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Characterization and nucleotide sequence of a chicken gene encoding an opal suppressor tRNA and its flanking DNA segments

(minor serine tRNA/gene cloning)

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ABSTRACT **A naturally occurring opal suppressor serine tRNA has been purified from chicken liver and used as a probe to isolate the corresponding gene from a library of chicken DNA in bacteriophage λ. This minor tRNA is encoded by a single-copy gene that is not part of a tRNA gene cluster. DNA sequence analysis of the gene and its flanking DNA segments shows that the gene is encoded in an 87-base-pair segment without intervening sequences and specifies a tRNA that reads the termination codon UGA. This gene has additional nucleotides in the 5′ internal promoter region but has a normal 3′ internal promoter sequence and the usual termination signal.**

Molecular cloning and DNA sequencing have increased our understanding of the structure and organization of eukaryotic tRNA genes. In higher eukaryotes many of the major tRNA species are encoded by multiple structural genes, and tRNA genes often have a clustered organization. In *Drosophila* four separate gene clusters have been studied and found to contain arrays of the same (1) or different (2–5) tRNA genes. An exception to tRNA clustering is the $tRNA_i^{Met}$ genes, which are dispersed (6). Human tRNA genes can be clustered (7) or dispersed (8), with the $tRNA_i^{Met}$ genes serving again as the example of dispersal. A unique organization has been described for a cluster of four tRNA genes in *Xenopus* (9), in which the entire 3.18-kilobase-pair DNA segment containing them is tandemly repeated. Some tRNA genes contain introns. Their presence or absence has not yet been correlated with any other structural feature, but when present they occur in the anticodon loop (4, 10). Pseudogenes, either with deletions of coding sequence (11) or with extensive fragmentation and rearrangement of coding sequence (6), have been described in rat and *Drosophila* genomes, respectively. There are short oligonucleotides homologous to the coding sequence occurring in the regions flanking some tRNA genes (8); the significance of these oligonucleotides is unknown.

Most published work deals with major tRNA species. Here we report the characterization of a gene encoding a minor tRNA species with an unknown but apparently specialized function: a naturally occurring opal suppressor tRNA (12) that has the unique property of forming phosphoseryl-tRNA (13). The following questions about the gene(s) encoding this minor tRNA have been addressed: Are they multiple copy and clustered with other tRNA genes? Do they have more or different locations of introns, suggesting alternative processing routes? Do they have the same promoter and terminator sequences as other tRNA genes? (If not, they might require different transcription and termination factors.) Are they vestiges of a larger set of functional genes that became pseudogenes through deletion or rearrangement? The experimental evidence providing answers to these questions is presented below.

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METHODS

Purification of tRNA. Total tRNA was prepared from 2.1 kg of fresh chicken liver (12). The $tRNA^{Ser}$ that recognizes the nonsense codon UGA (13), hereafter referred to as the opal suppressor tRNA, was purified by a published benzoylated DEAE-cellulose column chromatography procedure (12) modified as follows: tRNA was loaded in 0.7 M NaCl/10 mM sodium acetate/10 mM magnesium acetate/1 mM EDTA, pH 4.5, and the column was extensively washed with the same solution. When more than 95% of the material absorbing light at 260 nm had eluted, a linear gradient of 0.7–1.5 M NaCl in the same buffer was applied. Finally, the column was washed with 2.5 M NaCl in 30% (vol/vol) ethanol. The fractions eluting between 0.98 and 1.5 M NaCl were pooled with the final wash and the opal suppressor tRNA was further purified by RPC-5 chromatography (13). The final purification step was two-dimensional polyacrylamide gel electrophoresis. Two additional tRNA fractions, which together contained about 80% of the total tRNA population, were prepared from the material depleted of the opal suppressor. The first was enriched for the major serine isoacceptors and the second was depleted of them (14).

Miscellaneous Procedures. Library screening, subcloning in pBR322, phage and plasmid DNA isolation, restriction analysis, gel electrophoresis, Southern transfer and hybridization, and labeling of DNA by nick-translation were all as previously described (15). DNA sequence analysis was by the method of Maxam and Gilbert (16), except that the G+A reaction was in 60% formic acid. tRNA was dephosphorylated and 5′-end-labeled by using phage T4 polynucleotide kinase and [γ-^{32}P]ATP.

RESULTS

Characterization of Purified tRNA. An opal suppressor tRNA has been purified from chicken liver and several methods have been combined to establish its identity and characteristics. In these measurements material from the stage prior to the final purification by two-dimensional polyacrylamide gel electrophoresis was used. The semipurified tRNA accepted 1,036 pmol of serine per A_{260} unit. Chromatography of this material with total seryl-tRNA on an RPC-5 column showed that it eluted after the major seryl-tRNA species and that it represented a small portion of the total seryl-tRNA population (Fig. 1). It specifically recognized the nonsense codon UGA and suppressed this termination codon in an *in vitro* translation assay (13) (data not

Abbreviation: bp, base pair(s).

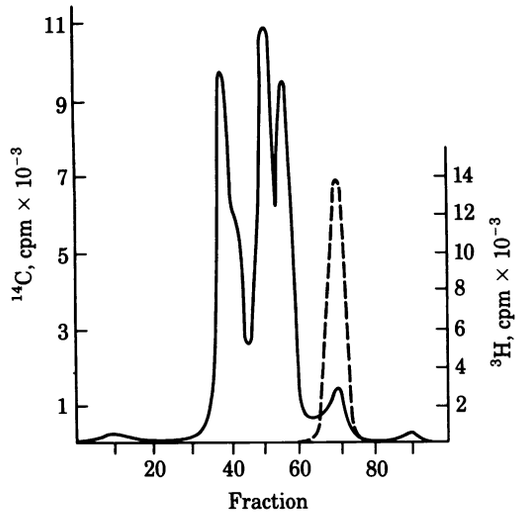

FIG. 1. Characterization of the opal suppressor tRNA probe. Total chicken liver tRNA aminoacylated with [^{14}C]serine (——) was chromatographed with purified opal suppressor tRNA aminoacylated with [^{3}H]serine (– – –). Aminoacylation of tRNA and RPC-5 chromatography were performed as described (12, 13).

shown). After final purification this tRNA was labeled with ^{32}P and used to screen a library of chicken DNA for the corresponding gene(s).

Isolation of a Recombinant Phage Carrying the tRNA Gene and Subcloning of the Gene Region. A total of 250,000 plaques from a chicken DNA library in bacteriophage λ Charon 4a were screened for the presence of DNA sequences complementary to the opal suppressor tRNA. A single positive plaque was identified, and from it phages were isolated, purified, amplified, and used as a source of DNA. This DNA was analyzed by restriction endonuclease analysis and the restriction fragments that hybridize to the tRNA were identified by Southern transfer hybridization. These results are shown in Fig. 2 *A* and *B*. Various restriction endonucleases were used to fragment the phage DNA and the tRNA hybridized to a single restriction fragment in each digest. The smallest restriction fragment detected by hybridization was about 300 base pairs (bp) in length (Fig. 2*A*, lane F). It is concluded that there is only one short region in this phage that hybridizes to the tRNA probe.

To construct a restriction map of the region homologous to the tRNA probe and to localize the homology region further, the 1,150-bp *Sau*3a fragment shown in Fig. 2*B*, lane M, was ligated into the *Bam*HI site of the plasmid pBR322. This re-

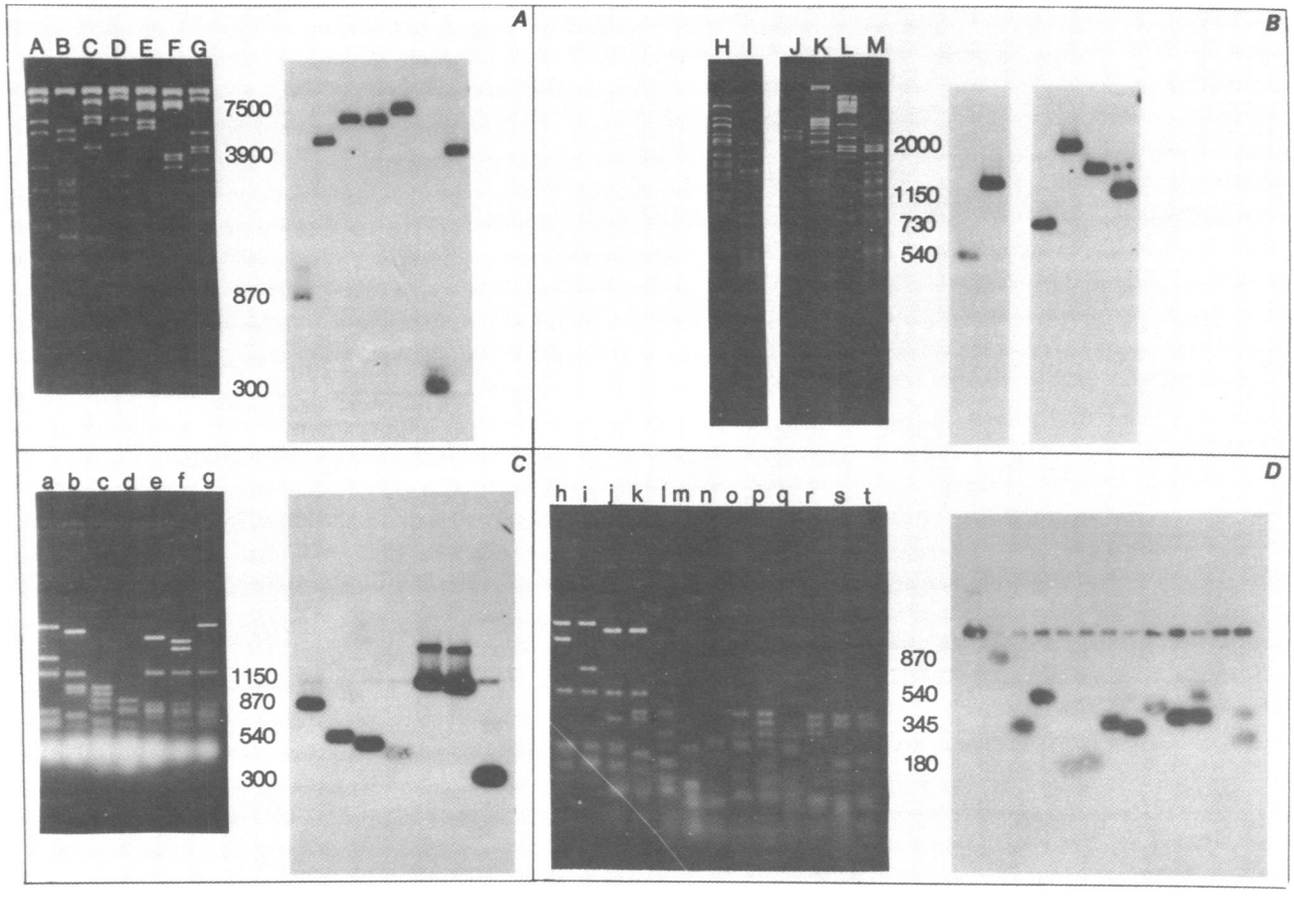

FIG. 2. Cloning and localization of the opal suppressor gene. DNA from the recombinant phage carrying the opal suppressor gene was digested with restriction nucleases. The fragments were separated in a 1% (*A*) or a 1.8% (*B*) agarose gel, transferred to nitrocellulose, and hybridized to purified opal suppressor tRNA labeled with ^{32}P. Ethidium bromide-stained gels and the corresponding autoradiograms are shown on the left, together with an indication of DNA fragment lengths (bp) derived by comparison with standard fragments on the same gel (not shown). Restriction nucleases were: A, *Ava* I; B, *Bgl* II; C, *Bam*HI; D, *Eco*RI; E, *Hin*dIII; F, *Kpn* I; G, *Sst* I; H, *Ava* II; I, *Hae* III; J, *Msp* I; K, *Pst* I; L, *Pvu* II; and M, *Sau*3a. The 1,150-bp *Sau*3a fragment that hybridized to this probe (lane M) was inserted into the *Bam*HI site of pBR322. This recombinant plasmid was digested with restriction endonucleases and the separated fragments were hybridized to the tRNA probe as above. *C* and *D* show the ethidium bromide-stained 2% agarose gels and the corresponding autoradiograms. All the DNA samples were digested first with *Sau*3a to release the inserted fragment and then with the following enzymes: a, *Ava* I; b, *Ava* II; c, *Hae* III; d, *Hha* I; e, *Hin*fI; f, *Pvu* II; g, *Kpn* I; h, *Sau*3a; i, *Ava* I; j, *Ava* I + *Ava* II; k, *Ava* II; l, *Ava* I + *Hae* III; m, *Ava* I + *Hha* I; n, *Ava* II + *Hha* I; o, *Ava* II + *Hae* III; p, *Hae* III; q, *Hha* I; r, *Msp* I; s, *Ava* I + *Msp* I; t, *Ava* II + *Msp* I.

combinant plasmid was digested with several different restriction endonucleases alone or in combination and the separated fragments were hybridized to the tRNA probe (Fig. 2 *C* and *D*). The homology region was further localized to a 540-bp *Ava* II fragment (Fig. 2*D*, lane k), and a restriction map of the entire subcloned region was derived (Fig. 3).

Sequence Analysis of the Opal Suppressor Gene and Its Flanking DNA Segments. Fig. 3 shows the strategy that was used to determine the sequence of the 540-bp *Ava* II fragment described above, the nucleotide sequence of the gene and its 5′ and 3′ flanking DNA segments, and the predicted secondary structure of the tRNA product. This tRNA is encoded in an 87-bp segment without intervening sequences. The C-C-A terminus of the mature tRNA is not encoded. The gene specifies a tRNA that would read the codon UGA (positions 36–38, Fig. 3), establishing its identity as an opal suppressor gene. By computer analysis (17) we were unable to locate any oligonucleotides longer than 7 bp homologous to the gene within the region whose sequence has been determined. It is concluded that this region contains only one opal suppressor gene.

The Chicken Genome Appears to Contain Only One Opal Suppressor Gene. During screening of the chicken library we found only a single positive phage, which contains only one opal suppressor gene. To determine whether the chicken genome

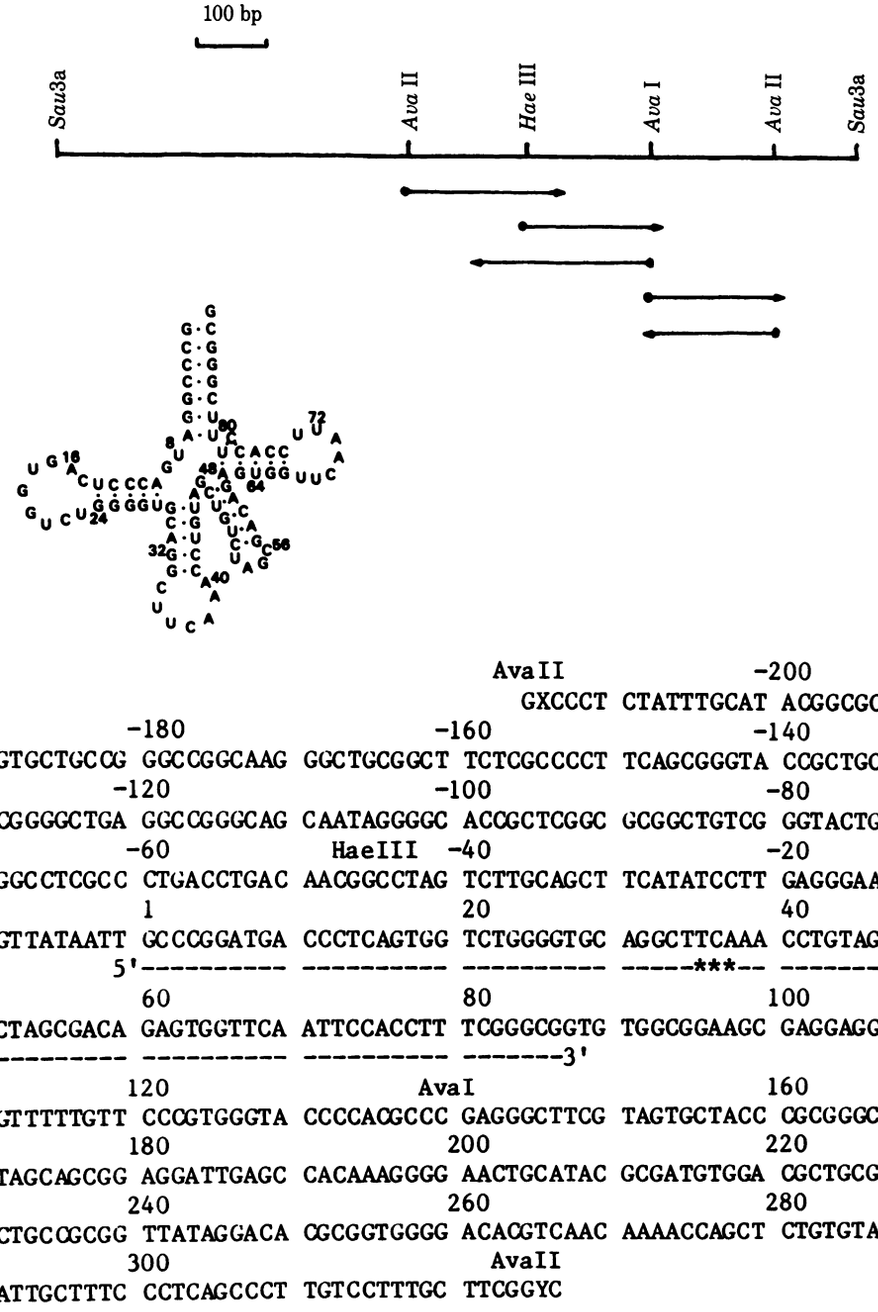

FIG. 3. Sequence analysis of the 540-bp *Ava* II fragment containing the opal suppressor gene. Restriction endonuclease cleavage sites within the 1,150-bp *Sau*3a fragment are shown together with the direction and extent of nucleotide sequence derived from end-labeling of these sites and sequencing by the method of Maxam and Gilbert (16). The nucleotide sequence of 540 bp containing the opal suppressor gene appears below. The noncoding strand is shown with the tRNA gene occupying positions 1–87 (underlined). Asterisks mark the tRNA anticodon position. The presumptive transcription termination signal is at position 112–119. (*Inset*) One possible secondary structure for the mature opal suppressor tRNA encoded by this gene.

contains additional opal suppressor genes or pseudogenes, total DNA was digested with restriction endonucleases and hybridized to the ^{32}P-labeled plasmid containing the 1,150-bp *Sau*3a fragment. The genomic restriction fragments positive with the probe were compared to those found in the isolated recombinant phage. These results are shown in Fig. 4. In several different restriction digests, the positive restriction fragments in the phage DNA are identical in length to those found in genomic DNA. No additional fragments were detected in genomic DNA. Thus it appears that the isolated recombinant phage bears the only genomic region that hybridizes to this plasmid probe. In addition, the labeled tRNA itself hybridizes to only a single fragment in genomic DNA digested with *Hin*dIII or *Eco*RI (data not shown), and these fragments correspond to those cloned in the recombinant phage. It may be that the chicken genome contains other genes or pseudogenes that fail to hybridize to this probe, but we appear to have cloned the only homology region detectable by standard hybridization procedures.

Genes Encoding Other Major tRNAs Do Not Occur in the Opal Suppressor tRNA Gene Region. Eukaryotic tRNA genes sometimes occur in clusters, which can be composed of isoaccepting species or of unrelated tRNA species. We have analyzed the 19,000-bp chicken DNA fragment inserted in the recombinant phage carrying the opal suppressor tRNA gene for the presence of a cluster of tRNA genes. With cloned DNA segments, major tRNA genes can usually be detected by using total tRNA as a probe (1, 10). The RNA probes we used included total tRNA, a tRNA fraction enriched in the major serine isoacceptors [corresponding to those eluting in fractions 35–60 (Fig. 1)], a tRNA fraction depleted of all serine isoacceptors, and preparations of opal suppressor tRNA from two stages of our purification procedure. The phage DNA was digested with restriction enzyme combinations that separate the opal suppressor gene from other segments of the inserted DNA. The hybridization results are shown in Fig. 5. The highly purified opal suppressor tRNA hybridized to a single restriction fragment in each digest (Fig. 5*B*). At an earlier stage of purification these same fragments were detected but the probe was weaker (Fig. 5*C*). Thus the intensity of hybridization is related to the content of the hybridizing species in the probe. Fig. 5*D* shows the hybridization to the tRNA probe enriched in major serine isoacceptors. No additional fragments were detected. Likewise, the tRNA fraction depleted in major serine isoacceptors did not detect additional fragments (Fig. 5*E*), nor did total tRNA (Fig. 5*F*). Upon longer radiographic exposure, the fragments positive with the purified opal suppressor tRNA were also detected weakly by using total tRNA as a probe (Fig. 5*G*, arrows), but again additional fragments were not detected. It is concluded that no major and probably no minor tRNA genes other than the opal suppressor gene occur in this recombinant phage. The opal suppressor gene is apparently not part of a tRNA gene cluster in the chicken genome.

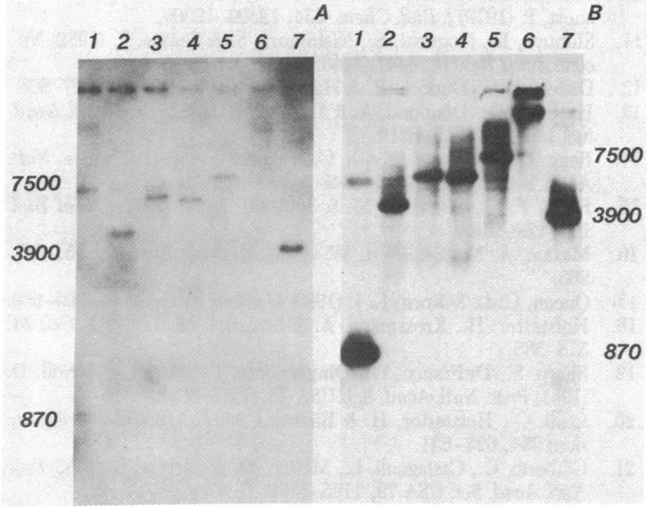

FIG. 4. The chicken genome contains only one region that hybridizes to the cloned opal suppressor tRNA gene. The recombinant plasmid bearing the opal suppressor gene was labeled with ^{32}P by nick-translation and hybridized to DNA from chicken liver or to DNA from the recombinant phage from which it was derived. (*A*) Each lane contains 10 μg of total chicken DNA digested with: 1, *Ava* I; 2, *Bgl* II; 3, *Bam*HI; 4, *Eco*RI; 5, *Hin*dIII; 6, *Sal* I; and 7, *Sst* I. Restriction fragments were separated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to 1×10^7 cpm of probe. A 2-week autoradiographic exposure is shown. (*B*) Each lane contains 1 μg of DNA from the recombinant phage digested with the same restriction endonucleases as in *A*, separated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to 5×10^6 cpm of probe. The autoradiogram was exposed for 20 hr. Fragment lengths are indicated in bp.

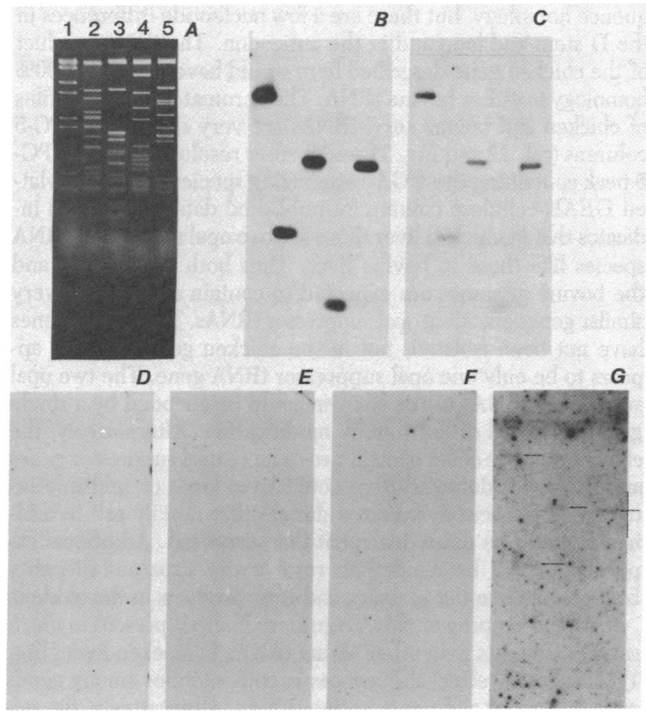

FIG. 5. Absence of other tRNA genes in the opal suppressor tRNA region. (*A*) DNA from the recombinant phage carrying the opal suppressor gene was digested with *Eco*RI, *Eco*RI + *Ava* I, *Eco*RI + *Bgl* II, *Eco*RI + *Kpn* I, or *Eco*RI + *Sst* I (lanes 1–5, respectively) and the fragments were separated in a 1.5% agarose gel and visualized by ethidium bromide staining. Fragments from duplicate gels were transferred to nitrocellulose filters and hybridized to 1.5×10^7 cpm of each of the following ^{32}P-labeled probes: *B* and *C*, highly and partially purified opal suppressor tRNA, respectively; *D* and *E*, tRNA fractions enriched or depleted in major serine isoacceptors, respectively; *F* and *G*, total tRNA. Autoradiographic exposures were 24 hr except for *G*, which was exposed for 7 days. The fragments that hybridized to the probe are indicated by arrows in *G*.

DISCUSSION

The opal suppressor tRNAs of bovine liver have been extensively studied (12). In bovine tissue there are two tRNAs that recognize the nonsense codon UGA. The primary sequence of the two tRNAs (13) shows that they have more than 90% se-

quence homology, but there are a few nucleotide differences in the D stem and loop and in the anticodon. The tRNA product of the chicken gene described here would have more than 90% homology to either bovine tRNA. The chromatographic profiles of chicken and bovine seryl-tRNAs are very similar on RPC-5 columns (ref. 12 and Fig. 1), and further resolution of the RPC-5 peak containing the UGA-recognizing species on a benzoylated DEAE-cellulose column (unpublished data and ref. 12) indicates that in chicken liver there are two opal suppressor tRNA species like those in bovine liver. Thus both the chicken and the bovine genomes are expected to contain at least two very similar genes encoding opal suppressor tRNAs. The bovine genes have not been isolated, but in the chicken genome there appears to be only one opal suppressor tRNA gene. The two opal suppressor tRNAs in the chicken could be encoded by a single gene and could differ only by modification. Alternatively, the chicken genome could contain two or more opal suppressor genes and those not detected here could have large or multiple introns, could occur as inverted dimers that rapidly self-hybridize, or could be more divergent than expected. Additional experiments will be needed to resolve the apparent disparity between the number of genes and gene products in the chicken.

The opal suppressor tRNA considered here is present in much smaller amounts than other serine tRNAs in chicken liver (Fig. 1). This could reflect differences in copy number among genes encoding major and minor serine tRNAs. Alternatively, the relative amounts of serine tRNAs could be controlled at the transcription level, with weak or altered promoters associated with minor tRNAs. The presumptive promoter regions of the chicken opal suppressor tRNA gene described here can be compared with published promoter sequences derived principally from studies of major tRNAs. Compilation of published 5′ internal promoter sequences (18–22) yields the consensus T-R-G-Y-N-N-A-R-T-G-G (R, purine nucleoside; Y, pyrimidine nucleoside; N, any nucleoside). The chicken gene has the nucleotide sequence T-G-*A*-C-C-C-*T*-*C*-A-G-T-G-G in this region (nucleotides 8–20, Fig. 3), differing by the substitution of an A for a G at position 10 and by insertion of two extra nucleotides after position 13. (These changes are italicized above.) These particular changes do not occur in any of 39 published eukaryotic tRNA gene sequences (22). In the 3′ promoter region the chicken gene sequence G-G-T-T-C-A-A-T-T-C-C (nucleotides 65–75, Fig. 3) corresponds closely to the published consensus sequence G-G-T-T-C-R-A-N-N-C-C (18–22). Thus this gene encoding a minor tRNA species has a normal 3′ promoter sequence but could have alterations in the 5′ part of the promoter that significantly affect transcription. *In vitro* or *in vivo* transcription experiments with the cloned gene will be required to test this hypothesis.

Transcription termination requires a cluster of T residues in the noncoding strand distal to the 3′-terminal nucleotide of a tRNA gene (9). Fig. 3 shows that the opal suppressor gene has such a cluster in the expected position (nucleotides 112–119).

The physiological function of naturally occurring nonsense suppressors is a subject of much interest, and among suppressors those that form phosphoseryl-tRNA are perhaps the most intriguing. If the insertion of phosphoserine directly into protein is mediated by them, then a fundamental undiscovered feature of eukaryotic protein synthesis may come to light as they are studied further. An opal suppressor tRNA found in rabbit reticulocytes is apparently responsible for the presence of a small amount of hemoglobin readthrough protein in those cells (23), demonstrating that a low level of nonsense suppression is a normal and perhaps even an essential cellular event. Normal termination codons are relatively resistant to the action of suppressor tRNAs even when these tRNAs are present in high amounts, as shown by oocyte injection experiments (24). Thus nonsense suppressors could be prevented from causing readthrough in most cases by the nucleotide context of the termination signal but could still be available for certain specialized readthrough functions. In contrast to those UGA codons that function as normal terminators, UGA codons generated internally in protein coding sequences through mutation can be efficiently suppressed (25), again indicating that these tRNAs can act selectively. These facts suggest that suppressor tRNAs may be important elements in the fine control of gene expression at the translation level.

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