Sec}$ presumably strengthened the U:U base pairing (i.e., between U6 and U67 as reported to occur at this site within the stem [32]) and this mutation had no effect. Changing G7 to C7 in $tRNA^{[Ser]Sec}$ destroyed possible pairing between G7 and C66, which is the proposed last base pair within the acceptor stem (31, 32); this mutation had a moderate effect (Table I). These observations show that the acceptor stem has a role in the aminoacylation process and that the base sequence apparently is not an absolute requirement. Further, it seems that the base pairings at the extreme ends of the acceptor stem of both tRNAs (i.e., 1:72 and 7:66) are the more important pairings involved in the recognition process.

Alteration of the bases between the acceptor and DHU stems in $tRNA^{Ser}$ (U8 → A8 and G9 → C9) had no effect on aminoacylation, while mutation of U9 to C9 in $tRNA^{[Ser]Sec}$ reduced aminoacylation slightly (Fig. 4A and Table I). It is of interest to note that the naturally occurring avian and *Xenopus* liver $tRNA^{[Ser]Sec}$ isoacceptors (35) have a C at position 9. This pyrimidine transition between *Xenopus* and mammalian Sec tRNAs (12) results in a conformational change in their structures (20, 36) which may account for the slightly reduced serylation. In any case, the bases between the acceptor and DHU stems have only a slight or no role in the identity process. Generally in tRNA, nucleotide 8 (normally a U in tRNA) and

14 (normally an A in tRNA) form a hydrogen bond that is important to the 3D structure. Mutation U8 → A8 in $tRNA^{Ser}$ had no effect on aminoacylation in our studies (Table I). This mutant would not be able to hydrogen bond in this region and it would seem from our mutant studies that the conformational change generated by U8–A14 is not involved in the identity process. Wu and Gross (22) have shown that G9 in $tRNA^{Ser}$ has some effect on aminoacylation. However, in our studies, the G9 → C9 mutation has no effect. The tRNA examined by Wu and Gross (22) also had changes in other parts of the molecule making the effect of the change at G9 difficult to assess in their studies.

We mutated C11 to G11 and G23 to C23 in the same $tRNA^{[Ser]Sec}$ which disrupted the proposed base pairings between positions 11:24 and 12:23 within the DHU stem (Table I). These changes had no affect on aminoacylation, suggesting that the sequence and orientation of base pairings in this region of the DHU domain are not involved in the identity process of $tRNA^{[Ser]Sec}$. Alteration of the C11:G24 base pair to G11:C24 in $tRNA^{Ser}$ had a slight effect on aminoacylation (Fig. 4B and Table I) suggesting that the DHU stem in this tRNA may have a role in the identity process. Wu and Gross (22) have shown that mutation of G22 to A22 in $tRNA^{[Ser]Sec}$ reduces aminoacylation quite substantially. Thus, it may be that the base pairs closer to the DHU loop may play a greater role in identity of both tRNAs.

Roles of the anticodon and TψC stems. Although the anticodon has been shown to be a critical element in the identity of numerous tRNAs (see 1–9 for review), the anticodon stem apparently does not play a significant role in tRNA:seryl-tRNA synthetase recognition. The isoacceptors within the tRNA populations of $tRNA^{Ser}$ and $tRNA^{[Ser]Sec}$ read a total of seven codons and none of the bases in the anticodon is the same in all isoacceptors. Furthermore, $tRNA^{[Ser]Sec}$ is capable of donating Sec to protein in mammalian systems after alteration of its anticodon to UUA or to UAA (37). Thus, the anticodon would not seem to be a critical element in $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ identity. However, since both tRNAs share a G:C base pair at positions 30 and 40 within the anticodon stem, we examined the possible influence of this common sequence on aminoacylation by making the reciprocal base pair (i.e., G:C → C:G) in $tRNA^{[Ser]Sec}$ (Table I). This alteration had only a slight effect on $tRNA^{[Ser]Sec}$ aminoacylation. We, therefore, conclude that this G:C base pair is not a critical sequence for $tRNA^{[Ser]Sec}$ identity. We were unable to make the complementary change in $tRNA^{Ser}$ as the new mutant results in a *Nsi*I restriction site which is the site used in the vector tRNA gene to generate the 3′ CCA terminus.

The TψC stem is characterized by containing several G and C base pairings in both $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ (see Fig. 3). Alteration of the first G:C base pair within the stem to the recriprocal C:G base pair (i.e., G49:C65 → C49:G65) in $tRNA^{Ser}$ had no effect on aminoacylation, while alteration of the second G:C base pair to the recriprocal C:G base pair (i.e., G52:C62) had a slight affect (Table I). The recriprocal G:C base pairings at positions G52 and C62 in $tRNA^{[Ser]Sec}$, on the other hand, had a moderate effect on aminoacylation. Mutation of G50 and C66 to C50 and G66, respectively, reduced aminoacylation substantially, and the level of serine attachment to this tRNA was lower than disruption of the base pairing between G7 and C66 alone (see Table I and mutation G7 → C7 above). These studies show that the DHU stem has some role in the identity process and that the base pairings toward the loop may have more influence on aminoacylation.

The crystal structure of *Thermus thermophilus* seryl-tRNA synthetase complexed with $tRNA^{Ser}$ has recently been resolved (38). On the basis of the crystal structure, the tRNA specificity depends primarily on the recognition of the shape of $tRNA^{Ser}$ through backbone contacts. The general conclusions on tRNA specificity appear to be true for the mammalian enzyme as well on the basis of the present and previous studies. The major identity elements are obviously the discriminator base and the orientation and length of the long extra arm-loop in both $tRNA^{Ser}$ and $tRNA^{[Ser]Sec}$. Several new insights are revealed in the present study. For example, the present study shows that $tRNA^{[Ser]Sec}$ may exhibit some degree of sequence specificity of the long extra arm–stem (46–47l and 47–47j) and long extra arm–loop (47d), while $tRNA^{Ser}$ does not. The difference probably reflects that $tRNA^{[Ser]Sec}$ bears unique secondary structural features such as a nine-base-pair acceptor stem and a four-base-pair TψC stem (32). These extra base pairs in the acceptor stem and TψC stem in $tRNA^{[Ser]Sec}$ altered the orientation and proximity of the long extra arm stem–loop relative to the 3′ end of tRNA and the discriminator base as compared to $tRNA^{Ser}$. As a result, seryl-tRNA synthetase could make contact with some of the bases in the long extra arm–stem and –loop in $tRNA^{[Ser]Sec}$ that results in the observed sequence specificity of $tRNA^{[Ser]Sec}$. Such flexibility in the tRNA recognition by seryl-tRNA synthetase is unique among all synthetases examined thus far. In addition, our data suggest, beyond earlier studies, that the base pairs at the extreme ends of the acceptor stem and those nearest the loop in the TψC stem may exert greater influence in the recognition process than the base pairs within the stems themselves.

Base modification plays a varying role in tRNA:synthetase recognition (see 1–9 for review). For example, there is an absolute requirement for the presence of a modified cytosine in the anticodon of an *E. coli* $tRNA^{Ile}$ for correct aminoacylation of this tRNA with isoleucine (39). On the other hand, methylation for the most part appears to be unimportant in the aminoacylation process (1–9). We observed that the $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$

transcripts have the same V_{max}/K_m as the corresponding naturally occurring tRNAs (data not shown). Thus, it seems that the modified bases in these tRNAs, including the methylated bases, do not have a role in the aminoacylation process.

Despite the proposed differences in the recognition between $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ by seryl-tRNA synthetase as described above, there are distinct similarities as well that are unique for seryl-tRNA synthetase among all other synthetases. The changes of about threefold in the k_{cat}/K_m ratios of mutant tRNAs relative to that of wild type were small compared to other synthetases (2). Additionally, the deletion mutants exhibited much greater effects on the k_{cat}/K_m ratios than the point mutations of either a single base or a base pair. These similarities between $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ may reflect the fact that seryl-tRNA synthetase recognizes the shape of the $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ molecules rather than the specific contacts as is the case in other synthetases that have been examined (38).

Our approach to examining the identity elements in $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ has been primarily to focus on the role of the identical bases in the recognition process. We rationalized that if the points of recognition were not sequence specific in a given region, but yet other mutations in that region affected aminoacylation, then the orientation of the nucleotides in that domain (and not their sequence) must play a more important role in identity. Furthermore, if a given base (or bases) is (are) found to be highly involved in synthetase recognition in one tRNA, but not the other, then such an observation would demonstrate that the synthetase interacts with these tRNAs differently at different regions in the respective molecule. As noted above, the results in this study show that the discriminator base is clearly sequence specific in both tRNAs and this base is essential for synthetase recognition (see also 22). Further, and on the other hand, the extra long arm is extremely important in the identity of both tRNAs, but synthetase recognition seems to operate in a different manner with respect to this region of both tRNAs; i.e., it seems to be orientation- (see also 22) and length-specific in $tRNA^{Ser}$ and, in addition, may manifest sequence specificity in $tRNA^{[Ser]Sec}$. The other domains of the tRNAs (acceptor, DHU, and TψC stems) affect aminoacylation to a lesser extent than G73 and the long extra arm. Further, $tRNA^{[Ser]Sec}$ and $tRNA^{Ser}$ were shown to be aminoacylated by the same synthetase through competition studies in the presence of an unfractionated synthetase and a highly purified seryl-tRNA synthetase preparation.

ACKNOWLEDGMENTS

The authors express their sincere appreciation to Drs. Mark G. Sandbaken, John F. Atkins, and Raymond F. Gesteland for their very helpful advice and support during the course of these studies. This research was supported in part by NIH Grant GM-25848 to D.C.-H.Y.

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