Introduction of an intervening sequence into a human serine suppressor tRNA gene: effects on gene expression *in vitro* and *in vivo*

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ABSTRACT

The 13 nucleotide *Xenopus laevis* tyrosine tRNA gene intervening sequence was introduced into a human serine suppressor tRNA gene which lacked an intron, by site-directed mutagenesis. Analysis of the products of *in vitro* transcription in a HeLa cell extract indicates that the intervening sequence is accurately removed to generate a mature sized RNA identical to that obtained from an intron-less gene. Analysis of the transcripts obtained *in vitro* and *in vivo* shows that the U in the CUA anticodon sequence is partially modified to ψ .

Total tRNA isolated from cells infected with recombinant SV40 viruses carrying the mutant tRNA genes is active in suppression of UAG codons in a reticulocyte cell-free system. Cotransfection of COS cells with the mutant tRNA genes and a mutant chloramphenicol acetyltransferase gene containing the termination codon UAG demonstrated that the tRNA functions as a UAG suppressor *in vivo*. Analysis of ^{32}P -labeled RNA obtained from infected cells showed, however, that cells infected with the intron-containing gene accumulate less mature tRNA than cells infected with the intron-less tRNA genes.

INTRODUCTION

Transfer RNA genes from a variety of eukaryotic organisms have been sequenced. One of the characteristics of many of the tRNA genes from the yeast and invertebrates is the preponderance of tRNA genes containing intervening sequences (1). The location of these introns is invariant, between nucleotides 37 and 38 in the anticodon loop. The size of these introns ranges from 10 to several hundred nucleotides. In the yeast *Saccharomyces cerevisiae*, tRNA genes for isoleucine, leucine, lysine, phenylalanine, proline, serine, tryptophan, and tyrosine have intervening sequences. In *Drosophila melanogaster*, genes for leucine and tyrosine tRNAs have introns. In vertebrates, however, only the genes encoding tyrosine tRNAs have been shown to contain intervening sequences (2,3).

Why do some tRNA genes have introns, whereas others do not? Recent work demonstrates that the presence of an intervening sequence in some tRNA precursors is required for introduction of certain base modifications with the anticodon. For example, in the case of a yeast tyrosine suppressor tRNA gene, the intron was required for modification of CUA to C ψ A (4). Similarly, an intron was required for modification of CUA to m 5 CUA in a yeast leucine suppressor tRNA (5). In each case, it was found that the modification improved translational efficiency of the tRNA, as measured by *in vivo* suppression.

A similar relationship between anticodon base modification and the presence of an intervening sequence within the precursor tRNA has been seen in higher eukaryotes. The presence of an intron in the human tyrosine tRNA genes is required for *in vitro* modification of the anticodon GUA in HeLa extracts (6). The *Drosophila melanoaster* tyrosine tRNA genes have been shown to require an intron for the same modification in *Xenopus laevis* oocytes (7). It is not known if the anticodon modification influences the translational activity of these tRNAs.

However, this phenomena is not universal. In the case of a yeast proline tRNA gene, removal of the intron had no effect on the base modification pattern of the anticodon (8). Also, the finding that injection of mature tRNAs possessing totally unmodified anticodons into *Xenopus laevis* oocytes results in introduction of certain types of base modifications at position 34 of the anticodon (9, 10) suggests that enzymes which introduce base modifications into the anticodon may be divided into two classes, those which require an intron in the transcript, and those which do not.

Studies of heterologous tRNA gene transcription and processing both *in vivo*, in *Xenopus laevis* oocytes, or *in vitro*, in HeLa and oocyte extracts, have demonstrated that vertebrates have the capacity to accurately remove introns from the transcripts of the tRNA genes of yeast and insects. Nevertheless, the only vertebrate tRNA gene found to have introns to date are the tyrosine tRNA genes (2,3). This might imply that most vertebrate tRNA genes lack introns either for transcriptional constraints, or incompatibility of the rest of the vertebrate pre-tRNAs with the intron processing enzymes.

In order to determine if there were constraints upon which tRNA genes could have introns, and also to see if the presence of an intron within a tRNA precursor would result in base modification not normally found in the anticodon, we used site-directed mutagenesis to insert a *Xenopus laevis* tyrosine tRNA gene intervening sequence into a human serine suppressor tRNA gene. Analysis of the transcripts obtained from either transcription reactions in HeLa extracts, or from CV1 cells infected with SV40 recombinant virus carrying the mutant genes indicates that the intron is correctly excised *in vitro* and *in vivo* to give a functional mature-sized product. The CUA anticodon of the intron-containing gene transcripts is partially modified to CwA both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Enzymes and Radioactive Compounds: α -[^{32}P]-GTP, -UTP, and -ATP, and γ -[^{32}P]-ATP were purchased from Amersham. [^{32}P]-orthophosphate, [^{14}C]-chloramphenicol, [^{35}S]-methionine, and [^3H]-serine were from NEN. Restriction enzymes were purchased from either Boehringer Mannheim, or new England Biolabs, and were used according to the manufacturer's instructions. T4 polynucleotide kinase, T4 DNA ligase, and alkaline phosphatase were from Boehringer. T1 and T2 RNase were obtained from Calbiochem, and pancreatic

RNase was from Cooper. Tobacco mosaic virus was kindly provided by Dr. M. Zaitlin, Cornell University, Ithaca. RNA was extracted from virus by adjusting the virus stock to 0.5% SDS, heating to 65°C for 10 minutes, followed by extraction by phenol/chloroform, pH 8. The RNA was recovered by ethanol precipitation.

Plasmids and Strains: M13HST-Su⁺ (amber), and its pSV1GT3 derivatives (herein called tS-Su⁺) have been previously described (11). pRSVCAT amb and wt were described in Capone, et al. (12). pSP-CAT^{amb}, and pSP-CAT^{wt} were constructed by subcloning the HindIII-EcoRI fragment containing the respective genes from the pRSVCAT vectors into the HindIII-EcoRI site of pSP64.

Site-Directed Mutagenesis: DNA oligonucleotides were made on an Applied Biosystems DNA synthesizer. The oligonucleotide, GGACTCTAAGTGTGATCGAG-CAATCCATTGGG was used as primer for mutagenesis by the gap duplex methods, essentially as described by Kramer, et al. (13). A 15 base oligonucleotide (AGTGTGATCGAGCAA), corresponding to the intron plus the two flanking anticodon nucleotides was used as a hybridization probe to screen plaque lifts (14). In control reactions without the mutagenic primer, no positive plaques were seen in the hybridization screen. The presence of the intron was confirmed by sequencing of the recombinant M13 virion DNA by the dideoxy chain termination method (15).

In addition to the desired mutant having the 13 nucleotide intervening sequence (tS-Su⁺-IVS2), a mutant was found which had an additional A residue at the 5' exon-intron junction (tS-Su⁺-IVS1). Since the extra nucleotide is not present in the mutagenic oligonucleotide, we presume that it was added by slippage of the DNA polymerase during replication of the synthetic heteroduplex DNA *in vivo*.

In Vitro Transcription: Preparation of the HeLa whole-cell extracts, transcription of the mutant genes carried in M13, and analysis of the transcription products was essentially as described previously (16).

Cloning of the Mutant tRNA Genes into SV40 Vectors: The mutant genes were excised from the M13mp9 sequence with HindIII and EcoRI. The fragment ends were filled in using the large fragment of *E. coli* DNA polymerase I as described in Maniatis, et al. (17). The fragment was then isolated by differential precipitation with polyethylene glycol (18). The vector pSV1GT3 was cut with BamHI, treated with calf alkaline phosphatase, and the fragment ends similarly filled in. The insert was then ligated to the vector. The ligation products were used to transform DH5 α cells. Potential positives were identified by the increase in size of the HindIII fragment originally possessing the BamHI site.

Preparation of Virus Stocks: Recombinants were digested with PstI and religated as described previously (19). The DNA was mixed with the helper vector pSVrINS7, previously digested with EcoRI, religated, and used to transfect CV-1 cells as described in Lopata, et al. (20).

In Vivo Expression: CV-1 cells infected with recombinant virus for 48 hours were labeled with $^{32}\text{PO}_4$ as described previously (19), except that the phosphate free media was supplemented with 25mM Hepes KOH, pH 7.2. Low molecular weight nucleic acids were isolated by the method of Hirt (21), and analyzed on polyacrylamide gels as described in (11). Analysis of the base modification pattern of the anticodon was done by isolating the large 15 nucleotide RNase T1 fragment containing the anticodon by polyacrylamide gel electrophoresis. The fragment was then digested with pancreatic RNase, and the digest analyzed by two-dimensional thin layer chromatography (22).

Assay for In Vivo Suppression: COS and CV-1 cells were co-transfected with the pSV1GT3 recombinants and pRSVCAT^{amb} as described previously (12). After 48 hours, cell extracts were prepared, and then assayed for chloramphenicol acetyltransferase activity (12).

Assays for In Vitro Suppression: Synthesis of Sp6 mRNAs was essentially as described in (23), except that reactions were supplemented with 50 $\mu\text{g/ml}$ of acetylated bovine serum albumin (Promega). Following digestion of the linear template DNA with DNase I, the RNA was recovered by phenol extraction, ethanol precipitation, and chromatography on spin columns (17).

Total tRNA was isolated from 15 cm dishes of CV-1 cells infected for 48 hours with SV40 recombinant virus carrying wild type (Su^-), amber, and mutant human serine tRNA genes. The media was removed and cell layers rinsed once with PBS (phosphate buffered saline). Then, 2 mls of buffer containing 1% NP40, 30mM Hepes KOH, pH 8.0, 0.2M sucrose, 40mM NaCl, 5mM MgCl_2 , 5mM EDTA (24) were added to each dish at room temperature. When the cell layer had dislodged (about 5 minutes), the fluid and cell layer were transferred to a plastic disposable centrifuge tube. The plate was rinsed once with PBS, and this was also added to the tube. The cell layer was then centrifuged in an IEC refrigerated centrifuge for 2 minutes at 600 x g. The supernatant was adjusted to 0.5% SDS, and then transferred to another centrifuge tube containing an equal volume of water-saturated phenol/chloroform (1:1). After vortexing, the aqueous phase was recovered by centrifugation. The interphase and organic phases were re-extracted with TE (10mM Tris HCl, pH 7.4, 1mM EDTA). The combined aqueous phases were re-extracted with phenol/chloroform. The nucleic acids were obtained by ethanol precipitation and centrifugation. This RNA was then further fractionated by DEAE cellulose column chromatography. Material eluted with 0.7M NaCl was concentrated by ethanol precipitation, and used for aminoacylation experiments as described below.

The tRNA was used to supplement a reticulocyte cell-free system programmed with mRNA obtained from transcription of an Sp64-CAT^{amb} gene, or TMV RNA. The products of translation were analyzed on 10% polyacrylamide SDS gels for the CAT proteins, and on 6% gels for the TMV polypeptides.

RESULTS

At the onset of these studies, the only sequenced vertebrate tRNA gene containing an intervening sequence was the *Xenopus laevis* tyrosine tRNA gene. Laski, et al. (25, 26) have demonstrated that CV-1 monkey cells were capable of accurately removing this intron to generate a functional tRNA in vivo and in vitro. This sequence was introduced into the human serine suppressor tRNA gene originally described by Capone, et al. (10). The reasons for selecting the human serine suppressor tRNA gene is that this gene is expressed well in CV-1 cells, and its activity as a suppressor allows convenient measurement of its translational properties. In addition, the anticodon of the mature tRNA does not contain any modified nucleotides.

The serine suppressor tRNA gene was used as a template for site-directed mutagenesis using a 32 nucleotide long primer. The oligonucleotide sequences included the intron, and 10 nucleotides flanking the point of intron insertion. The sequence of the serine tRNA amber suppressor, along with the two intervening sequences obtained are shown in Figure 1. As mentioned in the Materials and Methods, we presume that the extra nucleotide in tS-Su⁺-IVS1 was a result of slippage of the DNA polymerase during in vivo replication of the heteroduplex DNA used for transformation. Both tRNA genes were used in the following studies.

The Intervening Sequences Are Accurately Removed By A HeLa Cell-Free Extract

To assess the feasibility of successful in vivo expression of the mutant tRNA genes, their expression was first studied in a HeLa cell-free transcription system. Figure 2 shows a polyacrylamide gel separation of the products of transcription of the intron-less and intron-containing genes. Both tS-Su⁺-IVS1 and tS-Su⁺-IVS2 yield three main species. The first heterogeneous broad band (P) corresponds to the primary transcripts ending in differing numbers of U's. As expected, the average size of these transcripts is larger than those obtained from the intron-less gene. The second major species, which yields a discrete band (labeled M+IVS), is a transcript which has been processed at both 5'- and 3'- ends, but which still contains the intervening sequences. Finally, there is a band obtained from both intron-less and intron-containing genes, which is identified as fully matured serine suppressor tRNA. Minor species seen in the tS-Su⁺-IVS1 and tS-Su⁺-IVS2 transcription reactions include fragments near the bottom of the gel whose size is consistent with 5' and 3' half molecules, with and without intron sequences. These fragments were not characterized further. In addition, there are very minor species which most likely correspond to 5'-processed, intronless transcripts in both intron-containing and intron-less reaction products. Although the level of transcription from all three tRNA genes is similar, the presence of the intron reduces the amount of mature-sized product obtained. In three experiments, the mature species accounted for 57, 18, and 14% of the total transcripts for tS-Su⁺, tS-Su⁺-IVS1, and

Figure 1 Sequence of the human serine amber suppressor tRNA, and sequence of the two introns introduced by site-directed mutagenesis IVS-1 contains one additional A residue at the intron-exon junction, and IVS-2 contains the expected intervening sequence. The arrow points to the position of the intron within the tRNA coding sequence.

ts-Su⁺-IVS2 respectively. Since end processed intron containing transcripts accumulate, it is likely that intron removal is inefficient. It is not known if the conditions used here are optimal for intron removal and ligation. Removal of the same intron from the *Xenopus laevis* tyrosine tRNA gene transcript is also not efficient in this whole cell extract (data not shown). Presumably the enzymes responsible are labile, or difficult to extract, so that their concentration in the extract is low. However, removal of the intron from the serine tRNA gene transcript appears inefficient also *in vivo* compared to its removal from the *Xenopus laevis* tyrosine tRNA gene transcripts. The fact that the intron is excised at all demonstrates that the lack of an intron in the human serine tRNA gene is not due to incompatibility between the serine tRNA sequence and the enzymes involved in intron removal.

To verify the accuracy of intron removal, the labeled transcripts were digested with RNase T1, and the digests analyzed by two dimensional homochromatography (Figures 3A-3C) and polyacrylamide gel electrophoresis (Figure 3D). The two-dimensional RNase T1 fingerprint pattern of the mature-sized transcripts shows that the anticodon fragment from

Figure 2 Analysis of the products of transcription of M13-tS-Su⁺ (intron-less) (lane 1), M13-tS-Su⁺-IVS1 (lane 2), and M13-tS-Su⁺-IVS2 (lane 3) in a HeLa cell-free extract. M denotes the mature sized serine tRNA transcript. P denotes the putative primary transcript. M+IVS denotes the 5'- and 3'- processed intermediate which still contains the intervening sequences. * is an α -³²P-GTP labeled RNA species present in the absence of DNA template.

the three transcripts (circled spot in Figures 3A-3C) behave identically. This is also seen clearly by the identical size of the largest T1 fragment on denaturing polyacrylamide gels (Figure 3D, lanes 1, 3 and 5). From this data, we conclude that the intron is accurately removed *in vitro*.

The Anticodon Of In Vitro Transcripts From Intron-Containing tRNA Genes Contain Pseudouridine.

The HeLa cell extracts are capable of introducing several base modifications. The modification of U55 to ψ in loop IV could approach 100% efficiency in a mutant human initiator methionine tRNA gene transcript (15). In addition, m⁵C was also produced. In view

Figure 3 Analysis of T-1 RNase digests of α -[^{32}P]-GTP labeled transcripts by two-dimensional homochromatography (A-C) and 20% polyacrylamide gel electrophoresis (D). Panel A,B and C are digests of mature-sized transcript of M13-tS-Su $^{+}$, M13-tS-Su $^{+}$ -IVS1, and M13-tS-Su $^{+}$ -IVS2 genes, respectively. The circled nucleotide is the 15 nucleotide fragment expected from intron-processed RNA. The two spots seen in panel A, but not in B and C are most likely due to non-specific cleavage of the long oligonucleotide by the T1 RNase. Panel D: M, marker lane (Partial T-1 digest of 5'- ^{32}P -labeled rabbit initiator methionine tRNA) Lanes 1,3, and 5 are digests of mature-sized processed transcripts of the M13-tS-Su $^{+}$ (intron-less), M13-tS-Su $^{+}$ -IVS1, and M13-tS-Su $^{+}$ -IVS2 DNA. The large arrow points to the 15 nucleotide long T-1 fragment expected from an intron-processed RNA. Lanes 2, and 4 are digests of 5' and 3' processed transcripts of M13-tS-Su $^{+}$ -IVS1 and M13-tS-Su $^{+}$ -IVS2. The small arrows point to the T-1 fragments containing the intron-exon junction. As expected, the M13-tS-Su $^{+}$ -IVS1 transcript produces a T-1 fragment that is longer than the M13-tS-Su $^{+}$ -IVS2 transcript. Although the presence of a band in between the two T1 fragments derived from tS-Su $^{+}$ -IVS1 and tS-Su $^{+}$ -IVS2 transcripts indicates that they differ by 2 nucleotides, DNA sequence of the tRNA genes indicates that they differ by only a single nucleotide. The most likely explanation is that the oligonucleotide UCUCCCGp, which is present in digests of both tS-Su $^{+}$ -IVS1 and tS-Su $^{+}$ -IVS2 RNAs, is migrating anomalously between ACUCUAAGp, and CACUCUAAGp because of its asymmetric base composition.

of previous studies in yeast (4,5), the anticodon sequence CUA may be expected to be modified either at C to produce m^5C , or at U to produce ψ , although different enzymes from those responsible for the modifications mentioned above must participate in the reactions.

Analysis of transcripts containing ^{32}P -label derived from α - $[^{32}P]$ -ATP were used to detect modification of U35. Mature-sized and intron-containing, end-processed transcripts were isolated from polyacrylamide gels, and digested with RNase T2. The U35 is expected to be labeled by nearest neighbor transfer from the labeled α -phosphate of A36. The digests were analyzed by two-dimensional thin-layer chromatography. Figure 4 shows that ψ p was present only in digests of RNA from the intron-containing genes. Using the intensity of m^1A58 as a reference (labeled from transfer of label from A59, one per molecule), the amount of ψ 35 produced is less than stoichiometric. The exact ratios have not been determined due to the small amount of radioactivity available for the analysis. Similar analysis using α - ^{32}P -UTP labeled RNA failed to detect any m^5C (data not shown). This result indicates that the anticodon sequence in the transcripts originating from the intron-containing genes is partially modified to C ψ A in the HeLa extract, and encouraged us to proceed to study the *in vivo* expression of these genes.

Transcripts Of The Intron-Containing Genes Are Accurately Processed In Vivo

The mutant tRNA genes were excised from the M13 vectors and inserted into the SV40 shuttle vector pSV1GT3. The resulting constructs, together with helper vector pSVrINS7, were used to transfect CV-1 cells (11, 19). Virus stock generated from such transfections were used in the studies described below.

Cells infected for 48 hours with recombinant virus stocks were labeled with $^{32}PO_4$ for 5 hours. The low molecular weight nucleic acids were extracted by the method of Hirt (21), and the tRNAs were analyzed on denaturing polyacrylamide gels (Figure 5). A prominently labeled RNA species, not present in mock-infected cells (Lane 1), is present in cells infected with the intron-less serine amber suppressor tRNA gene (Lane 2), as shown previously (10). Cells infected with the intron-containing tRNA genes (Lanes 3 and 4) have a similar sized RNA. Analysis of T1 digests of this RNA demonstrated that it is the mature serine tRNA (data not shown). The amount of mature serine suppressor tRNA produced from the intron-containing tRNA genes is significantly lower than that produced from the intron-less tRNA gene. Analysis of the yield of viral DNA from these cultures indicated that each culture was infected to the same extent (not shown). Thus, it is unlikely that the low yield of mature tRNA in the cells infected with the intron-containing tRNA genes is due to poor infection of these cells.

Along with the lower level of mature RNA, cells infected with the intron-containing genes contain an RNA species corresponding to the *in vitro* transcript which has been processed at both 5'- and 3'- ends, but which still contains the intron (Band M+IVS). The

Figure 4. Analysis of T2 RNase digestion products of α - ^{32}P -ATP labeled *in vitro* transcripts by 2D TLC. A., mature sized M13-tS-Su $^{+}$ transcripts; B, mature sized M13-tS-Su $^{+}$ -IVS1 transcript; C, mature sized M13-tS-Su $^{+}$ -IVS2 transcript; D, end processed intron-containing M13-tS-Su $^{+}$ -IVS1 transcript (M+IVS). m 1 Ap is seen more clearly in shorter exposures, which do not show ψ p well.

slower migration of this species in tS-Su $^{+}$ -IVS1 compared to tS-Su $^{+}$ -IVS2 is consistent with this interpretation, since the intron in tS-Su $^{+}$ -IVS1 is one nucleotide longer than the intron of tS-Su $^{+}$ -IVS2. These results suggest that while the removal of introns is accurate *in vivo*, it is slow, and this leads to the accumulation of end-processed, intron containing transcripts.

For analysis of base modification in the anticodons of the mature-sized transcripts, the large RNase T1 oligonucleotide fragment containing the anticodon was purified by polyacrylamide gel electrophoresis, and digested with pancreatic RNase. The digestion products were then analyzed by two-dimensional thin-layer chromatography (Figure 6). Although the RNase T1 fragment containing the anticodon has a ψ at the beginning of the 3'-anticodon stem (See Figure 1), pancreatic RNase will liberate ψ p only from the anticodon sequence itself. As shown in Figure 6A, no ψ p is liberated from the anticodon oligonucleotide derived from the serine amber suppressor tRNA isolated from cells infected with the intron-less tRNA gene. However, the serine amber suppressor tRNA from cells infected with tS-Su $^{+}$ -IVS1 has ψ p in the anticodon fragment which is liberated by pancreatic RNase. A similar result was seen upon analysis of tRNA obtained from cells infected with tS-Su $^{+}$ -IVS2 (data

Figure 5. Analysis of RNA expressed during infection of CV-1 cells by recombinant SV40 viruses carrying serine tRNA genes. Cells infected for 48 hours were labeled with $^{32}\text{PO}_4$ for 5 hours, and the tRNAs analyzed on denaturing 15% 7M urea polyacrylamide gels. Lane 1, mock infected; Lane 2, SV-tS-Su⁻; Lane 3, SV-tS-Su⁺-IVS1; Lane 4, SV-tS-Su⁺-IVS2. M denotes the mature sized serine tRNA, M+IVS indicates end processed intron-containing transcripts.

not shown). Comparison of the intensity of the Up and/or UmpGp oligonucleotide spots, which should be present in one mole per T1 fragment, with that of ψ p shows that the modification of U to ψ is partial. Quantitation of radioactivity in the thin layer spots indicates that the level of modification in the tS-Su⁺-IVS1 anticodon is between 30-35% of the expected amount. Therefore, *in vivo*, as well as *in vitro*, the presence of an intron in the tRNA precursor results in modification of the U in the anticodon to ψ , although this modification occurs only partially.

Transfer RNA From The Mutant Suppressor tRNA Genes Is Functional In Vivo And In Vitro.

To determine whether or not functional suppressor tRNA was made from the intron-containing genes *in vivo*, COS-1 cells were co-transfected with pSVGT3 plasmids carrying the tRNA genes and a plasmid carrying a chloramphenicol acetyl-transferase (CAT) gene containing an amber codon. After 48 hours, extracts were prepared from the transfected cells and assayed for CAT activity as described previously (11). Results of such an assay are shown in Figure 7.

Extracts prepared from cells transfected with either the serine amber suppressor (tS-Su⁺), or tS-Su⁺-IVS1 or tS-Su⁺-IVS2 had significant CAT activity (lanes 1-3 of each set), whereas extracts prepared from the wild type serine tRNA (tS-Su⁻) do not (lane 4). Fur-

Figure 6. Analysis of RNase A digests of the T1 RNase fragment containing the anticodon fragments of *in vivo* labeled serine tRNAs. Panel A, tS-Su⁺; B, tS-Su⁺-IVS1; ψ is found only in tRNA obtained from the intron-containing genes.

thermore, the level of CAT activity in the extracts from cells infected with the intron-containing tRNA genes is as high as those from cells infected with the intron-less tRNA gene. This is in spite of the anticipated lower amount of mature tRNA in cells infected with intron-containing tRNA genes.

To further investigate the function of the mutant tRNAs, total tRNA was isolated from large-scale cultures of CV-1 cells infected with the recombinant virus stocks described above for 48 hours. The tRNA was then added to a reticulocyte cell-free protein synthesis system programmed with CAT mRNA carrying an amber mutation. The products of these reactions were then analyzed on SDS polyacrylamide gels (Figure 8). A band corresponding to the chloramphenicol acetyl-transferase polypeptide is seen in reactions programmed with the wild type Sp6-Cat mRNA (Lane 2). In reactions programmed with the Sp6-CAT^{amb} mRNA, this band is present when the reactions are supplemented with the tRNA

Figure 7. Thin layer chromatography of reaction products of chloramphenicol acetyl-transferase assays. Extracts were prepared from COS cells transfected with 5 μ g of pRSVCAT^{amb}, and increasing amounts of either pSV-tS-Su⁺, pSV-tS-Su⁺-IVS1, or pSV-tS-Su⁺-IVS2. Lanes 1,2 3 used extracts from cells transfected with 2,5, and 10 μ g respectively of plasmid carrying the tRNA gene; lane 4 used an extract from cells transfected with pSV-tS-Su⁺; lane 5 used an extract from cells transfected with pRSVCAT^{amb} only; lane 6, minus extract. "cam" is [¹⁴C]-chloramphenicol; "3-ac-cam" is 3-acetyl-chloramphenicol.

Figure 8 Polyacrylamide gel analysis of reticulocyte lysate translation products. Total tRNA was extracted from CV1 cells infected for 48 hours with the recombinant virus, and added to a reticulocyte cell-free protein synthesis system programmed with chloramphenicol acetyl-transferase mRNA (wild type or amber mutant). Lane 1, -mRNA; Lane 2, + wt mRNA; Lane 3,4,5,6,7, + amb mRNA; Lanes 1-3, -tRNA; Lane 4, + tS-Su⁻ tRNA; Lane 5, + tS-Su⁺-tRNA; Lane 6, + tS-Su⁺-IVS1 tRNA; Lane 7, + tS-Su⁺-IVS2 tRNA.

from cultures infected with any of the serine amber suppressor tRNA gene constructs (Lanes 5-7), but not with the wild type Su⁻ serine tRNA gene (Lane 4).

Likewise, the 160 kDa polypeptide readthrough protein translated from the TMV RNA is only seen when reactions are supplemented with the suppressor tRNAs whereas when the reactions are supplemented with Su⁻ tRNA, a 110 kDa polypeptide is seen (data not shown).

Thus, in vitro as in vivo, the tRNA derived from intron-containing tRNA^{ser} genes is active in suppression of amber codons.

DISCUSSION

This report describes the introduction for the first time of an intervening sequence into a human tRNA gene which does not normally have one. The mutant tRNA genes produce transcripts which are accurately processed in vitro and in vivo to yield fully functional tRNAs. Thus, the absence of intervening sequences in the case of human serine tRNA genes is not due to specific features of those tRNA genes which would make possession of an intron incompatible with gene function. Furthermore, the introduction of the *Xenopus laevis* tyrosine tRNA gene intron into the human serine suppressor tRNA gene resulted in the partial base modification of the anticodon from CUA to CψA both in vitro and in vivo.

The structural features of an intron-containing pre-tRNA required for efficient intron removal has been most well characterized for yeast pre-tRNAs, using homologous and heterologous systems. A common, albeit not universal feature of tRNA introns is the potential for base pairing to the anticodon itself. This feature has been found not to be required for efficient intron removal (4). The *Xenopus laevis* intron used in this study is not predicted to interact with the anticodon either within the tyrosine tRNA or the human ser-

ine tRNA used here. The results presented here confirm that this feature is not a requirement for accurate intron removal.

It is not clear at this time why the modification of U to ψ in the anticodon sequence is incomplete. Based on the ratio of pseudouridine to uridine in the anticodon fragments, it is estimated that less than 35% of the mature serine tRNA derived from the intron-containing genes is modified at this position. Laski, et al. (25, 26) showed that when the *Xenopus laevis* tyrosine tRNA gene containing the same intron was expressed in CV1 cells, the anticodon sequence was fully modified. Additionally, no end-processed intron-containing transcripts were found to accumulate, as is the case with the intron-containing serine tRNA genes studied here. This suggests that in the case of the serine tS-Su⁺-IVS1 and tS-Su⁺-IVS2 transcripts, the intron sequence is not within the most favorable context, such that both intron excision and the modification of the anticodon are slow. If only the intron-containing transcripts are substrates for the modification enzymes, the finding that mature tRNA is only partially modified suggests that base modification is a slow process relative to intron removal.

All eukaryotic tyrosine tRNA genes sequenced to date have intervening sequences, and all have ψ in the anticodon sequence. Since there is little homology among all of these intron sequences, and more homology between the tRNAs themselves, it could be argued that the enzyme responsible for modification of the anticodon actually recognized a feature common to all of the tyrosine tRNAs, for example features which the tyrosyl-tRNA synthetase recognizes. However, in the studies presented here, the base modification imparted to the serine suppressor tRNA was ψ , and not an m⁵C, which is found in the anticodon (m⁵CUA) of the yeast leucine suppressor tRNA. Therefore, it is possible that the sequence of the intron may affect the pattern of modification in the anticodon. Alternatively, it is possible that mammalian cells lack an enzyme which modifies C34 to m⁵C. It would be of interest to determine if insertion of a yeast leucine tRNA intron into the human serine tRNA gene results in modification of C34 and/or of U35 in the anticodon.

The features within the intervening sequence of either the yeast pre-tRNA_{3^{leu}} or pre-tRNA^{tyr} which direct the modification of the CUA anticodon sequence to either m⁵CUA or to C ψ A are not known. Since efficient anticodon base modification, as well as efficient removal of the *Xenopus laevis* intron was achieved when the intron was within the *Xenopus* tRNA^{tyr}, but not within the human tRNA^{ser}, some type of interaction between the intron domain and the tRNA itself must occur in order to present the ideal substrate to the enzymes responsible for these reactions.

Finally, Abelson and co-workers have shown with two yeast tRNA genes that anticodon modification results in a more active suppressor tRNA in vivo (4,5). Since little mature-sized, fully anticodon-modified tRNA is made from the intron-containing genes in vivo, it is not technically feasible at this time to obtain pure tRNAs for use in direct measure-

ments of suppressor efficiency in vitro. As mentioned, the same intron in the *Xenopus* tyrosine tRNA transcript appears to be more efficiently removed, as similar in vivo labeling experiments show only the accumulation of the fully matured product, and no significant amounts of end-processed, intron-containing transcript (25, 26). The observation that more mature tRNA is obtained from the intron-containing gene with an additional nucleotide in the intron suggests that improvements in expression can be obtained by further alteration of this sequence. This should allow us to determine whether in mammalian cells as in yeast, tRNA with ψ in the anticodon sequence is more active in suppression in vivo compared to one with U35. Should this prove to be the case, it opens up a way for making more active suppressors.

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REFERENCES

1. Sprinzl, M., Hartman, T., Meissner, F., Moll, J., and Vorderwulbecke, T. (1987) *Nuc. Acids Res.* **15**, r53-188.
2. MacPherson, J.W., and Roy, K.L. (1986) *Gene* **42**, 101-106.
3. Muller, F. and Clarkson, S.G. (1980) *Cell* **19**, 345-353.
4. Johnson, J. and Abelson, J. (1983) *Nature* **303**, 681-687.
5. Strobel, M. and Abelson, J. (1986) *Mol. Cell. Bio.* **6**, 2663-2673.
6. Van Tol, H. and Beier, H. (1988) *Nuc. Acids Res.* **16**, 1951-1966.
7. Kubli, E., Suter, B., Doerig, R., and Choffat, Y. (1988) Suppression, Introns, and Stage Specific Expression of tRNA^{tyr} Genes in *Drosophila melanogaster*. *Genetics of Translation* (NATO ASI 14), Springer Verlag, Berlin.
8. Winey, M., Mendenhall, M.D., Cummins, C.M., Culbertson, M.R., and Knapp, G. (1986) *J. Mol. Biol.* **192**, 49-63.
9. Fournier, M., Haumont, E., deHanau, S., Gangloff, J., and Grosjean, H. (1983) *Nuc. Acids Res.* **11**, 707-718.
10. Droogmans, L., Haumont, E., deHanan, S., and Grosjean, H. (1986) *EMBO J.* **5**, 1105-1109.
11. Capone, J.P., Sharp, P.A., and RajBhandary, U.L. (1985) *EMBO J.* **4**, 213-221.
12. Capone, J.P., Sedivy, J.M., Sharp, P.A., and RajBhandary, U.L. (1986) *Mol. Cell. Biol.* **6**, 3059-3067.
13. Kramer, W., Drutsa, V., Jansen, H.W., Kramer, B., Pflugfelder, M., and Fritz, H.J. (1984) *Nuc. Acids Res.* **12**, 9441-9456.
14. Zoller, M.J. and Smith, M. (1982) *Nucleic Acids Research* **10**, 6487-6500.
15. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
16. Drabkin, H.J. and RajBhandary, U.L. (1985) *J. Biol. Chem.* **260**, 5580-5587.
17. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor, N.Y.

18. Lis, J.T. (1986) **Methods in Enzymology**, 65, 347-353.
19. Drabkin, H.J. and RajBhandary, U.L. (1985) **J. Biol. Chem.** 260, 5588-5595.
20. Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. (1984) **Nuc. Acids Res.** 12, 5707-5717.
21. Hirt, B. (1966) **J. Mol. Biol.** 54, 145-154.
22. Nishimura, S. (1972) **Prog. Nucleic Acid Res. Mol. Biol.** 12, 49-85.
23. Krieg, P.A. and Melton, D. (1984) **Nuc. Acids Res.** 12, 7057-7070.
24. Leeds, J.M., Slabaugh, M.B., and Matthews, C.K.. (1985) **Mol. Cell. Biol.** 5, 3443-3450.
25. Laski, F.A., Alzner-DeWeerd, B., RajBhandary, U.L., and Sharp, P.A. (1982) **Nuc. Acids Res.** 10, 4609-4626.
26. Laski, F.A., Belagaje, R., RajBhandary, U.L., and Sharp, P.A. (1982) **Proc. Natl. Acad. Sci. USA** 79, 5813-5817.