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The length and the secondary structure of the D-stem of human selenocysteine tRNA are the major identity determinants for serine phosphorylation*

Xin-Qi Wu and Hans J.Gross[1]

Institut für Biochemie, Bayerische Julius-Maximilians-Universität, Biozentrum, Am Hubland, D-97074 Würzburg, Germany
[1]Corresponding author

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*Dedicated to Professor Friedrich Cramer on the occasion of his 70th birthday

Selenocysteine tRNA [tRNA$^{(Ser)Sec}$] has been shown to be serylated by tRNA^{Ser} synthetase. The serine moiety of seryl-tRNA$^{(Ser)Sec}$ in vertebrates is further phosphorylated by a kinase, in addition to being converted into selenocysteine. Using site-directed mutagenesis we have introduced a number of mutations into T7 RNA polymerase transcripts of human tRNA$^{(Ser)Sec}$. Our results show that most of the unique structural features of tRNA$^{Ser(Sec)}$, like the 5′-triphosphate, the 9 bp long acceptor stem and the anticodon, are not identity elements for phosphorylation of human seryl-tRNA$^{(Ser)Sec}$. However, the length and secondary structure of the D-stem (6 bp in contrast with 4 bp in the canonical serine tRNA) of human tRNA$^{(Ser)Sec}$, but not its sequence, are the major identity determinants which discriminate this tRNA from common tRNA^{Ser} and identify it as the substrate for phosphorylation by seryl-tRNA$^{(Ser)Sec}$ kinase. This notion is confirmed by the fact that normal seryl-tRNA^{Ser}, which is not a substrate for serine phosphorylation, becomes a substrate if two additional base pairs are introduced into its D-stem.

Key words: human selenocysteine tRNA/identity elements/serine phosphorylation/seryl-tRNA$^{(Ser)Sec}$ phosphorylation

Introduction

A number of proteins containing selenium in the form of selenocysteine have been characterized in both prokaryotes and eukaryotes (for a review see Stadtman, 1990; Böck *et al.*, 1991). In *Escherichia coli*, the selenocysteine residues are co-translationally incorporated into the polypeptides directed by specific UGA stop codons (Zinoni *et al.*, 1987; Leinfelder *et al.*, 1988). This incorporation mechanism has also been suggested for mammalian cells (Lee *et al.*, 1989b). Decoding of UGA as selenocysteine requires a stem–loop structure immediately downstream of the UGA in the *E.coli* formate dehydrogenase mRNA (Zinoni *et al.*, 1990), or a stem–loop structure in the 3′-untranslated regions of mammalian type I deiodinase mRNA (Berry *et al.*, 1991) and glutathione peroxidase mRNA (Shen *et al.*, 1993). A specific elongation factor required for the process has been characterized in *E.coli* (Forchhammer *et al.*, 1989).

Selenocysteine tRNA is the molecule which decodes the UGA stop codon as selenocysteine (Leinfelder *et al.*, 1988). It is first charged with L-serine by the seryl-tRNA synthetase which also charges the canonical serine tRNAs (Mizutani *et al.*, 1984; Leinfelder *et al.*, 1988, 1989). This seryl-tRNA is further converted into selenocysteyl-tRNA$^{(Ser)Sec}$ in the presence of selenocysteine synthase and other proteins (Leinfelder *et al.*, 1990; Forchhammer *et al.*, 1991; Mizutani *et al.*, 1991). In contrast to *E.coli* seryl-tRNA$^{(Ser)Sec}$ and all seryl-tRNAs^{Ser}, the serine moiety of mammalian seryl-tRNA$^{(Ser)Sec}$ has been shown to be phosphorylated to form phosphoseryl-tRNA$^{(Ser)Sec}$ (Mäenpää and Bernfield, 1970; Sharp and Stewart, 1977; Hatfield *et al.*, 1982; Lee *et al.*, 1989b). The kinase has been purified from bovine liver (Mizutani and Hashimoto, 1984). Phosphoseryl-tRNA$^{(Ser)Sec}$ was once thought to be the intermediate for the conversion from seryl- to selenocysteyl-tRNA$^{(Ser)Sec}$ (Lee *et al.*, 1989b; Mizutani, 1989). However, it was later shown that selenocysteine was formed *in vitro* in the absence of the kinase responsible for the phosphorylation of seryl-tRNA$^{(Ser)Sec}$ (Mizutani *et al.*, 1991). Therefore, the function of phosphoseryl-tRNA$^{(Ser)Sec}$ remains unknown.

tRNAs$^{(Ser)Sec}$ from *E.coli* (Schön *et al.*, 1989), bovine liver (Diamond *et al.*, 1981; Hatfield *et al.*, 1982), HeLa and mouse cells (Kato *et al.*, 1983) have been characterized. tRNA$^{(Ser)Sec}$ genes have also been isolated from *E.coli* (Leinfelder *et al.*, 1988), *Proteus vulgaris* (Heider *et al.*, 1989) and a number of animals (O'Neill *et al.*, 1985; Lee *et al.*, 1990). Three models for the secondary structure of vertebrate tRNA$^{(Ser)Sec}$ have been suggested (Diamond *et al.*, 1981; Hatfield *et al.*, 1982; Böck *et al.*, 1991). One is favoured by the data from structure probing and computer modelling (Sturchler *et al.*, 1993). According to this model and that for *E.coli* tRNA$^{(Ser)Sec}$ (Baron *et al.*, 1993), the most striking features of tRNA$^{(Ser)Sec}$, compared with cytoplasmic tRNA^{Ser} (Dock-Bregeon *et al.*, 1989; Steinberg *et al.*, 1993), are: (i) tRNA$^{(Ser)Sec}$ has an extended acceptor stem of 8 bp in the case of *E.coli* tRNA$^{(Ser)Sec}$ and 9 bp in vertebrate tRNA$^{(Ser)Sec}$, including a UxU mismatch. In contrast, tRNA^{Ser} has a 7 bp acceptor stem; (ii) tRNA$^{(Ser)Sec}$ has a 6 bp D-stem, while tRNA^{Ser} has a D-stem of 3 bp; (iii) the sequence, length and orientation of the long extra arm of tRNA$^{(Ser)Sec}$ is different from that of tRNA^{Ser}; (iv) the T-stem of vertebrate tRNA$^{(Ser)Sec}$ is 4 bp long, whereas that of tRNA^{Ser} contains 5 bp; (v) tRNA$^{(Ser)Sec}$ from vertebrates has a 5′-triphosphate end (Lee *et al.*, 1987). Here we show that the length and secondary structure of the D-stem are the major determinants for the phosphorylation of human seryl-tRNA$^{(Ser)Sec}$.

Results

Phosphorylation of seryl-tRNA$^{(Ser)Sec}$

For the phosphorylation assay, tRNA$^{(Ser)Sec}$ (Figure 1) was first aminoacylated with [^{3}H]serine and an S100 extract which had been frequently used and had little kinase activity (see procedure I in Materials and methods). After deacylation and paper chromatography, only [^{3}H]serine could be detected, as expected (Figure 2). However, incubation of

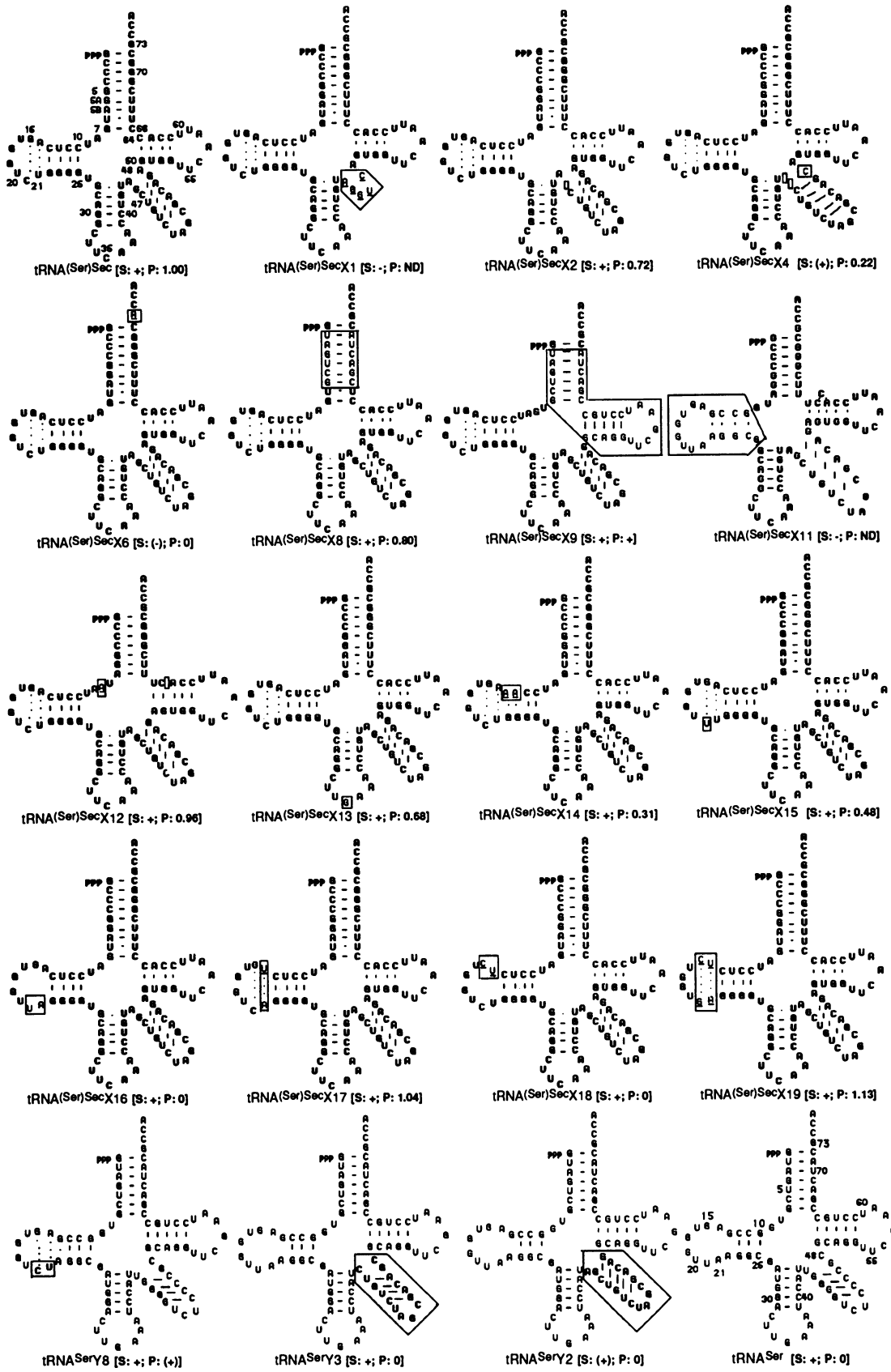

Fig. 1. Secondary structures of $tRNA^{(Ser)Sec}$, $tRNA^{Ser}$ and their derivatives. Mutated sequences are boxed. Sequences from $tRNA^{(Ser)Sec}$ are in bold; sequences from $tRNA^{Ser}$ are not bold and not underlined; other mutations are underlined. Empty boxes (□) indicate nucleotide deletions. The secondary structure and numbering of $tRNA^{(Ser)Sec}$ are according to Sturchler *et al.* (1993). The numbering of $tRNA^{Ser}$ is according to Sprinzl *et al.* (1991). Relative serylation (S) and phosphorylation (P) efficiencies, respectively, are indicated by +, (+), (−) and −, and by numbers [1.00 = efficiency of wild-type $tRNA^{(Ser)Sec}$ transcript].

[^{3}H]seryl-tRNA$^{(Ser)Sec}$ (procedure I) with an S100 extract freshly thawed from storage at −83°C gave rise, on paper chromatographic analysis, to two radioactive peaks: the fast one comigrated with authentic serine and the slow one with authentic phosphoserine (Figure 2). The concentration of seryl-tRNA$^{(Ser)Sec}$ used in procedure I was ~20 nM, close to the K_m reported by Mizutani and Hashimoto (1984), and 60% of the seryl-tRNA$^{(Ser)Sec}$ was converted into phospho-seryl-tRNA$^{(Ser)Sec}$ after incubation for 60 min (Table I, row a). Detailed studies of the time course for a series of substrates revealed that there is a linear increase in phosphorylation during this time period and that the phosphorylation efficiencies determined here are therefore proportional to the initial rates of this reaction. Phospho-[^{3}H]seryl-tRNA$^{(Ser)Sec}$ is also formed from the direct incubation of tRNA$^{(Ser)Sec}$ with [^{3}H]serine and the freshly thawed S100 extract (see procedure II in Materials and methods). The absolute amount of phospho-[^{3}H]seryl-tRNA$^{(Ser)Sec}$ is increased, but the proportion of phospho-[^{3}H]-serine is reduced to ~2% because of the larger increase of [^{3}H]seryl-tRNA$^{(Ser)Sec}$, since about 80-fold more tRNA substrate is present in this procedure (Table II, row a). A similar phosphorylation efficiency of 5% was reported by Mizutani (1989) using native tRNA$^{(Ser)Sec}$ from bovine liver.

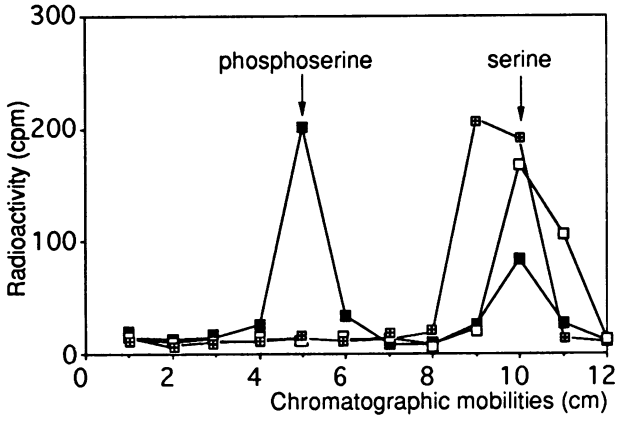

Fig. 2. Chromatographic patterns of [^{3}H]serine and phospho-[^{3}H]serine resulting from the incubation of [^{3}H]seryl-tRNA$^{(Ser)Sec}$ (■) and [^{3}H]seryl-tRNA^{Ser} (□) with S100 extract and deacylation. The positions of authentic serine and phosphoserine are indicated by arrows. Serylation of tRNA$^{(Ser)Sec}$ with the frequently used S100 extract does not yield any phosphoserine ([⊞]).

The incubation of [^{3}H]seryl-tRNA^{Ser} with the freshly thawed S100 extract used above produced only one radioactive peak of [^{3}H]serine (Figure 2; Table I, row n). Phospho-[^{3}H]serine did not appear in the case of seryl-tRNA^{Ser}, even if the reaction was performed using procedure II (Table II, row e) by which even poor phosphorylation could be measured (e.g. Table II, row h). These results are consistent with earlier reports that only seryl-tRNA$^{(Ser)Sec}$ is phosphorylated and tRNA^{Ser} is not (Mäenpää and Bernfield, 1970; Sharp and Stewart, 1977; Hatfield *et al.*, 1982; Mizutani and Hashimoto, 1984; Lee *et al.*, 1989b). The fact that seryl-tRNA$^{(Ser)Sec}$ is phosphorylated, but seryl-tRNA^{Ser} is not, indicates that identity elements for phosphorylation must be located in regions specific for tRNA$^{(Ser)Sec}$.

The 5′-triphosphate group is not required for phosphorylation

tRNA$^{(Ser)Sec}$ is transcribed directly from the 5′-terminal G (+1) *in vivo* and possesses a 5′-triphosphate which remains intact after 3′-terminal maturation and transport into the cytoplasm (Lee *et al.*, 1987). tRNA$^{(Ser)Sec}$ transcribed in the presence of 20 mM GMP and 2 mM GTP, which therefore carries a 5′-monophosphate, was as efficiently phosphorylated as tRNA$^{(Ser)Sec}$ with 5′-triphosphate (data not shown). To remove all 5′-phosphates, tRNA$^{(Ser)Sec}$ was dephosphorylated with calf intestinal alkaline phosphatase (CIP). The 5′-dephosphorylated tRNA was efficiently phosphorylated by polynucleotide 5′-kinase in the presence of γ-[^{32}P]ATP (data not shown). Table I (row b) shows that 34% of the CIP-treated seryl-tRNA$^{(Ser)Sec}$ is phosphorylated

Table I. Phosphorylation of seryl-tRNA according to procedure I

Row letter	tRNA	[^{3}H]Serine (c.p.m.)[a]	Phospho-[^{3}H]serine (c.p.m.)[b]	Efficiency of phosphorylation (%)[c]	Relative efficiency
a	tRNA$^{(Ser)Sec}$	76	113	59.8	1.00
b	tRNA$^{(Ser)Sec}$deP[d]	189	97	33.9	0.57
c	tRNA$^{(Ser)Sec}$X2	142	108	43.2	0.72
d	tRNA$^{(Ser)Sec}$X4	215	33	13.3	0.22
e	tRNA$^{(Ser)Sec}$X8	101	92	47.7	0.80
f	tRNA$^{(Ser)Sec}$X12	90	121	57.3	0.96
g	tRNA$^{(Ser)Sec}$X13	143	98	40.7	0.68
h	tRNA$^{(Ser)Sec}$X14	150	34	18.5	0.31
i	tRNA$^{(Ser)Sec}$X15	187	76	28.9	0.48
j	tRNA$^{(Ser)Sec}$X16	228	0	0.0	0.00
k	tRNA$^{(Ser)Sec}$X17	61	101	62.3	1.04
l	tRNA$^{(Ser)sec}$X18	247	0	0.0	0.00
m	tRNA$^{(Ser)Sec}$X19	68	136	67.6	1.13
n	tRNA^{Ser}	759	0	0.0	0.00

[a,b]Amount of [^{3}H]serine and phospho-[^{3}H]serine, respectively, released from [^{3}H]seryl-tRNA after a phosphorylation assay.
[a]Obtained by subtracting the background (15 c.p.m. for each 1 cm long paper piece) and the ^{3}H counts due to endogenous tRNA^{Ser} in S100 extract from the values measured by scintillation counting.
[b]Background counts were subtracted from the values measured by scintillation counting.
[c]Efficiency (%) = [b/(a + b)] × 100.
[d]tRNA$^{(Ser)Sec}$ without the 5′-triphosphate group.

Table II. Phosphorylation of tRNA according to procedure II[a]

Row letter	tRNA	[^{3}H]Serine (c.p.m.)[b]	Phospho-[^{3}H]serine (c.p.m.)[c]
a	water	1 678	77
b	$tRNA^{(Ser)Sec}$	65 732	1 225
c	$tRNA^{(Ser)Sec}X16$	43 485	105
d	$tRNA^{(Ser)Sec}X18$	32 341	134
e	$tRNA^{Ser}$	56 194	81
f	$tRNA^{Ser}Y3$	19 542	95
g	$tRNA^{Ser}Y7$[d]	64 711	86
h	$tRNA^{Ser}Y8$	57 634	205

[a]1.6 μM tRNA was incubated with 0.65 μM [^{3}H]serine (31 Ci/mmol) and 8% of S100 extract in a total volume of 25 μl (see Materials and methods).
[b,c]Values shown were obtained by scintillation counting without subtraction of the background.
[d]Mutant $tRNA^{Ser}$ with an anticodon UCA.

Table III. Phosphorylation of tRNA which is weakly charged with serine[a]

Row letter	tRNA	[^{3}H]Serine (c.p.m.)[b]	Phospho-[^{3}H]serine (c.p.m.)[c]
a	water	5 997	133
b	$tRNA^{(Ser)Sec}X1$	7 000	107
c	$tRNA^{(Ser)Sec}X4$	11 453	381
d	$tRNA^{(Ser)Sec}X6$	8 902	84
e	$tRNA^{(Ser)Sec}X9$	28 791	449
f	$tRNA^{Ser}Y2$	12 892	102

[a]1.6 μM tRNA was incubated at 37°C for 1 h with 0.65 μM [^{3}H]serine (31 Ci/mmol) and 8% S100 extract in a volume of 100 μl (procedure II, see Materials and methods).
[b,c]Values obtained by scintillation counting without subtraction of the background.

at the serine moiety. It should be noted that $tRNA^{Ser}$ transcribed by T7 RNA polymerase contains also a 5′-triphosphate, and that no phosphoserine is formed from this seryl-$tRNA^{Ser}$ (Figures 1 and 2).

The acceptor stem does not play a role in the discrimination of seryl-$tRNA^{(Ser)Sec}$ from seryl-$tRNA^{Ser}$ for serine phosphorylation

Sequence comparison of $tRNA^{Ser}$, $tRNA^{(Ser)Sec}$ and $tRNA^{(Ser)Sec}$ genes thus far characterized from animals (Lee *et al.*, 1990; Steinberg *et al.*, 1993) shows that a stretch of three C:G base pairs is highly conserved at the top end of the acceptor stem of $tRNA^{(Ser)Sec}$, but does not appear in $tRNA^{Ser}$ (Figure 1). To verify the possible importance of the acceptor stem for phosphorylation, the whole stem of $tRNA^{(Ser)Sec}$ was replaced by that of $tRNA^{Ser}$, as shown in $tRNA^{(Ser)Sec}X8$ (Figure 1). Mutant seryl-$tRNA^{(Ser)Sec}X8$ was phosphorylated with an efficiency of 48% (Table I, row e), only slightly less than seryl-$tRNA^{(Ser)Sec}$ (Table I, row a).

Another structural feature concerning the acceptor stem of $tRNA^{(Ser)Sec}$ is its length. It is 9 bp long, including a UxU mismatch, as shown by structure probing and computer modelling (Sturchler *et al.*, 1993). The change of G_7:C_{66} to A_7xC_{66} did not affect serylation and phosphorylation efficiency (data not shown). Even further deletion of one of the two Cs (i.e. C_{64} or C_{66}) between the acceptor stem and T-stem, as shown in $tRNA^{(Ser)Sec}X12$ (Figure 1), did not reduce phosphorylation; $tRNA^{(Ser)Sec}X12$ is phosphorylated with an efficiency of 57% (Table I, row f), identical to that of the wild-type substrate.

The anticodon is not an identity element for the phosphorylation of seryl-$tRNA^{(Ser)Sec}$

Although four anticodons (i.e. U*GA, CGA, GCU and IGA) are assigned to serine tRNA isoacceptors, all $tRNAs^{(Ser)Sec}$ so far characterized have U*CA (where U* is a modified U) as their anticodon (Steinberg *et al.*, 1993). Therefore, the role of the anticodon for serine phosphorylation was investigated. $tRNA^{(Ser)Sec}$ with an anticodon UGA (Figure 1, X13) was only slightly less efficiently phosphorylated, with an efficiency of 41% (Table I, row g) as compared with the wild-type. Consistent with this result, $tRNA^{Ser}Y7$ with the anticodon UCA is not phosphorylated at all (Table II, row g).

Effect of the long extra arm on the phosphorylation of $tRNA^{(Ser)Sec}$

The extra arms of $tRNA^{(Ser)Sec}$ and $tRNA^{Ser}$ differ in sequence, length and orientation (Figure 1). When the long extra arm of $tRNA^{(Ser)Sec}$ was replaced by that of $tRNA^{Val}$, an insufficient amount of seryl-tRNA was formed for the phosphorylation assay, even if procedure II was applied (Figure 1, X1; Table III, row b).

Although there are some sequence variations in the extra arms of $tRNA^{(Ser)Sec}$ within the animal kingdom, the length and orientation of the extra arm are highly conserved (Lee *et al.*, 1990; Steinberg *et al*, 1993) and different from those of $tRNAs^{Ser}$, which are also conserved in all eukaryotic cytoplasmic $tRNAs^{Ser}$ (Steinberg *et al.*, 1993). Deletion of G_{45} in $tRNA^{(Ser)Sec}X2$ (Figure 1) only slightly reduced phosphorylation to an efficiency of 43% (Table I, row c). Mutant

tRNA$^{(Ser)Sec}$X4 was obtained by deletion of $A_{44}G_{45}$ and insertion of $C_{47:M}$ (Figure 1). It was very poorly charged with serine. However, ~5000 c.p.m. of [^{3}H]seryl-tRNA were synthesized from 4 μg of tRNA$^{(Ser)Sec}$X4 using procedure II (Table III, row c). Seryl-tRNA$^{(Ser)Sec}$X4 was significantly phosphorylated with an efficiency of 13% (Table I, row d), only 4-fold less than the wild-type tRNA$^{(Ser)Sec}$. Replacement of the extra arm of tRNA^{Ser} by that of tRNA$^{(Ser)Sec}$, as in tRNA^{Ser}Y2 (Figure 1), dramatically reduced serylation. There was no phosphoserine formed from ~6000 c.p.m. of [^{3}H]seryl-tRNA^{Ser}Y2 (Table III, row f). Changing the orientation of the extra arm of tRNA^{Ser}Y2 to that of wild-type tRNA^{Ser}, as shown in tRNA^{Ser}Y3 (Figure 1), significantly increased serylation (not shown). However, [^{3}H]tRNA^{Ser}Y3 was still not phosphorylated (Table II, row f).

Effect of the T-stem on the phosphorylation of seryl-tRNA$^{(Ser)Sec}$

Selenocysteine tRNA from vertebrates has a 4 bp long T-stem, in contrast to the canonical tRNAs^{Ser} with 5 bp (Dock-Bregeon *et al.*, 1989; Steinberg *et al.*, 1993; Sturchler *et al.*, 1993). To study the significance of this difference for phosphorylation, tRNA$^{(Ser)Sec}$X9 was constructed. It has the acceptor stem and the T-stem from tRNA^{Ser} (Figure 1), and its charging activity is greatly reduced (not shown). An assay using procedure I did not show any phosphorylation of tRNA$^{(Ser)Sec}$X9; however, from ~25 000 c.p.m. of [^{3}H]seryl-tRNA$^{(Ser)Sec}$X9, some 300 c.p.m. of phosphoserine were measured using procedure II for phosphorylation (Table III, row e).

Interruption of the extra base pairs in the D-stem dramatically reduces the phosphorylation of seryl-tRNA$^{(Ser)Sec}$

tRNA$^{(Ser)Sec}$ and tRNA^{Ser} have different D-stem−loop structures due to the sequence difference at positions 20:A and 21. With $C_{20:A}U_{21}$, tRNA$^{(Ser)Sec}$ has a 6 bp D-stem and 4-base D-loop (Sturchler *et al.*, 1993); while with $U_{20:A}A_{21}$, tRNA^{Ser} has a 4 bp D-stem and 8-base D-loop (Dock-Bregeon *et al.*, 1989). The change of $U_{12}C_{13}$ to $A_{12}A_{13}$ reduces the phosphorylation by a factor of three (Figure 1, X14; Table I, row h). Substitution of the whole D-arm of tRNA$^{(Ser)Sec}$ with that of tRNA^{Ser}, as shown in tRNA$^{(Ser)Sec}$X11 (Figure 1), abolished serylation completely (not shown). The change of $C_{20:A}$ to $U_{20:A}$ (Figure 1, X15) reduced phosphorylation by a factor of two (Table I, row i). However, mutation of $C_{20:A}U_{21}$ to $U_{20:A}A_{21}$ to create a D-loop and D-stem, like that of tRNA^{Ser} as shown in tRNA$^{(Ser)Sec}$X16 (Figure 1), dramatically reduced phosphorylation: there was no detectable phosphorylation produced using procedure I (Table I, row j), and only a trace of phosphoserine was formed using procedure II (Table II, row c).

Changing $U_{20:A}A_{21}$ to $C_{20:A}U_{21}$ in the D-loop of seryl-tRNA^{Ser} allows it to be weakly phosphorylated

The results from tRNA$^{(Ser)Sec}$X16 suggested that the nucleotides in the D-stem play a critical role, possibly as an identity element, for phosphorylation. To confirm this notion, the mutation of $U_{20:A}A_{21}$ to $C_{20:A}U_{21}$ was introduced into the D-loop of tRNA^{Ser}, as shown in tRNA^{Ser}Y8 (Figure 1). Interestingly, the serine moiety of seryl-tRNA^{Ser}Y8 is phosphorylated, albeit with a low efficiency (Table II, row h).

The length and secondary structure, but not the sequence of the D-stem, determine the phosphorylation of seryl-tRNA$^{(Ser)Sec}$

To verify whether secondary structure (base pairing) and/or the nature of the nucleotides which form the last 2 bp of the D-stem are responsible for phosphorylation, three other mutants, i.e. tRNA$^{(Ser)Sec}$X17, X18 and X19, were constructed (Figure 1). tRNA$^{(Ser)Sec}$X17 has U_{14}:A_{21} instead of A_{14}:U_{21} as in the wild-type; in tRNA$^{(Ser)Sec}$X19, $A_{14}G_{15}$ were changed to $U_{14}C_{15}$ and $C_{20:A}U_{21}$ were changed to $G_{20:A}A_{21}$ (Figure 1). These two mutants maintain a 6 bp D-stem but with different sequence. tRNA$^{(Ser)Sec}$X18 has $U_{14}C_{15}$ which cannot pair with $C_{20:A}U_{21}$ any more; thus, it has a 4 bp D-stem and an 8-base D-loop (Figure 1). Seryl-tRNA$^{(Ser)Sec}$X17 and X19 are as efficiently phosphorylated (Table I, rows k and m) as the wild-type, while seryl-tRNA$^{(Ser)Sec}$X18, like seryl-tRNA$^{(Ser)Sec}$X16, is only very weakly phosphorylated by procedure II (Table II, row d); there is no phosphoserine detected in seryl-tRNA$^{(Ser)Sec}$X18 using procedure I (Table I, row l).

Effect of mutation at the discriminator base G_{73} on the phosphorylation of seryl-tRNA$^{(Ser)Sec}$

All eukaryotic cytoplasmic tRNAs^{Ser} and tRNAs$^{(Ser)Sec}$ have a G at the discriminator position 73 (Steinberg *et al.*, 1993). The change of G_{73} to A_{73} (Figure 1, X6) markedly reduced charging of tRNA$^{(Ser)Sec}$ (not shown). Nevertheless, some 3000 c.p.m. of [^{3}H]seryl-tRNA$^{(Ser)Sec}$X6 over background were measured after paper chromatography (Table III, rows a and d). However, no phosphoserine could be detected in this case (Table III, row d), while a measurable amount of phospho-[^{3}H]serine was obtained from a comparable mutant, tRNA$^{(Ser)Sec}$X4, which is also only weakly serylated (Figure 1; Table III, row c).

Discussion

Using pure tRNA$^{(Ser)Sec}$ and tRNA^{Ser} transcribed *in vitro* by T7 RNA polymerase, we first confirmed earlier reports that seryl-tRNA$^{(Ser)Sec}$ is phosphorylated, but seryl-tRNA^{Ser} is not (Mäenpää and Bernfield, 1970; Sharp and Stewart, 1977; Hatfield *et al.*, 1982; Lee *et al.*, 1989b). This observation (Figure 2) clearly indicates that identity elements for the specific and exclusive phosphorylation of seryl-tRNA$^{(Ser)Sec}$ must be present in the tRNA$^{(Ser)Sec}$ domain. Rather surprisingly, most of the unique features of tRNA$^{(Ser)Sec}$, such as the 5′-terminal triphosphate, the longer acceptor stem, the U*CA anticodon, the long extra arm which differs from that of tRNAs^{Ser} by its sequence, length and orientation, and the shorter T-stem, do not function as major identity elements for seryl-tRNA$^{(Ser)Sec}$ phosphorylation.

However, the phosphorylation of seryl-tRNA$^{(Ser)Sec}$ is profoundly affected by mutations in the D-stem of tRNA$^{(Ser)Sec}$. Mutation of $C_{20:A}U_{21}$ to $U_{20:A}A_{21}$ in the D-stem of tRNA$^{(Ser)Sec}$ dramatically reduced the phosphorylation (Figure 3, X16; Table I, row j; Table II, row c). tRNA$^{(Ser)Sec}$X16 has a 4 bp D-stem and a D-loop like that of tRNA^{Ser}. Thus, the loss of phosphorylation of tRNA$^{(Ser)Sec}$X16 might be due to: (i) the presence of a short

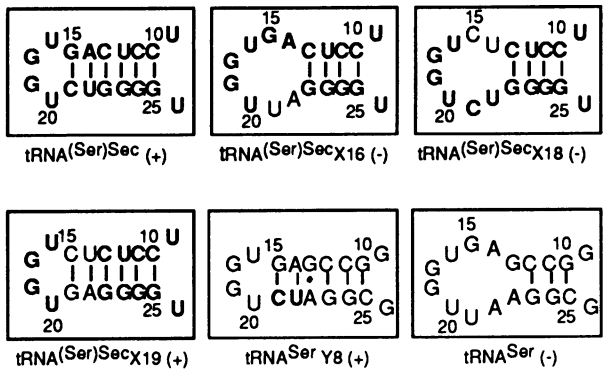

Fig. 3. D-stem−loop structures of tRNA$^{(Ser)Sec}$, derivatives X16, X18, X19 of tRNA$^{(Ser)Sec}$, tRNA^{Ser} and the derivative Y8 of tRNA^{Ser} (see Figure 1). (+) and (−) indicate whether the tRNA is phosphorylated or not, respectively.

D-stem; (ii) a negative effect of $U_{20:A}A_{21}$, which is highly conserved in all animal tRNAs^{Ser} (Steinberg *et al.*, 1993); or (iii) the loss of the positive effect of $C_{20:A}U_{21}$. The fact that tRNAs$^{(Ser)Sec}$X17 and X19 are as efficiently phosphorylated as the wild-type (Figures 1 and 3; Table I, rows k and m), but that tRNA$^{(Ser)Sec}$X18 is very weakly phosphorylated (Figures 1 and 3; Table I, row l; Table II, row d), clearly shows that the length rather than the sequence of the D-stem is the major cause for the dramatic reduction of the phosphorylation of tRNA$^{(Ser)Sec}$X16. The change of $U_{20:A}A_{21}$ to $C_{20:A}U_{21}$ in the D-loop of tRNA^{Ser} made it apparently a substrate for phosphorylation, albeit at a low efficiency (Figure 3, Y8; Table II, row h). G_{13}:A_{22} in the D-stem of tRNA^{Ser} was suggested by structure probing to be stacked and paired through O-6(G_{13})...N-6(A_{22}) and N-1(G_{13})...N-1(A_{22}) hydrogen bonding, similar to G_{26}:A_{44} in tRNA^{Phe} and tRNA^{Asp} (Dock-Bregeon *et al.*, 1989). Therefore, $C_{20:A}U_{21}$ in the D-stem may permit tRNA^{Ser}Y8 to have a 6 bp D-stem. Similarly, A:G pairing may also occur in tRNA$^{(Ser)Sec}$X14 (Figure 1), since it was significantly phosphorylated (Table I, row h). Thus, the length and secondary structure, but not the sequence of the D-stem, are the major determinants for serine phosphorylation. It should be noted that the sequences in the D-stem−loop region of all tRNAs$^{(Ser)Sec}$ or tRNA$^{(Ser)Sec}$ genes so far characterized in the animal kingdom can form a 6 bp helix with a loop of four nucleotides (Lee *et al.*, 1990; Steinberg *et al.*, 1993).

The low phosphorylation efficiency of tRNA^{Ser}Y8 may be mainly due to the presence of a 5 bp T-stem, as observed in tRNA$^{(Ser)Sec}$X9 (Figure 1; Table III, row e). The sequences in the acceptor stem do not specify phosphorylation of tRNA$^{(Ser)Sec}$, as revealed by tRNA$^{(Ser)Sec}$X8, which has an acceptor stem of tRNA^{Ser} and is efficiently phosphorylated (Figure 1; Table I, row e). Therefore the reduction in phosphorylation of tRNA$^{(Ser)Sec}$X9 may be mainly due to the presence of a T-stem from tRNA^{Ser}, and thus the 3-D structure might be altered somehow. tRNA$^{(Ser)Sec}$X9 may have another secondary structure, but that shown in Figure 1 has maximal base pairing. Similarly, tRNA$^{(Ser)Sec}$X12 (Figure 1) has one G:C and one A:U base pair more than another possible form. Therefore, tRNA$^{(Ser)Sec}$X9 and X12 are likely to have an acceptor stem of 7 bp. tRNA$^{(Ser)Sec}$X12 is efficiently phosphorylated (Table 1, row f). Thus, the length of the acceptor stem appears not to be important for phosphorylation of tRNA$^{(Ser)Sec}$. More probably, an appropriate D-/T-stem−loop interaction or 3-D structure is required for efficient phosphorylation.

The discriminator base G_{73} seems to be another structural element important for phosphorylation of seryl-tRNA$^{(Ser)Sec}$. The change from G_{73} to A_{73} reduced serylation to a very low, but measurable, level; however, phosphoserine was no longer detected in our assay (Table III, row d). Because G_{73} is also highly conserved in cytoplasmic tRNAs^{Ser} (Steinberg *et al.*, 1993), it may be a contact site for the kinase, without contributing to the discrimination of seryl-tRNA$^{(Ser)Sec}$ from seryl-tRNA^{Ser} by the kinase for phosphorylation. Alternatively, the discriminator base may not be a contact site for the kinase, but may influence the local structure around the CCA 3′-end, like the discriminator A_{73} in *E.coli* initiator tRNA^{fMet} for transformylase (Lee *et al.*, 1993).

The phosphorylation of seryl-tRNA$^{(Ser)Sec}$ is not the only case of modification of an activated amino acid (for a review see Söll, 1993). The formylation of *E.coli* Met-tRNA^{fMet} (Marcker and Sanger, 1964) is another example of the post-aminoacylation modification of amino acids. The prominent determinants for the formylation of Met-tRNA^{fMet} are clustered in the acceptor stem including C_1xA_{72}, G_2:C_{71} and C_3:G_{70}, while the T-/D-stems play only a minor role in this process (Lee *et al.*, 1991; Varshney *et al.*, 1991). Only the mutation of the discriminator base A_{73} to G_{73} has a detrimental effect on the formylation (Lee *et al.*, 1993). This led to the proposal that the discriminator base may not be a site of direct contact, rather the deleterious effect of G_{73} may be due to an alteration in the local structure (Lee *et al.*, 1993).

Very little is known about the determinants for the conversion from Glu-tRNA^{Gln} to Gln-tRNA^{Gln} by the amidotransferase which is found in gram-positive bacteria, archaebacteria, chloroplasts and mitochondria (Wilcox and Nirenberg, 1968; Schön *et al.*, 1988). The recognition of Glu-tRNA^{Glu} by the Glu-tRNA reductase, which converts the glutamate moiety to glutamate-1-semialdehyde [the early precursor for porphyrin synthesis (for a review see Söll, 1993)], also remains to be determined.

Although the sequences of the extra arms of tRNAs$^{(Ser)Sec}$ from lower and higher animals are not highly conserved (Lee *et al.*, 1990), they are always 17 nucleotides long, forming a helix of 6 bp (including a AxG mismatch) with a loop of four nucleotides (Sturchler *et al.*, 1993). In contrast, tRNAs^{Ser} have a 14 nucleotide long extra arm which forms a helix of 4 bp and a loop of three nucleotides (Dock-Bregeon *et al.*, 1989; Steinberg *et al.*, 1993). Whether the long extra arm is required for phosphorylation of seryl-tRNA$^{(Ser)Sec}$ remains unclear, because tRNA$^{(Ser)Sec}$ with a short extra arm from tRNA^{Val} did not produce sufficient seryl-tRNA for a phosphorylation assay. Introduction of the extra arm of human tRNA$^{(Ser)Sec}$ into tRNA^{Ser} did not allow its phosphorylation (Table II, row f and Table III, row f). The orientation of the extra arm of tRNA$^{(Ser)Sec}$ is also highly conserved (Lee *et al.*, 1990; Steinberg *et al.*, 1993). However, the orientation does not play a critical role in the phosphorylation, as demonstrated by tRNA$^{(Ser)Sec}$X2 and X4 (Figure 1; Table I, rows c and d).

Interestingly, the A box of tRNA$^{(Ser)Sec}$ genes has two extra nucleotides (Lee *et al.*, 1989a). It is inactive and the transcription of tRNA$^{(Ser)Sec}$ genes is regulated by the internal B box and the upstream promoter elements such as

TATA box and PSE (Lee *et al.*, 1989a; Carbon and Krol, 1991). These two extra nucleotides are not highly conserved (Lee *et al.*, 1989a) and can be mutated without abolishing phosphorylation (Table I, row h). They may, however, be necessary to provide a tRNA$^{(Ser)Sec}$ molecule with a 6 bp D-stem and a 9 bp acceptor stem, so that phosphorylation can occur and the function of the acceptor stem remains.

Although the acceptor stem of human tRNA$^{(Ser)Sec}$ has several unique structural properties, such as the 5′-triphosphate group, three C:G base pairs and a length of 9 bp (Lee *et al.*, 1987; Sturchler *et al.*, 1993), it does not seem to contribute to the specificity of serylation and phosphorylation of tRNA$^{(Ser)Sec}$, as revealed by tRNA$^{(Ser)Sec}$ without 5′-phosphate and tRNAs$^{(Ser)Sec}$X8, X12 and X9 (Table I, rows b, e and f; Table III, row e). The three C:G base pairs in the acceptor stem or the 5′-triphosphate group may be the recognition sites for the selenocysteine synthase or other proteins involved in the synthesis of selenoproteins. Little is known about the recognition of tRNA$^{(Ser)Sec}$ by these proteins.

Phosphoseryl-tRNA$^{(Ser)Sec}$ was thought to be the intermediate for the synthesis of selenocysteine from seryl-tRNA$^{(Ser)Sec}$ (Lee *et al.*, 1989b; Mizutani, 1989). However, selenocysteine has been synthesized *in vitro* in the absence of the kinase (Mizutani *et al.*, 1991). Phosphoseryl-tRNA binds to ribosomes in the presence of UGA trinucleotide (Hatfield *et al.*, 1982). In the presence of seryl-tRNA$^{(Ser)Sec}$ or phosphoseryl-tRNA$^{(Ser)Sec}$, the UGA codon of glutathione peroxidase mRNA is translated to yield a protein product that reacts with antibodies against glutathione peroxidase (Lee *et al.*, 1989b). However, the direct incorporation of phosphoserine into protein via phosphoseryl-tRNA$^{(Ser)Sec}$ remains to be demonstrated (Mizutani and Tachibana, 1986). Another possible role of phosphoseryl-tRNA concerns its function in the biosynthesis of serine (Mäenpää and Bernfield, 1970; Sharp and Stewart, 1977) or in the reverse metabolic pathway from serine to 3-phosphohydroxypyruvate (Mizutani and Hashimoto, 1984). Thus far, *in vitro* studies of the function of phosphoseryl-tRNA$^{(Ser)Sec}$ have been unsuccessful (Mäenpää and Bernfield, 1970; Mizutani and Tachibana, 1986; Mizutani, 1989). After the major identity element for serine phosphorylation has been identified, the biological function of phosphoseryl-tRNA$^{(Ser)Sec}$ should be elucidated.

Materials and methods

Enzymes and reagents

T7 RNA polymerase was prepared in our laboratory (Zawadzki and Gross, 1991) from an overproducing strain kindly provided by Dr W.Studier. S100 extract from HeLa cells used as the source of the aminoacyl synthetase and the kinase for seryl-tRNA$^{(Ser)Sec}$ phosphorylation was prepared according to Dignam *et al.* (1983). [^{3}H]Serine (31 Ci/mmol) was obtained from Amersham. Phosphoserine was acquired from Sigma. Other enzymes and reagents were purchased from commercial suppliers.

Bacterial strains and plasmids

Escherichia coli JM 109 was used as a host for propagation of plasmid pUC19 DNA. *Escherichia coli* strains CJ 236 and TG 1 were used for site-directed mutagenesis in M13 vectors. pHtU was constructed by ligating the end-filled 0.5 kb *Eco*RI–*Ava*I DNA fragment containing the selenocysteine tRNA gene from human placenta (O'Neill *et al.*, 1985) into the *Sma*I site of plasmid pUC19. A T7 RNA polymerase promoter sequence was introduced directly in front of this tRNA gene, and a CCAGG *Bst*NI site at the 3′-end. pHtS was obtained by cloning a synthetic human tRNA^{Ser}(UGA) gene, deduced from the published human tRNA^{Ser}(UGA) sequence (Capone *et al.*, 1985) which is preceded by a T7 promoter and followed by the CCAGG *Bst*NI recognition sequence, into the *Hin*dIII and *Sma*I sites of pUC19.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out according to Kunkel (1985) in ssM13mp18 or M13mp19. Mutations were confirmed by dideoxynucleotide chain termination DNA sequencing (Sanger *et al.*, 1977).

In vitro transcription of tRNA genes with T7 RNA polymerase

In vitro transcription with T7 RNA polymerase was performed essentially as published by Himeno *et al.* (1989). tRNA transcripts were purified on a 10% polyacrylamide–8 M urea gel and eluted from the gel slices with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 10% phenol.

Dephosphorylation of tRNA

15 μg of tRNA$^{(Ser)Sec}$, transcribed by T7 RNA polymerase in the presence of 20 mM GMP and 2 mM GTP, were incubated with 0.06 U of calf intestinal phosphatase in 50 mM Tris–HCl, pH 8.0, at 50°C for 1 h to remove the 5′-phosphate group (Beier and Gross, 1991). The dephosphorylated tRNA was purified as described above.

In vitro phosphorylation of seryl-tRNA

Two procedures were used for the phosphorylation assay according to the charging and phosphorylation efficiencies of the tRNA. Procedure I was applied to tRNA, which was efficiently serylated and phosphorylated. tRNA (0.33 μM) was first aminoacylated in 20 μl of aminoacylation buffer (20 mM imidazole–HCl, pH 7.5; 150 mM KCl, 8 mM $MgCl_2$, 0.5 mM DTT), 5 mM ATP, 0.5 mM CTP, 0.65 μM [^{3}H]serine (31 Ci/mmol) and 4% (v/v) S100 extract which had been used frequently, i.e. thawed and frozen. Up to 80% serylation was achieved for certain transcripts. Relative serylation efficiencies are indicated in Figure 1. [^{3}H]Seryl-tRNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 1 μg of total yeast tRNA. The pellet was washed with 70% ethanol to remove free [^{3}H]serine, dried and redissolved in water. Some 2500 c.p.m. of [^{3}H]seryl-tRNA were then incubated in 25 μl of the same solution used for aminoacylation and 8% (v/v) of HeLa S100 extract (kept at −83°C), except that [^{3}H]serine was omitted. After incubation at 37°C for 1 h, RNA was recovered as above.

For tRNA which was efficiently charged with serine, but inefficiently or not phosphorylated, an alternative procedure (procedure II) was used. Serylation and phosphorylation were coupled in a single incubation volume of 25 μl containing 1.6 μM tRNA, 0.65 μM [^{3}H]serine (31 Ci/mmol), 5 mM ATP, 0.5 mM CTP, aminoacylation buffer and 8% (v/v) S100 extract. For tRNA which was poorly charged, the reaction was simply scaled up to 100 μl. Further treatment was as described in procedure I (above).

Determination of phosphoseryl-tRNA formation

The formation of phosphoseryl-tRNA was determined by paper chromatography as described by Mäenpää and Bernfield (1970). Briefly, the mixture of phospho-[^{3}H]seryl-tRNA and [^{3}H]seryl-tRNA was first deacylated in fresh ammonia solution (0.15 M) at 37°C for 1 h. The resulting phospho-[^{3}H]serine and [^{3}H]serine were then separated by paper (Whatman 3 MM) chromatography with 1-butanol/acetic acid/H_2O (12:3:5, v/v) at room temperature for 26 h. The paper was dried at 65°C and cut into 1 cm long pieces. The radioactivity on each piece was measured by scintillation counting. To confirm the position of [^{3}H]serine and phospho-[^{3}H]serine, authentic serine and phosphoserine were chromatographed in parallel and stained with 0.25% ninhydrin in ethanol (Stepka, 1957).

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