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Unique pathway of expression of an opal suppressor phosphoserine tRNA

(tRNA transcription/tRNA processing/tRNA transport/tRNA fingerprinting)

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ABSTRACT **An opal suppressor phosphoserine tRNA gene is present in single copy in the genomes of higher vertebrates. We have shown that the product of this gene functions as a suppressor in an *in vitro* assay, and we have proposed that it may donate a modified amino acid directly to protein in response to specific UGA codons. In this report, we show through *in vitro* and *in vivo* studies that the human and *Xenopus* opal suppressor phosphoserine tRNAs are synthesized by a pathway that is, to the best of our knowledge, unlike that of any known eukaryotic tRNA. The primary transcript of this gene does not contain a 5′-leader sequence; and, therefore, transcription of this suppressor is initiated at the first nucleotide within the coding sequence. The 5′-terminal triphosphate, present on the primary transcript, remains intact through 3′-terminal maturation and through subsequent transport of the tRNA to the cytoplasm. The unique biosynthetic pathway of this opal suppressor may underlie its distinctive role in eukaryotic cells.**

Transcription initiation of eukaryotic tRNAs begins at a purine nucleotide in the 5′ flank near the coding sequence of the gene (1, 2). The 5′-leader sequence must, therefore, be removed during maturation. Transcription termination occurs at a cluster of thymidine residues in the 3′ flank of tRNA genes (3), and the 3′-trailer sequence must also be removed. tRNAs are transcribed by RNA polymerase III. Following transcription and maturation, tRNAs are rapidly transported from the nucleus to the cytoplasm (4, 5).

In this study, we have determined the transcription, processing, and transport of an opal suppressor phosphoserine tRNA, which has been described in higher vertebrates (6–8). In fact, two opal suppressor phosphoserine tRNAs that arise from a single gene (9–11) have been identified (7). These isoacceptors manifest several distinctive characteristics that set them apart from all other eukaryotic tRNAs. (*i*) They are aminoacylated by seryl-tRNA synthase even though one of the isoacceptors has a tryptophan anticodon (6). (*ii*) They are 90 nucleotides long and thus are the longest tRNAs sequenced to date (6–8). (*iii*) They are highly undermodified (6–8). (*iv*) They contain two extra nucleotides between the universal uridine at position 8 and an adenosine at position 14 (6–8). (*v*) They contain an extra unpaired nucleotide within the stem of loop IV (6–8). (*vi*) They differ in their primary sequences by several pyrimidine transitions including one in the wobble position of their anticodons (7), and these transitions must occur post-transcriptionally since both species are encoded by the same gene (9–12). (*vii*) They are phosphorylated on their serine moiety by a kinase to form phosphoseryl-tRNA (7, 13). The genes corresponding to the human (10), rabbit (11), chicken (9), and *Xenopus* (unpublished results) opal suppressor phosphoserine tRNAs have been isolated and sequenced. The human and rabbit genes are identical in sequence and differ from those of *Xenopus* and chicken by a single pyrimidine transition at position 11. As shown in this study, transcription of the opal suppressor phosphoserine tRNAs begins at the first nucleotide within the coding sequence of the gene, the 3′-trailer sequence that is produced during transcription is processed by a purified 3′-processing enzyme (3′ pre-tRNase) (14), and the mature tRNA is transported from the nucleus to the cytoplasm demonstrating that it is a cytoplasmic species.

MATERIALS AND METHODS

Materials. HeLa cell extracts that were rich in RNA polymerase III were purchased from Bethesda Research Laboratories, *Xenopus* females were from Nasco, Fort Atkinson, WI, [α-^{32}P]GTP (specific activity, 400 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]UTP (specific activity, 400 Ci/mmol) were from Amersham, T1 RNase was from Bethesda Research Laboratories, P1 RNase was from P-L Biochemicals, cellulose acetate strips were from Schleicher & Schuell, and DEAE-cellulose plates were from Machery & Nagel.

tRNA Transcription, Processing, Transport, and Fingerprinting. Transcription of opal suppressor tRNA genes was carried out in HeLa cell extracts in the presence of labeled nucleoside triphosphates (14). Preparation of *Xenopus* oocytes, microinjection of plasmids and of labeled nucleoside triphosphates into *Xenopus* oocytes, and microdissection of *Xenopus* oocytes have been described elsewhere (4, 5). Preparation of transcription products from HeLa cell extracts and from *Xenopus* oocytes, separation of precursor and mature tRNAs on polyacrylamide gels, and precursor and mature tRNA isolations were carried out as described (4, 5, 14). Digestion of labeled precursor and mature tRNAs with T1 RNase, fingerprinting the resulting products, and identification of nucleotides and oligonucleotides on fingerprints were the procedures of Silberklang *et al.* (15). The 5′ processing enzyme (5′ pre-tRNase) (16) and 3′ pre-tRNase (14) were purified from *Xenopus laevis* ovaries.

Plasmid Constructions. A 193-base-pair *Ava* I–*Hin*dIII fragment encoding the human opal suppressor phosphoserine tRNA gene was subcloned into the *Hin*dIII–*Xma* I cloning site of pUC 18 and a 900-base-pair *Sph* I–*Acc* I fragment encoding the *Xenopus* opal suppressor tRNA gene was subcloned into the *Sph* I–*Acc* I cloning site of pUC 18 (unpublished results), and the resulting plasmids were used in transcription assays.

RESULTS

Primary Transcript of the Opal Suppressor tRNA Gene. The primary transcripts of all eukaryotic tRNA genes studied to

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Abbreviations: pppGp, guanosine 3′-phosphate 5′-triphosphate; pre-tRNase, tRNA processing enzyme.

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date contain sequences at both the 5′ and 3′ termini that are removed endonucleolytically to yield the mature sequence (2). To establish the structure of the primary transcript of the vertebrate opal suppressor tRNA gene, the *Xenopus* gene was transcribed *in vitro* in HeLa cell extracts in the presence of [α-^{32}P]GTP. The products were separated on a polyacrylamide gel, and a number of discrete bands were observed (Fig. 1, lane 1). To determine which bands corresponded to opal suppressor tRNA gene transcripts, each of the major bands designated a–e in lane 1 was extracted from the gels and incubated in HeLa cell extracts. Only the band designated d was altered by further incubation. This band was processed to band e suggesting that band d was the primary transcript and that band e is the mature transcript. This observation was confirmed by fingerprinting (see below); therefore, band d is also designated pre-tRNA^{Ser}, and band e is tRNA^{Ser} in the figure. The larger bands (designated a, b, and c) most certainly represent transcripts from the insert and vector that initiate and terminate at sites other than those involving the opal suppressor tRNA gene.

Bands d and e in Fig. 1 were subjected to RNase T1 fingerprint analysis. The result of this analysis demonstrated that these two bands were derived from transcription of the *Xenopus* gene (Table 1). Both species display the expected oligonucleotides based on the DNA sequence of the tRNA gene. Surprisingly, each species yielded guanosine 3′-phosphate 5′-triphosphate (pppGp) on T1 RNase digestion (Table 1). The presence of a triphosphate on the 5′ terminal nucleotide was confirmed by digestion with nuclease P1, followed by electrophoresis on cellulose acetate and by chromatography on PEI-cellulose plates. The amount of pppGp was less than the expected amount in each digest. Although the possibility exists that the opal suppressor tRNA may be transcribed from different initiation sites (17), which would account for the less than expected amount of pppGp observed in T1 digests, it seems more likely that a portion of the 5′-terminal triphosphate may be modified during maturation or may be degraded during digestion (see below). Similar results as those observed with the *Xenopus* gene transcripts were also obtained from the fingerprint analysis of the human tRNA gene transcripts (see Table 1).

The 3′ terminus was established by RNase T1 digestion of the transcription products synthesized in the presence of [α-^{32}P]UTP. RNA fingerprint analysis revealed that only the band corresponding to that designated 3′ pre-tRNA^{Ser} in Fig. 1 contains a 3′ terminus, and about 90% of this material has a 3′ terminus that extends from the end of the mature coding region to the first thymidine residue within the termination tract (i.e., at the 99th base; see Table 1). Minor species corresponding to transcripts terminating at each of the four thymidine residues within the major termination sequence and at the thymidine residue at position 93 were also identified. The primary transcript of the *Xenopus* opal suppressor deduced from these analyses is shown in Fig. 2. The most striking feature of this RNA species is that it does not contain a 5′-leader sequence. *In vitro*, furthermore, the mature tRNA that is cleaved of its 3′ terminus still retains the 5′ terminus of the primary transcript.

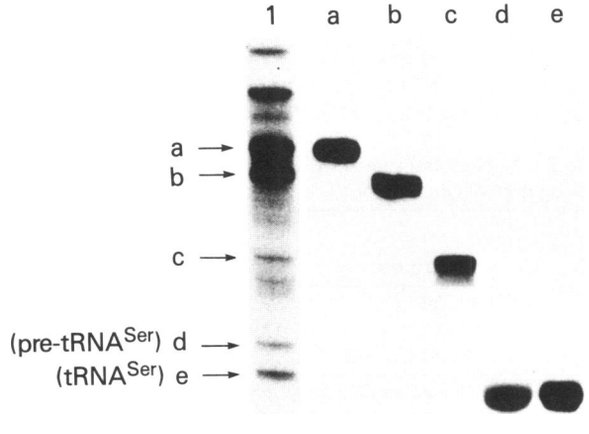

FIG. 1. Analysis of the transcription products generated in HeLa cell extracts. After incubation of the *Xenopus* opal suppressor tRNA gene in HeLa cell extracts, the products were electrophoresed on polyacrylamide gels, and an autoradiogram of the gel was prepared as shown in lane 1. The major bands in lane 1, designated bands a–e, were cut out, eluted from the gels, subjected to further incubation in HeLa cell extracts, and electrophoresed as shown in lanes a–e.

Table 1. Ratios of ^{32}P in oligonucleotides

Nucleotide or oligonucleotide	Mature tRNA Obs.	Mature tRNA Exp.	Precursor tRNA Obs.	Precursor tRNA Exp.
pppGp	0.7	1.0	0.6	1.0
Gp	2.6	3.0	3.3	4.0
UpGp	4.8	5.0	4.6	5.0
CpGp	2.1	2.0	2.0	2.0
ApGp	1.3	1.0	3.1	3.0
CpUpGp	1.1	1.0	1.0	1.0
CpApGp	1.7	2.0	1.9	2.0
ApUpGp	1.0	1.0	0.8	1.0
UpApGp	1.1	1.0	1.2	1.0
CpCpCpGp	1.6	2.0	1.7	2.0
UpCpUpGp	1.8	2.0	1.9	2.0
ApCpApGp	1.0	1.0	1.0	1.0
UpCpUpApGp	1.0	1.0	1.0	1.0
ApCpCpCpUpCpApGp	1.0	1.0	1.0	1.0
CpUpUpCpApApCpCpUpGp	1.0	1.0	1.0	1.0
UpUpCpApApUpUpCpCp-ApCpCpUpUpUpCpGp	1.7	2.0	1.9	2.0

The *Xenopus* opal suppressor phosphoserine tRNA gene was incubated in HeLa cell extracts with [α-^{32}P]GTP. After 1 hr at 30°C, reaction mixtures were electrophoresed in 10% denaturing polyacrylamide gels. The mature (87 bases long) and precursor (≈90% of which was 99 bases long and the remaining 10% was 93 and 100–103 bases long) tRNA transcripts were eluted from gels, digested with T1, and fingerprinted. The 87th (and last 3′) base in the *Xenopus* opal suppressor tRNA gene is a guanosine and the 3′-flanking sequence is AGGGCTTCTCCTTTTT where TTTTT represents the primary RNA polymerase III termination sequence. Location of resolved nucleotides and oligonucleotides on fingerprints was detected by autoradiography. Areas corresponding to the spots on autoradiograms were circled on chromatograms (DEAE-cellulose plates) and then removed; radioactivity was measured in a scintillation counter. The number of counts in ApCpApGp was standardized to 1.0. Nucleotides and oligonucleotides were identified by standard techniques from DNA sequences. Transcription assays were also carried out with the human opal suppressor tRNA gene. The mature tRNA was isolated from transcription assays and fingerprinted. Similar results as those given in the table were observed with the human gene with the exception of the oligonucleotide which was identified as ApUpCpUpApGp (see Fig. 2).

The Primary Transcript Is Processed Exclusively at Its 3′ End. All eukaryotic tRNA species studied to date are derived from nucleolytic processing of their corresponding primary transcripts by the action of endonucleases that remove both 5′ and 3′ termini (2). The enzymes that process these precursors have been purified from *X. laevis* ovaries (14, 16). A striking feature of these enzymes is that they process pre-tRNA transcripts in a distinct order, 5′ processing preceding 3′ processing (2, 14, 16). The order derives from the substrate specificity of the 3′-processing enzyme, which requires prior 5′ processing of the primary transcript before the species can be utilized as substrate (2, 14, 16).

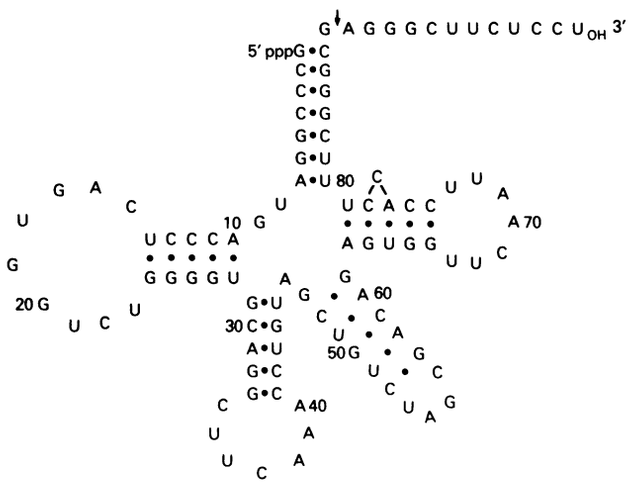

FIG. 2. Primary transcript of the *Xenopus* opal suppressor phosphoserine tRNA gene. The sequence of the *Xenopus* primary transcript is shown. The arrow shows the site of processing at the 3' end of the primary transcript. The sequence was deduced from the corresponding DNA sequence and is consistent with that determined by analysis of the transcription products described in the text. The termination site shown is the principal point of termination of the *Xenopus* opal suppressor tRNA gene. The only difference in the 3'-processed sequence of *Xenopus* and human transcripts is a pyrimidine transition at position 11.

When the primary transcript of the *Xenopus* opal suppressor gene was incubated in the presence of purified *Xenopus* 5' pre-tRNase, no processing of the species was observed (Fig. 3, lanes 2–4). In contrast, purified *Xenopus* 3' pre-tRNase readily cleaved the precursor to the mature species (lanes 5 and 6). Simultaneous exposure of the substrate to both enzymes had the same effect as seen with the 3' enzyme alone (lanes 7 and 8). These data demonstrate that the opal suppressor primary transcript may be processed by the same endonuclease that removes the 3' trailer from other tRNAs (14). Furthermore, 5' processing of the primary transcript is not required for 3'-end processing of this species, demonstrating that the 5'-terminal guanosine 5'-triphosphate (pppG) need not be removed for nucleolytic maturation of the primary transcript to occur.

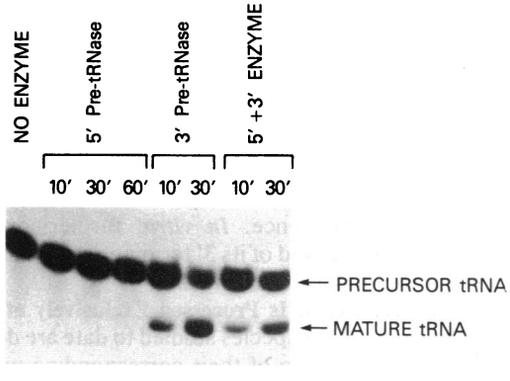

FIG. 3. Processing of opal suppressor phosphoserine precursor tRNA with purified pre-tRNases. Precursor tRNA was prepared from the *Xenopus* opal suppressor tRNA gene in HeLa cell extracts and isolated as in Table 1. Precursor tRNA was incubated with purified 5' or 3' pre-tRNase as follows. Lanes: 1, no enzyme; 2–4, 5' pre-tRNase; 5 and 6, 3' pre-tRNase; and 7 and 8, a mixture of 3' and 5' pre-tRNase. Aliquots were removed at the time intervals shown and electrophoresed on a 10% denaturing polyacrylamide gel. The gel was autoradiographed. The autoradiogram shown was an 18-hr exposure.

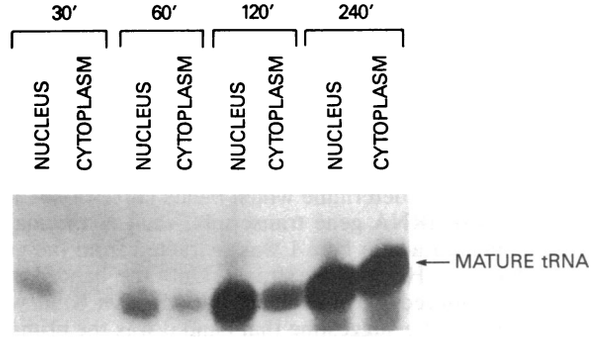

FIG. 4. Intracellular distribution in *Xenopus* oocytes of the opal suppressor phosphoserine tRNA. *Xenopus* opal suppressor tRNA gene (see Table 1) was injected into the nucleus of *Xenopus* oocytes and [α-^{32}P]GTP was injected into the cytoplasm. At the time intervals shown, intact nuclei and cytoplasm that was free of nuclei were prepared, and tRNAs were extracted and electrophoresed. The autoradiogram shown was a 14-hr exposure of the gel.

Opal Suppressor tRNA Is Transported to the Cytoplasm. Through manual microdissection of *Xenopus* oocytes, the intracellular distribution of RNA species generated on transcription can be studied (4). By such methods, regions of the tRNA molecule critical for tRNA nuclear transport have been mapped (5). To define the transport behavior of the *Xenopus* opal suppressor tRNA, the gene was introduced into the nucleus of the *X. laevis* oocyte, and the cell was subsequently dissected into nuclear and cytoplasmic fractions. As shown in Fig. 4, the opal suppressor tRNA is transported into the cytoplasm, establishing it as a cytoplasmic species. It is curious, however, that considerable intranuclear tRNA accumulates, in marked contrast to what is observed when $tRNA^{Met}$ is studied by analogous methods (4, 5). In the latter case, <10% of human $tRNA^{Met}$ remains in the nucleus 2–3 hr after microinjection of the gene (4, 5). The basis for this difference remains uncertain. However, the intracellular distribution of the opal suppressor shown in Fig. 4 is not a measure of the transport properties of this tRNA. tRNA nuclear transport can be measured by introduction of the mature tRNA into the nucleus of the *Xenopus* oocyte (4). To define the transport properties of the opal suppressor tRNA, we compared the nuclear transport behavior of the bovine opal $tRNA^{Ser}$ and a major species of bovine $tRNA^{Ser}$ by our standard technique (4). As shown in Table 2, after direct introduction into the oocyte nucleus, both tRNA species displayed comparable rates of nuclear transport. The kinetics of transport observed for the opal suppressor in this system ($t_{1/2}$ is $\approx$11 min) is very similar quantitatively to that observed for vertebrate $tRNA^{Met}$ (4, 5). We conclude that the

Table 2. Nuclear transport of the opal suppressor tRNA and a major species of serine tRNA

Compartment	tRNA, cpm	
	Opal	Ser
Cytoplasm	9,322	151,531
Nucleus	12,430	228,721
% transported	43%	40%

Opal and Ser designate opal suppressor tRNA and serine $tRNA_1$ (18), respectively. These tRNAs were purified first by RPC-5 chromatography and then by polyacrylamide gel electrophoresis (6, 7). Each tRNA was labeled with ^{32}P at its 5' end as described (9). ^{32}P-labeled tRNAs were injected into the nucleus of *Xenopus* oocytes, and, after 10 min, oocytes were fixed and microdissected. tRNA content in each compartment was determined by liquid scintillation counting as described (4). % transport from the nucleus represents that fraction of the total tRNA (nucleus + cytoplasm) present in the cytoplasm.

opal $tRNA^{Ser}$ has transport properties characteristic of a cytoplasmic tRNA.

***In Vivo* Transcription of the Opal Suppressor tRNA Gene.** The striking preservation of the 5′ terminus of the primary transcript in the mature tRNA observed *in vitro* was further examined on the mature tRNA species produced in the *X. laevis* oocyte after gene introduction. The tRNA accumulating in the cytoplasm of the oocyte was recovered, gel purified, and subjected to RNase T1 fingerprint analysis (Fig. 5). Each nucleotide and oligonucleotide is identified on the fingerprint. It is important to note the occurrence of the 5′ nucleotide pppGp in the cytoplasm. No guanosine 3′-phosphate 5′-phosphate (pGp) or guanosine 3′-phosphate 5′-diphosphate (ppGp) was detected on the fingerprints. The spot that runs slightly above pppGp in the second dimension was not identified. It may be a degradation product of pppGp that occurs during the transfer of the digest from cellulose acetate strips to DEAE-cellulose plates following electrophoresis or that occurs during homochromatography in the second dimension. It is also possible that this material could represent a modification of a portion of the 5′ nucleotide. Since the amount of radioactivity in this spot coupled with that found in pppGp closely approximates the expected value of 1.0 shown in Table 1, it most certainly appears that this material is part of the 5′ nucleotide.

DISCUSSION

The opal suppressor phosphoserine tRNAs manifest several distinctive characteristics compared to other eukaryotic tRNAs (see the introduction). In this study, we have observed additional properties specific to these tRNAs. For example, unlike any known tRNA, transcription starts at the 5′ nucleotide within the opal suppressor tRNA gene. Additionally, the triphosphate on the 5′ nucleotide of the tRNA remains intact in the cytoplasm suggesting that the triphosphate may have a role in the function of these tRNAs. On the other hand, the transport of the mature tRNA from the nucleus to the cytoplasm occurs with kinetic properties similar to those of other tRNAs (Table 2 and refs. 4 and 5). It should also be noted that the transcription and transport properties of the opal suppressor tRNAs resemble those of the *Alu* family members. The *Alu* family members are transcribed by RNA polymerase III beginning at the first base within the gene, contain a trailer sequence that is processed, and are transported efficiently from the nucleus to the cytoplasm (17).

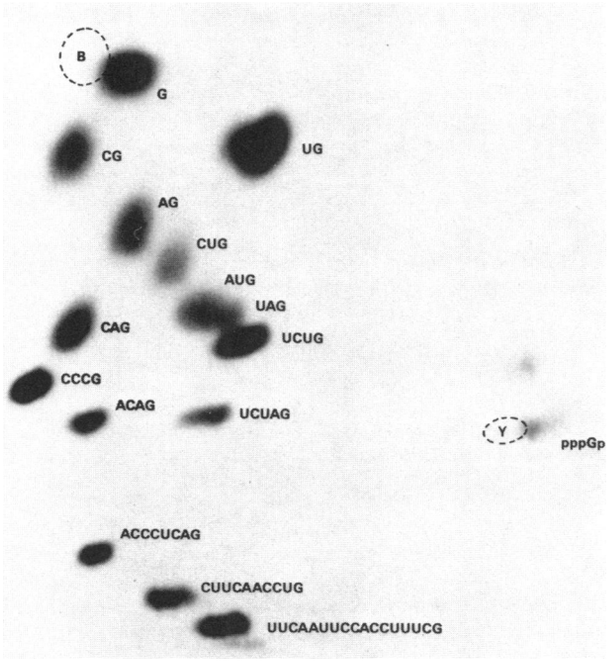

FIG. 5. Fingerprint of the opal suppressor phosphoserine tRNA isolated from the cytoplasm. *Xenopus* opal suppressor tRNA gene (see Table 1) was injected into the nuclei of *Xenopus* oocytes and [α-^{32}P]GTP into the cytoplasm. After 5 hr of incubation the transcript was isolated from the cytoplasm, digested with T1 RNase, and fingerprinted. The resulting nucleotides and oligonucleotides were identified as in Table 1. B, bromophenol blue; Y, methyl orange.

The *Xenopus* gene is severalfold more actively transcribed than the human gene in both HeLa cell extracts (homologous system for the human gene) and in *Xenopus* oocytes (homologous system for the *Xenopus* gene) (data not shown). In fact, in a study to be published elsewhere, we have determined that the order of transcription efficiencies in HeLa cell extracts of the four opal suppressor tRNA genes that have been sequenced thus far is *Xenopus* > human = rabbit > chicken; and we noted a correlation between the number of "TATA boxes" in the 5′-flanking regions of the opal suppressor tRNA genes and their relative transcription efficiencies in HeLa cell extracts. *Xenopus* has several TATA boxes in the 5′-flanking region of its gene, while the human (10) and rabbit (11) genes have a single TATA box and the chicken gene a TATA-like box (7).

In addition to the number of distinctive characteristics manifested by the opal suppressor phosphoserine tRNAs that were noted above, these isoacceptors may also have an specific cellular function. The fact that they read the termination codon UGA in protein synthesis (6, 7) and are phosphorylated on their serine moiety (7, 13) suggests that these tRNAs donate a modified amino acid directly to protein in response to specific UGA codons. A mRNA from Sindbis and from Middleburg viruses (19) and mouse glutathione peroxidase mRNAs (20) are the only ones from vertebrate sources that are known to contain UGA in an open reading frame. The amino acid at the read-through site in the viral protein is not known. However, the UGA in glutathione peroxidase mRNA codes for selenocysteine, which occurs at the active site of the protein. The backbone of the selenocysteine moiety at the active site of glutathione peroxidase arises from serine (21), which agrees with the possibility that the opal suppressor phosphoseryl-tRNA may be involved in the occurrence of selenocysteine in glutathione peroxidase. Whether the opal suppressor phosphoseryl-tRNA may be involved in the occurrence of selenocysteine in glutathione peroxidase remains to be established.

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