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Opal suppressor serine tRNAs from bovine liver form phosphoseryl-tRNA

(nucleotide sequence of a minor species of tRNA/protein synthesis *in vitro*/protein modification)

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ABSTRACT **An unusual minor species of bovine liver serine tRNA has previously been isolated, sequenced, and found to suppress the UGA termination codon in protein synthesis *in vitro* [Diamond, A., Dudock, B. & Hatfield, D. (1981) *Cell* 25, 497-506]. We have now found that this tRNA can be a substrate in a specific phosphorylation reaction in which phosphoseryl-tRNA is formed. Moreover, bovine liver contains a second UGA suppressor serine tRNA ($tRNA^{Ser}_{NCA}$; N is a modified nucleoside) which also forms phosphoseryl-tRNA. The nucleotide sequence and coding properties of $tRNA^{Ser}_{NCA}$ are presented.**

The suppression of termination codons by naturally occurring suppressor tRNAs has been best studied in prokaryotic systems. The genomes of both bacteriophage $Q\beta$ (1, 2) and λ (3) code for proteins that arise as a consequence of partial readthrough of UGA termination signals by the normal tryptophan tRNA of the host. In eukaryotes, tRNAs that suppress nonsense codons have been observed in yeast and *Drosophila* (4) as well as in mammalian cells (5, 6). A tyrosine tRNA from *Drosophila* that lacks the highly modified Q base in its anticodon is responsible for suppressing the termination codon UAG, whereas the corresponding tyrosine tRNA with a fully modified Q base does not read UAG (4). In mammalian cells a naturally occurring suppressor tryptophan tRNA has been shown to read the UGA stop codon of β-hemoglobin mRNA both in rabbit reticulocyte lysates and in intact reticulocytes (5). In addition, we have reported the characterization and nucleotide sequence of an unusual bovine liver UGA suppressor serine tRNA ($tRNA^{Ser}_{CmCA}$) (6).

In this paper we report that $tRNA^{Ser}_{CmCA}$ can be a substrate in a specific phosphorylation reaction in which phosphoseryl-tRNA is formed. In addition, there exists another bovine liver suppressor serine tRNA ($tRNA^{Ser}_{NCA}$, in which N is a modified nucleoside whose structure has not yet been determined) which also forms phosphoseryl-tRNA. The nucleotide sequence and coding properties of $tRNA^{Ser}_{NCA}$ are presented here. This tRNA, like $tRNA^{Ser}_{CmCA}$, is aminoacylated with serine and suppresses the UGA termination codon *in vitro*. The nucleotide sequences of $tRNA^{Ser}_{NCA}$ and $tRNA^{Ser}_{CmCA}$ differ in only six positions, all located in the 5′ 40% of the molecule.

MATERIALS AND METHODS

tRNA was isolated from bovine liver (7), estrogen-treated rooster liver (8), and rabbit reticulocytes (7). Aminoacyl-tRNA synthetases were prepared from rabbit reticulocytes as described (7). Conditions of aminoacylation were the same as those described (6, 7) except that [^{3}H]serine (14-28 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was used. Bovine liver $tRNA^{Ser}_{CmCA}$ was purified as described (6). $tRNA^{Ser}_{NCA}$ was isolated from fresh bovine liver (9) and subsequently purified by three successive chromatographic runs on RPC-5 columns, first in a linear 0.525-0.725 M NaCl gradient as described (7), followed by the same NaCl gradient run in the absence of Mg^{2+}, and finally in a 0.45 M NaCl (150 ml) to 1.0 M NaCl (75 ml) concave gradient. The final purification was by preparative electrophoresis on a 20% polyacrylamide gel (10).

Extracts of rooster liver (8) and bovine mammary tissue (11) were prepared as described except that the tissues were homogenized in 0.01 M Tris·HCl, pH 7.5/0.01 M $MgCl_2$/0.01 M 2-mercaptoethanol/0.01 M sodium acetate/10% (vol/vol) glycerol/0.25 M sucrose and that the fraction eluting from a DEAE-cellulose column (DE-52, Whatman) in 0.25 M KCl was used immediately as a source of seryl-tRNA synthetase and kinase. Reaction conditions for phosphoseryl-tRNA formation were the same as for aminoacylation of tRNA (6, 7) except that in one experiment 10 mCi of [γ-^{32}P]ATP (obtained from New England Nuclear and diluted to 10 Ci/mmol) was used. [^{32}P]Phospho[^{3}H]seryl-$tRNA^{Ser}_{NCA}$ and [^{32}P]phospho[^{3}H]seryl-$tRNA^{Ser}_{CmCA}$ were prepared with [^{3}H]serine and [γ-^{32}P]ATP in the presence of an enzyme extract from mammary tissue. Seryl-tRNA and phosphoseryl-tRNA were deacylated (11) and the resulting amino acid products were identified by chromatography in 1-butanol/acetic acid/water, 12:3:5 (vol/vol), and by high-voltage electrophoresis at pH 3.5 (8, 11). Assays for the suppression of the UGA termination signal of rabbit β-globin mRNA and the ribosomal binding of seryl-tRNAs were performed as described (6). Nucleotide sequence analysis of $tRNA^{Ser}_{NCA}$ was performed essentially as described (6).

RESULTS

Seryl-$tRNA^{Ser}_{NCA}$ and Seryl-$tRNA^{Ser}_{CmCA}$ in the Total Bovine Liver Serine tRNA Population. Seryl-$tRNA^{Ser}_{NCA}$ and seryl-$tRNA^{Ser}_{CmCA}$ were each chromatographed together with total bovine liver seryl-tRNA on an RPC-5 column. Cochromatography of total bovine liver [^{14}C]seryl-tRNA with [^{3}H]seryl-$tRNA^{Ser}_{NCA}$ and [^{3}H]seryl-$tRNA^{Ser}_{CmCA}$ are shown in Fig. 1 *A* and *B*, respectively. These tRNA profiles show that seryl-$tRNA^{Ser}_{NCA}$ and seryl-$tRNA^{Ser}_{CmCA}$ represent only a small proportion of the total serine tRNA population in bovine liver, that there is slightly more seryl-$tRNA^{Ser}_{NCA}$ than seryl-$tRNA^{Ser}_{CmCA}$, and that the seryl-$tRNA^{Ser}_{CmCA}$ elutes slightly later than seryl-$tRNA^{Ser}_{NCA}$ from the RPC-5 column. Both isoacceptors recognize only the nonsense codon UGA in a ribosomal binding assay (see Table 1, experiments 1 and 2).

Nucleotide Sequence. We have previously shown that the

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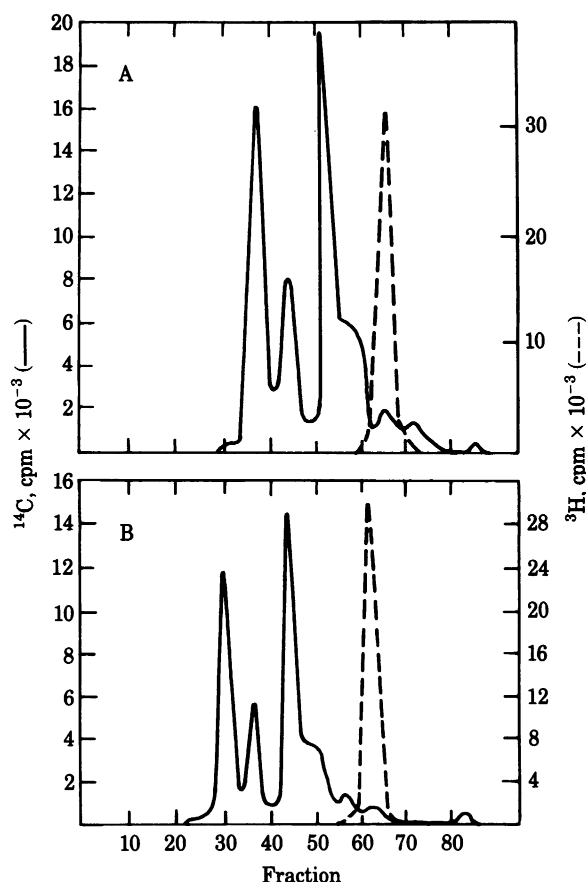

FIG. 1. Cochromatography of total bovine liver [^{14}C]seryl-tRNA with [^{3}H]seryl-tRNA$^{Ser}_{NCA}$ (*A*) and [^{3}H]seryl-tRNA$^{Ser}_{CmCA}$ (*B*). Total bovine liver tRNA was aminoacylated with [^{14}C]serine (171 mCi/mmol) and the purified seryl-tRNAs were each aminoacylated with [^{3}H]serine (14 Ci/mmol) by using rabbit reticulocyte synthetase. Each [^{3}H]seryl-tRNA (- - -) was cochromatographed with total bovine liver [^{14}C]seryl-tRNA (——) on an RPC-5 column as described (6, 7).

nucleotide sequence of a UGA suppressor serine tRNA (tRNA$^{Ser}_{CmCA}$) exhibits a number of unusual structural features (6). Among these are a dihydrouridine stem region containing two extra nucleotides compared to other tRNAs, a non-base-paired extra nucleotide within the double-stranded loop IV stem, only six modified residues, and a tryptophan anticodon, CmCA. The nucleotide sequence of tRNA$^{Ser}_{CmCA}$ was determined by using three independent methods as described (6). Similar methods were used to determine the structure of tRNA$^{Ser}_{NCA}$ (Fig. 2). The arrows in Fig. 2*A* indicate the differences between tRNA$^{Ser}_{NCA}$ and tRNA$^{Ser}_{CmCA}$. The two sequences are very similar, differing in only six positions. Both tRNAs share the above-mentioned unusual features except that tRNA$^{Ser}_{NCA}$ has only four modified residues and does not have a 2′-*O*-methylated C in the wobble position of the anticodon. The absence of this modified residue was confirmed by the absence of gaps in autoradiograms of "ladders" generated by either formamide or RNase digestion of end-labeled tRNA$^{Ser}_{NCA}$ electrophoresed on polyacrylamide gels. The residue in the wobble position of the anticodon of tRNA$^{Ser}_{NCA}$ is a modified nucleotide whose structure is unknown.

Fig. 2 shows tRNA$^{Ser}_{NCA}$ in two different cloverleaf forms. In Fig. 2*A* the tRNA has five base pairs in the dihydrouridine stem and nine nucleotides in the dihydrouridine loop but it lacks the universally present unpaired base between the dihydrouridine and anticodon stems. In Fig. 2*B* the tRNA is shown with maximal base pairing in the dihydrouridine stem. Both of these cloverleaf forms, as well as several additional ones which can be drawn, show the very unusual structural characteristics of this tRNA.

tRNA$^{Ser}_{NCA}$ Can Suppress UGA Termination. We have previously demonstrated that tRNA$^{Ser}_{CmCA}$ can suppress the opal termination codon, UGA, in an *in vitro* translation assay using rabbit globin mRNA (6). In Fig. 3, we show that tRNA$^{Ser}_{NCA}$ can also suppress UGA. A yeast opal suppressor (14), tRNA$^{Ser}_{CmCA}$, and tRNA$^{Ser}_{NCA}$ all result in the appearance of readthrough bands when added to reticulocyte lysates programmed with rabbit globin mRNA (see lanes a, b, and c, respectively).

Phosphoseryl-tRNA$^{Ser}_{NCA}$ and Phosphoseryl-tRNA$^{Ser}_{CmCA}$. Formation of phosphoseryl-tRNA from a minor species of seryl-tRNA was reported in 1970 in rooster and rat liver (8) and subsequently in lactating bovine mammary glands (11). To determine if tRNA$^{Ser}_{CmCA}$ and tRNA$^{Ser}_{NCA}$ could be phosphorylated, total tRNA from bovine liver was aminoacylated with [^{3}H]serine in the presence of ATP and an enzyme preparation from rooster liver. The resulting [^{3}H]seryl-tRNA was chromatographed on an RPC-5 column (see *Inset* of Fig. 4). Fractions from the column were pooled as shown and designated with a roman numeral in the order of elution. Fraction VI, which contained the serine isoacceptors recognizing UGA, was chromatographed again on an RPC-5 column. Three peaks and a trailing shoulder of [^{3}H]seryl-tRNA were obtained (Fig. 4). Each was pooled, and they were designated A–D in the order of elution. Ribosomal binding and deacylation studies were performed on each of the latter fractions. As shown in Table 2, fraction VI A responded to UCU but not to UGA, fractions VI B and VI C responded strongly to UGA and slightly to UCU, and fraction VI D responded only slightly to UCU and UGA. Fractions VI B and VI C correspond to seryl-tRNA$^{Ser}_{NCA}$ and to seryl-tRNA$^{Ser}_{CmCA}$, respectively. The responses of fractions VI B and VI C to UCU, however, demonstrate that they were not completely separated from a serine isoacceptor that recognizes UCU, as well as UCC and UCA (data for UCC and UCA binding not shown). Descending paper chromatography of the products of deacylation show that, although all seryl-tRNA species except those likely to be contaminated with tRNA$^{Ser}_{NCA}$ and tRNA$^{Ser}_{CmCA}$ contained a very small amount of phosphoserine (<1%), fractions VI B and VI C contained 39% and 35% phosphoserine, respectively. These studies clearly demonstrate that an enzyme preparation from rooster liver forms phosphoseryl-tRNA in a phosphorylation reaction with the isoacceptors recognizing UGA.

We have also prepared tRNA from rooster liver, aminoacylated it with [^{3}H]serine in the presence of an enzyme extract from rooster liver, and isolated the isoacceptor corresponding to seryl-tRNA$^{Ser}_{CmCA}$. Approximately 65% of this isoacceptor responded to UGA in the ribosomal binding assay, and it contained 57% phosphoserine (Table 1, experiment 3). In confirmation of the work of Sharp and Stewart (11), we also obtained an enzyme extract from lactating bovine mammary tissue that is capable of forming phosphoseryl-tRNA. Aminoacylation of bovine liver tRNA with mammary tissue extract demonstrated that approximately 50% of the isoacceptors corresponding to seryl-tRNA$^{Ser}_{NCA}$ and seryl-tRNA$^{Ser}_{CmCA}$ responded to UGA in the binding assay and contained approximately 65% phosphoserine (Table 1, experiment 4). The relatively high levels of phosphoseryl-tRNA formed with seryl-tRNA$^{Ser}_{NCA}$ and seryl-tRNA$^{Ser}_{CmCA}$ in the presence of enzyme extracts from rooster liver and mammary tissue in conjunction with the strong response of these isoacceptors to UGA strongly suggest that phosphoseryl-tRNA can also recognize UGA in the ribosomal binding assay. [^{32}P]Phospho[^{3}H]seryl-tRNA$^{Ser}_{CmCA}$ and [^{32}P]phospho[^{3}H]seryl-tRNA$^{Ser}_{NCA}$ were also prepared and their binding to ribosomes was

Table 1. Binding of seryl-tRNA$^{Ser}_{NCA}$, seryl-tRNA$^{Ser}_{CmCA}$, and phosphoseryl-tRNA to ribosomes

Exp.	[^{3}H]Ser-tRNA*	Codon	Δ cpm† ^{3}H	Δ cpm† ^{32}P	cpm added ^{3}H	cpm added ^{32}P	A_{260} units of tRNA added	% bound‡ ^{3}H	% bound‡ ^{32}P	% phosphoserine§
1	tRNA$^{Ser}_{NCA}$	UGA	8,849		12,748		0.007	69.4		ND
		Other¶	<4%		12,748		0.007			
		None	(703)		12,748		0.007			
2	tRNA$^{Ser}_{CmCA}$	UGA	8,689		11,496		0.002	75.6		ND
		Other¶	<4%		11,496		0.002			
		None	(614)		11,496		0.002			
3	tRNA$^{Ser}_{CmCA}$	UGA	7,400		11,420		0.008	64.8		57.6
		UCU	143		11,420		0.008	1.3		
		None	(1,469)		11,420		0.008			
4	tRNA$^{Ser}_{NCA}$	UGA	6,039		12,798		0.015	47.2		67.9
		UCU	153		12,798		0.015	1.2		
		None	(1,096)		12,798		0.015			
	tRNA$^{Ser}_{CmCA}$	UGA	7,865		14,045		0.020	56.0		61.7
		UCU	290		14,045		0.020	2.1		
		None	(2,282)		14,045		0.020			
5	[^{32}P]Phospho[^{3}H]-seryl-tRNA$^{Ser}_{NCA}$	UGA	885	363	2,423	2,385	0.015	36.5	15.2	>50%‖
		UCU	56	−20	2,423	2,385	0.015	—	—	
		None	(617)	(335)	2,423	2,385	0.015			
	[^{32}P]Phospho[^{3}H]-seryl-tRNA$^{Ser}_{CmCA}$	UGA	1,129	562	2,970	2,115	0.008	38	26.6	>50%‖
		UCU	28	−28	2,970	2,115	0.008	—	—	
		None	(597)	(346)	2,970	2,115	0.008			

* The source of the tRNA was bovine liver except in experiment 3, in which rooster liver was the source. The source of the enzymes used were experiments 1 and 2, rabbit reticulocyte; experiment 3, rooster liver; experiments 4 and 5, mammary tissue.

† cpm bound to ribosomes in the presence of codon minus cpm bound in the absence of codon. The latter values are given in parentheses after "None" in the codon column.

‡ Percent of cpm bound to ribosomes in response to codon. Value was obtained by dividing cpm bound to ribosomes in response to codon (column 4) by cpm added to assay (column 5).

§ Samples were deacylated after coding studies and percent of phosphoserine was determined as given below. ND, not determined.

¶ Other codons tested in the binding studies were the serine codons (UCU, UCC, UCA, UCG, and AGU) and codons containing a single substituted base in UGA (UGU, UGC, UGG, UUA, UAA, AGA, and CGA), except codon GGA, which was not tested. The amount of [^{3}H]seryl-tRNA bound to ribosomes in response to these codons was less than 4% of that bound in the presence of UGA.

‖ Percent phosphorylation was estimated from the specific activities of [^{32}P]phosphate and [^{3}H]serine in phosphoserine and from experiments carried out at the same time in which phosphoseryl-tRNA$^{Ser}_{NCA}$ and phosphoseryl-tRNA$^{Ser}_{CmCA}$ were prepared with unlabled ATP (see experiment 4).

examined. These studies show conclusively that the binding of phosphoseryl-tRNA to ribosomes is stimulated by UGA (Table 1, experiment 5).

DISCUSSION

We have shown that in higher eukaryotes there is a unique class of minor serine tRNAs. Thus far we have characterized two of these tRNAs, tRNA$^{Ser}_{NCA}$ and tRNA$^{Ser}_{CmCA}$. Both suppress UGA, both form phosphoseryl-tRNA, and both have unusual structural features. It is striking that the differences between these two sequences involve pyrimidine transitions (with the possible exception of residue 36) and that they all occur within the 5′ 40% of the molecules. The unusual structure of these tRNA molecules raises an interesting question concerning the expression of minor species of tRNA. Transcripts made by RNA polymerase III have been shown to be controlled from regions within the actual gene. This has been demonstrated for the *Xenopus laevis* 5S RNA gene (16, 17) and the VA1 gene of adenovirus (18). Galli *et al.* (19) have pointed out that the 5′ promoter sequences of tRNA^{Met} and tRNA^{Leu} from *Xenopus laevis* coincide with a conserved sequence found in all eukaryotic tRNA genes. This sequence block is located between residues 8 and 18, and the consensus sequence is T-G-G-C-N-N-A-G-T-G-G (N here represents ordinary nucleosides). It is interesting to note that tRNA$^{Ser}_{CmCA}$ and tRNA$^{Ser}_{NCA}$, both minor species of serine tRNA, contain two extra nucleotides within this important sequence. It is therefore an enticing hypothesis that the relatively low levels of certain minor species of tRNA are the result of the alteration of the 5′ internal sequences that function as a promoter for tRNA genes.

Isoacceptors corresponding to tRNA$^{Ser}_{NCA}$ and tRNA$^{Ser}_{CmCA}$ have been found in a variety of organisms, including *Xenopus* liver, rabbit liver, and human reticulocytes (data not shown). These tRNAs have been identified both by their chromatographic properties on an RPC-5 column and by their specific response to UGA in a ribosome-binding assay. Rabbit reticulocytes contain an isoacceptor corresponding to seryl-tRNA$^{Ser}_{NCA}$, but little or none of the isoacceptor corresponding to seryl-tRNA$^{Ser}_{CmCA}$. The seryl-tRNA population of chicken liver contains substantially more of the isoacceptor corresponding to seryl-tRNA$^{Ser}_{CmCA}$ than that corresponding to seryl-tRNA$^{Ser}_{NCA}$ (data not shown).

A number of questions are raised by this work. Are nonsense codons being used in eukaryotes as signals for events other than the termination of translation? The presence of two naturally occurring UGA suppressor serine tRNAs and one naturally occurring tryptophan tRNA (5) in higher eukaryotes would suggest

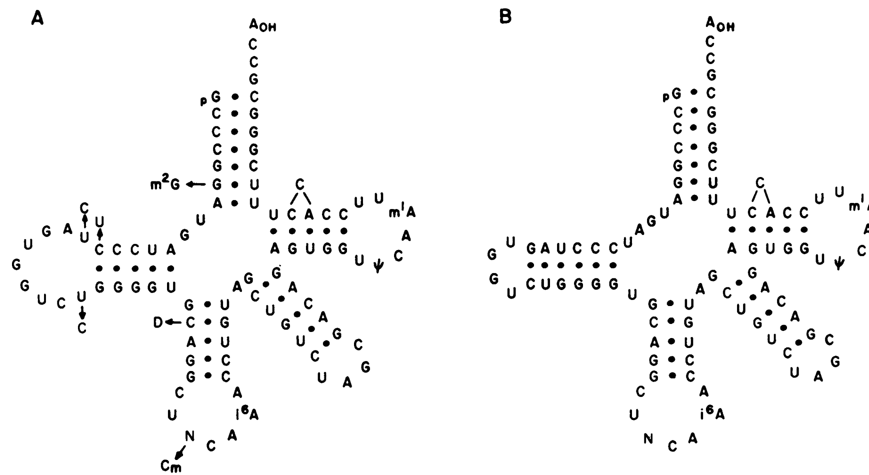

FIG. 2. Nucleotide sequence of $tRNA^{Ser}_{NCA}$ shown in two different cloverleaf forms. Arrows in *A* show the corresponding residues in $tRNA^{Ser}_{CmCA}$. Residue N is a modified nucleoside found in the wobble position of the anticodon. pNp, obtained from the formamide fragment analysis, migrated with an R_{pUp} = 1.18 in the ammonium sulfate system of Gupta and Randerath (12). pN migrated like pT in the two-dimensional thin-layer system of Silberklang *et al.* (13). The tRNA is cleaved at residue N by RNase U2 but not by RNase T1. Partial cleavage is observed with the pyrimidine-specific RNase from *Bacillus cereus*. Although a small amount of m^2G was observed at residue 6 by formamide fragment analysis, it is most likely that this is the result of a low level of contamination by $tRNA^{Ser}_{CmCA}$ rather than partial modification of residue G-6 in $tRNA^{Ser}_{NCA}$.

that the incorporation of an amino acid in response to UGA does occur. We do not know if the consequence of having these suppressor tRNAs is occasional readthrough of all UGA codons or if there exists a certain subset of UGA codons for which readthrough is tolerated. The second question concerns the role of phosphoseryl-tRNA. Does this molecule function in protein synthesis to insert phosphoserine into a growing polypeptide chain and, if so, for what function? Indeed, our data showing that [^{32}P]phosphoseryl-tRNA can bind to ribosomes in response to UGA would indicate that this possibility is likely. We hypothesize that the modifications found in proteins are of two

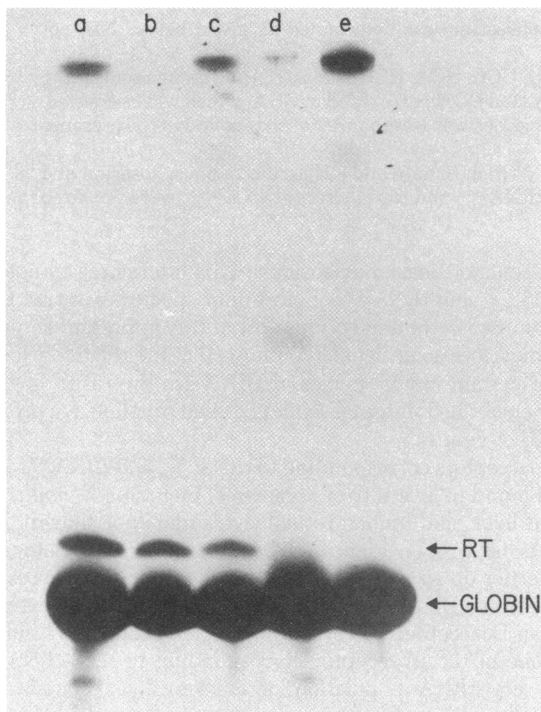

FIG. 3. Suppression of UGA by $tRNA^{Ser}_{CmCA}$ and $tRNA^{Ser}_{NCA}$. Translation reactions were performed and the resulting products were identified as described (6). Reaction mixtures contained the following: lane a, 10 μg of tRNA containing a yeast opal suppressor tRNA (14); lane b, 0.875 μg of $tRNA^{Ser}_{CmCA}$ (6); lane c, 0.25 μg of $tRNA^{Ser}_{NCA}$; lane d, 10 μg of crude bovine liver tRNA; and lane e, no additions. RT indicates the position of the β-globin readthrough product.

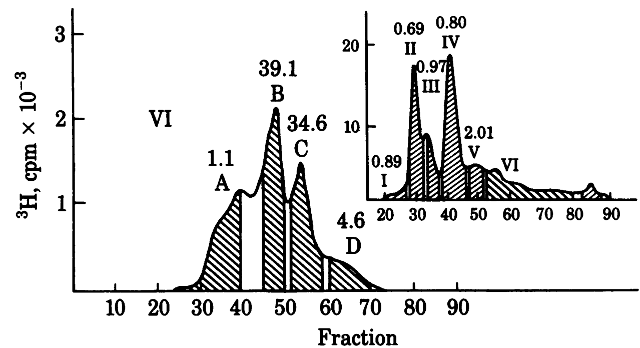

FIG. 4. Identification of phosphoseryl-tRNA in the total population of bovine liver serine tRNAs. Total bovine liver tRNA was aminoacylated with [^{3}H]serine (17 Ci/mmol) in the presence of an enzyme extract from rooster liver and the [^{3}H]seryl-tRNA was chromatographed on an RPC-5 column. The resulting fractions were pooled as designated by the hatched areas in the *Inset* and fractions I–V were deacylated. Fraction VI was rechromatographed on an RPC-5 column with a linear 0.575–0.70 M NaCl gradient as described (6, 7), and the resulting fractions were pooled as shown by hatched areas on the main graph and prepared for coding studies. The results of the coding studies are presented in Table 2. The remainders of column fractions VI A–D were then deacylated. Deacylated fractions were chromatographed in 1-butanol/acetic acid/water, 12:3:5 (vol/vol), for 30 hr and areas corresponding to serine (relative R_f = 1.0) and phosphoserine (relative R_f = 0.46) were cut from the developed chromatograms and their radioactivities were measured. Percent of total ^{3}H cpm detected as phosphoserine in fractions I–V is shown in the *Inset* above each peak and the values for fractions VI A–D are shown in the main graph. Total cpm detected on the chromatograms in each fraction were I, 48,984; II, 400,356; III, 150,935; IV, 457,600; V, 133,621; VI A, 47,574; VI B, 52,532; VI C, 32,130; and VI D, 10,916. Whatman paper containing the ^{3}H-labeled material corresponding to phosphoserine was rinsed in toluene to remove scintillation fluid and then dried, and the ^{3}H-labeled material was eluted with water. This material was divided into two equal portions, one of which was incubated with alkaline phosphatase. Aliquots of both samples were rechromatographed in the same solvent. ^{3}H migrated with authentic serine from the sample treated with alkaline phosphatase and with authentic phosphoserine from the untreated sample.

Table 2. Binding of column fractions to ribosomes

Fraction	Codon	Δ cpm	cpm added	A_{260} units of tRNA added
VI A	UCU	4,471	11,954	0.016
	UGA	9	11,954	0.016
	None	(4,052)	11,954	0.016
VI B	UCU	1,176	13,530	0.018
	UGA	7,113	13,530	0.018
	None	(2,447)	13,530	0.018
VI C	UCU	346	11,980	0.023
	UGA	5,784	11,980	0.023
	None	(1,744)	11,980	0.023
VI D	UCU	237	4,308	0.018
	UGA	144	4,308	0.018
	None	(1,267)	4,308	0.018

Binding studies were carried out as described in ref. 15. See Table 1 for explanations.

origins, those that are put in at the post-translational level, which probably make up the majority of protein modifications and, in addition, some that are put in during the process of protein synthesis itself by the direct incorporation of an "odd" amino acid in response to a specific codon. A study of the role of phosphoseryl-tRNAs in protein synthesis may provide direct support for this hypothesis.

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