Expression of a *X. laevis* tRNA^{Tyr} gene in mammalian cells

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ABSTRACT

Expression of a *X. laevis* tRNA^{Tyr} gene has been studied in mammalian cells. This tRNA^{Tyr} gene has a 13 base intervening sequence adjacent to its anticodon. A fragment containing the tRNA^{Tyr} gene was cloned into the late region of SV40. Cells infected with a recombinant virus stock vastly overproduce a tRNA^{Tyr} that is properly spliced, processed and modified. It was also found that the *X. laevis* tRNA^{Tyr} is identical or nearly identical to an endogenous tRNA^{Tyr} of monkey kidney cells. The possibility of using the *X. laevis* tRNA^{Tyr} gene to create an amber suppressor for mammalian cells is discussed.

INTRODUCTION

All three types of nonsense codons are known to be utilized for terminating translation in mammalian cells. Development of systems that suppress termination at one of these codons would permit the isolation of viral and cellular conditional nonsense mutations. In yeast, this was accomplished by selection of nonsense suppressor tRNAs (1,2). Selection of equivalent mutations in endogenous tRNA genes in mammalian cells has been difficult; such mutations may be deleterious for cellular function. Alternatively, a single tRNA suppressor gene per cell may not be adequate for efficient suppression. Another approach for development of a suppressor tRNA is the application of site-specific mutagenesis (3-5) to an isolated tRNA gene. The suppressor tRNA gene could then be introduced into mammalian cells with a convenient vector such as SV40. Synthesis of large amounts of the suppressor-tRNA from the replicating SV40 vector would ensure efficient suppression. In addition, it might also provide a permissive state for growth of nonsense mutants of rapidly replicating virus.

Expression of a functional tRNA from a replicating SV40-tRNA gene recombinant has so far not been demonstrated although SV40 vectors have been used for expression of a number of other genes in mammalian cells (6, 7). Most of these genes are transcribed by RNA polymerase II; tRNAs are RNA polymerase III products. Goff and Berg (8) inserted the gene for a yeast tRNA^{Tyr} in the late region of SV40. The yeast gene was transcribed, probably by a RNA polymerase III, but the precursor RNA was not processed to mature stable tRNA. The incomplete processing may have been the result of either a defect in base modification, RNA splicing or cleavage. A suppressor tRNA gene from *E.coli* has also been cloned into an SV40 vector (9). However, the tRNA gene was apparently not expressed.

Tyrosine tRNA genes are good sources of amber suppressors since a single point mutation in the anticodon is necessary for their conversion to a suppressor. A gene for tRNA^{Tyr} has been isolated in a fragment of *X.laevis* DNA along with a number of other tRNA genes (10, 11). This gene contains a 13 bp intervening sequence and lacks the 3' terminal CCA coding sequence. The sequence predicted for the mature tRNA from the DNA sequence is 76 nucleotides long and should be very similar to the corresponding mammalian tRNA^{Tyr} (12). Before attempting site-specific mutagenesis to convert this gene to an amber suppressor, it was important to show that the tRNA gene would be expressed and the precursor tRNA processed correctly in mammalian cells.

This paper reports on the cloning of the *X.laevis* tRNA^{Tyr} gene into the late region of SV40 and on its efficient expression in monkey cells (CV-1 cells).

MATERIALS AND METHODS

Construction of pSV-tT

The 263 bp HaeII-HhaI restriction fragment of pt201, which contains the *X.laevis* tRNA^{Tyr} gene, was ligated into the late region of HaeII-HhaI cut SV40. This recombinant was then cleaved with BamHI and ligated into the BamHI site of pBR322 (13). This DNA was transfected into *E.coli* C600 by the method of Dager and Ehrlich (14). Colonies were screened for the presence

of *X. laevis* DNA by the method of Grunstein and Hogness (15). Clones which hybridized to the probe were screened by restriction mapping.

Preparation of Virus Stock

Virus stocks of the SV40 recombinants were prepared by co-transfection of CV-1 cells with two complementing recombinants, SV-tT and SV-rINS-7 (see Fig.2 for structure). Plasmid DNAs (10 μ g) of pSV-tT and pSV-rINS-7 were digested to completion with BamHI and EcoRI, respectively. The DNAs were separately phenol and ether extracted, ethanol precipitated, resuspended in 200 μ l TE pH 8.0 (10mM Tris pH 8.0, 1mM EDTA) and diluted to 2ml with ligation buffer (50mM Tris pH 7.5, 10mM MgCl₂, 15mM DTT, 1mM ATP). Ligase reaction was overnight at 15°C after addition of 1500u of T4 DNA ligase (New England Biolabs). CV-1 cells were transfected by the method of Graham and van der Eb (16) as modified by Chu and Sharp (17). DNA pellets were resuspended in 10 μ l TE pH 8.0 and 0.5ml 2x HeBS (Hepes 10g/l; NaCl 15g/l; KCl 0.74g/l; Na₂HPO₄·H₂O 0.25g/l; dextrose 2g/l; pH 7.05) was added. 0.5ml of 0.25M CaCl₂ was added, vortexed for 5 seconds and incubated for more than 20 minutes at room temperature.

CV-1 cells from 3 plates (10cm) were trypsinized, mixed and pelleted. The cell pellet was resuspended in the DNA solution and left 15 minutes at room temperature. Fourteen ml of DME containing 10% calf serum, 1/30x HeBS and 4.2mM CaCl₂ was added and the mixture was divided into two (15cm) plates, each plate contained in addition 4.5ml of DME containing 10% calf serum, 1/10x HeBS and 12.5mM CaCl₂. After 4 hours at 37° the medium was aspirated off and 2.25ml of 25% glycerol - 75% DME was added for one minute. The cells were washed with 20ml DME and 20ml of DME containing 10% calf serum, 100 μ g/ml penicillin and 10 μ g/ml streptomycin; the cells were incubated at 37°C. Twenty-four hours later the medium was changed to DME containing 2% calf serum, 100 μ g/ml penicillin and 10 μ g/ml streptomycin.

The virus stock was harvested 14 days after transfection when CPE was observed. All but 1ml of medium was removed and cells were scraped off the plate, freeze/thawed three times and stored at -70°.

Infection of CV-1 Cells with SV-tT/SV-rINS-7 and Purification of Viral DNA and Cytoplasmic RNA

A 10cm dish of subconfluent CV-1 cells was infected with the SV-tT/SV-rINS-7 virus stock. Forty-six hours after infection the cells were washed with 5ml of DME(-PO₄) and labeled with 500 μ Ci of carrier free ³²PO₄ (New England Nuclear) in 3ml of DME(-PO₄) and 2% dialyzed calf serum. After 6 hours at 37° the medium was removed and cells were scraped off into 4ml cold PBS (phosphate buffered saline). The cells were pelleted at 800 RPM for 10 minutes at 0°C and resuspended in 1ml lysis buffer (0.65% NP40, 0.01M Tris pH 7.8, 0.15M NaCl, 1.5mM MgCl₂). Nuclei were removed by centrifugation at 2000 RPM for 3 minutes and the cytoplasmic supernatant was poured off. One ml of urea buffer (7M urea, 10mM Tris pH 7.4, 10mM EDTA, 0.35M NaCl, 1% SDS) was added to the supernatant. This mixture was extracted twice with phenol and the RNA was then precipitated with ethanol. The pelleted nuclei were resuspended in 0.25ml of PBS and the SV40 DNA purified by the method of Hirt (18).

Purification of tRNA^{Tyr} by Gel Electrophoresis

This consisted of two steps. The ³²P-labeled RNA was electrophoresed first through a 7.5% polyacrylamide gel (29:1, acrylamide:bisacrylamide; 8.3M urea; 1/2x TBE) at 360V until xylene cyanol had migrated 26cm. Following autoradiography the appropriate RNA band was eluted from the gel, ethanol precipitated and electrophoresed on a second gel (10% polyacrylamide; 29:1 acrylamide:bisacrylamide; 4M urea; 1/2x TBE) at 360V until xylene cyanol had migrated 20cm. The appropriate RNA band was then eluted and ethanol precipitated.

Purification of tRNA^{Tyr} by Hybrid Selection

Hybrid selection was as described by Weiner (19) with the following modification. After the three washes in hybridization buffer the filters were also washed for 15 minutes in 25ml TE pH 8.0 at 37°C. RNA obtained by hybrid selection was further purified by 7.5% polyacrylamide gel electrophoresis as described above.

Fingerprint Analysis of tRNA

T1-RNase fingerprint. ³²P-labeled RNA in TE (pH 7.4) was digested with one unit RNase T1 at 37°C for 4 hours. The mix-

ture of oligonucleotides was lyophilized, resuspended in 2-3 μ l H₂O and subjected to two-dimensional homochromatography fingerprinting (20). A cellulose acetate strip (Schleicher and Schuell, No. 250) was wet in 5% pyridinium acetate (pH 3.5), 2mM EDTA, 7M urea (deionized). The sample was applied onto the strip and electrophoresed in 5% pyridinium acetate (pH 3.5), 2mM EDTA buffer at 5000V until bromophenol blue dye migrated 8cm. The oligonucleotides were then transferred to a DEAE plate (Macherey-Nagel & Co., Polygram Cell 300 DEAE/HR-2/15), the plate was washed with H₂O at 65°C and then chromatographed in homomix (7M urea, 30% yeast tRNA hydrolyzed in 50mM KOH overnight at 65°C, then neutralized to pH 4.5) at 65°C. The plate was dried and autoradiographed.

The ³²P-labeled oligonucleotides were recovered as described previously (20).

Analysis of Nucleotide Composition by Digestion with T2-RNase

³²P-labeled tRNA or oligonucleotide was mixed with 0.5 A₂₆₀ unit of carrier yeast tRNA and digested with 1 unit RNase T2 for 6 hours at 37°C in 20mM NH₄ acetate pH 4.5. The sample was lyophilized five times, then resuspended in 1-2 μ l H₂O and chromatographed in two dimensions on a pre-coated cellulose TLC plate (Merck 5757). Solvent for the first dimension was isobutyric acid: NH₄OH:H₂O, 48:3.2:32 vol/vol and for the second dimension, t-butyl alcohol: Conc. HCl:H₂O, 70:15:15 vol/vol. The plates were dried and autoradiographed.

RESULTS

Generation of SV40-tRNA^{Tyr} Recombinant and Preparation of Virus Stock

The recombinant plasmid pt201 was obtained from Dr. Stuart Clarkson (10,11). pt201 contains 3.2kb of X.laavis DNA which has been largely sequenced and is thought to encode 8 tRNA genes. The sequence of a 263bp HaeII-HhaI fragment containing the tRNA^{Tyr} gene is shown in Figure 1. This fragment was excised from pt201 and ligated to HaeII-HhaI digested SV40 DNA. Ligation of the complementary restriction endonuclease cohesive ends placed the tRNA^{Tyr} gene in the late region of SV40 in the same polarity as late SV40 transcription. The SV40-tRNA^{Tyr} recombinant DNA was linearized by digestion with BamHI and then

Figure 1. DNA sequence of the HhaI-HaeII restriction fragment of pt201 containing the gene for tRNA^{Tyr} of *X. laevis*. This fragment was inserted into the late region of SV40 in the construction of pSV-tT. The boxed sequence is the coding region for the mature tRNA^{Tyr}. The two coding regions are separated by a 13bp intervening sequence. The arrow signifies the direction of transcription. The sequence was determined by Muller and Clarkson (10).

further ligated to BamHI cleaved pBR322 DNA. Bacterial colonies containing the recombinant of pBR322-SV40-tRNA^{Tyr} (pSV-tT) were identified by colony filter hybridization (15).

The SV40-tRNA^{Tyr} recombinant (SV-tT) is defective due to the insertion/deletion in the late region of SV40. Virus stocks of this recombinant were prepared by co-transfection of monkey cells with a SV40-rat preproinsulin gene recombinant (SV-rINS-7) (21; Fig.2A) which has intact late genes but an insertion/deletion in the early region. Monkey cells co-transfected with these two SV40 recombinants underwent CPE in 14 days yielding high titer virus stocks of SV-tT/SV-rINS-7 (Materials and Methods). The approximate titer and homogeneity of the virus stocks were determined by characterization of viral DNA extracted by the method of Hirt (18) 52 hours postinfection. Figure 2B shows a HindIII digestion of viral DNA extracted from either cells infected with the SV-tT/SV-rINS-7 stock or SV40 (3 m.o.i.). The two infections yielded comparable amounts of total viral DNA and the digestion pattern shows that the SV-tT/SV-rINS-7 DNA contains less than 1% w.t. recombinant virus. In addition no aberrant viral DNA forms appear in the recombinant stock.

Figure 2. HindIII restriction map of SV-rINS-7, SV40 and SV-tT. Solid vertical lines represent the position of HindIII sites in SV40 and the map units of the sites are shown. SV-rINS-7 has an insertion/deletion in the early region of SV40 creating the new restriction fragment A*. SV-tT has an insertion/deletion in the late region of SV40 creating the new restriction fragment C*. The origin of replication (Ori) and the direction of early and late transcription are shown. The plasmid DNAs pSV-tT and pSV-rINS-7 were formed by insertion of pBR322 into the BamHI site of SV-tT and EcoRI of SV-rINS-7 site, respectively. Restriction sites used in construction of the recombinant: Δ = HhaI site; $\blacktriangle$ = TaqI site; $\circ$ = BclI site.

2B. Autoradiogram of a HindIII digest of viral DNA from (lane 1) SV-tT/SV-rINS-7 infected cells and (lane 2) SV40 infected cells. 10cm plates of CV-1 cells were infected with 0.1ml of either the SV-tT/SV-rINS-7 virus stock or SV40 (10^8 pfu/ml) virus stock. 46 hours post-infection cells were labeled with 32 P- and the viral DNA was harvested 6 hours later (see Materials and Methods). The viral DNA was digested with HindIII and the fragments separated by electrophoresis on a 1.4% agarose gel. Bands are labeled according to Fig.2A.

X. laevis tRNA^{Tyr} Synthesis in CV-1 Cells

To determine whether the *X. laevis* tRNA^{Tyr} gene could be expressed in mammalian cells, CV-1 cells were infected with SV-tT/SV-rINS-7 virus and labeled 46 hours later with 32 P. Cytoplasmic RNA was prepared 6 hours later and electrophoresed in a 7.5% acrylamide-urea gel. A prominent 32 P-labeled tRNA band was observed in the recombinant infected cells that was not present in either mock- or SV40-infected cells (Fig.3). The prominent band comigrated with purified yeast tRNA^{Tyr} (77 bases in length as it is missing the terminal A base) and migrated faster than *E. coli* tRNA^{Tyr} (85 bases) (data not shown). We assume that the overproduced tRNA species observed after infection with the SV-tT/SV-rINS-7 virus is the mature *X. laevis* tRNA^{Tyr}. The sequence of the gene suggests the mature tRNA would have a length of 76

Figure 3A. Autoradiogram of ^{32}P -RNA purified from CV-1 cells and analyzed on a 7.5% polyacrylamide 8.3M urea gel. Cells were either mock infected, infected with SV40 or infected with the SV-tT/SV-rINS-7 virus stock. 46 hours post infection cells were labeled with ^{32}P - and 6 hours later cytoplasmic RNA was harvested and electrophoresed on the gel.

3B. The overproduced tRNA^{Tyr} band in the lane marked SV-tT/SV-rINS-7 of Fig. 3A was excised and the RNA eluted. A co-migrating band in the mock infected lane was also eluted. These RNAs were then electrophoresed on a 10% polyacrylamide 8.3M urea gel. The overproduced tRNA^{Tyr} band and the co-migrating band in the mock lane were excised and the RNA eluted and analyzed by digestion with T1-RNase.

3C. Autoradiogram of hybrid selected RNA. ^{32}P -labeled RNA from uninfected CV-1 cells was hybridized to filters containing 20 μg of denatured pSV-tT DNA, p201 DNA, pBR322-SV40 DNA or no DNA. After washing, the hybridized RNA was recovered and electrophoresed after melting on a 7.5% acrylamide 8.3M urea gel. The hybrid selected tRNA^{Tyr} was eluted from the gel and used for T1 fingerprinting. Cyto RNA lane is total cytoplasmic RNA from uninfected cells in (B) and SV-tT/SV-rINS-7 infected cells in (C).

nucleotides and the overproduced tRNA species migrates with a mobility expected of a tRNA of this length. Thus, the precursor to the *X. laevis* tRNA^{Tyr} is processed by splicing in mammalian cells (see below for analysis). The overproduced tRNA was purified by electrophoresis (Fig. 3B; and see Materials and Methods). Equivalent samples of mock and SV40 infected cytoplasmic RNAs were similarly prepared for analysis as controls (Fig. 3B).

The corresponding endogenous monkey tRNA^{Tyr} species was purified by hybridization. ³²P-labeled RNA from uninfected monkey cells was hybridized to filters on which 20µg of either pSV-tT, pt201 or pBR322-SV40 denatured DNA was immobilized. After hybridization, RNA was released and resolved by gel electrophoresis (Fig. 3C). Both pt201 DNA (the original pBR322-*X. laevis* plasmid DNA) and pSV-tT DNA (the pBR322-SV40-tRNA^{Tyr} plasmid DNA) selected tRNA size RNAs. The pSV-tT DNA selected RNA should be the endogenous monkey tRNA^{Tyr}.

Analysis of *Xenopus* tRNA^{Tyr} Synthesized in CV-1 Cells

Modified base composition. The extent of modification of the ³²P-labeled *Xenopus* tRNA^{Tyr} produced in CV-1 cells was examined by RNase T2 digestion followed by two dimensional thin layer chromatography (Fig. 4). Besides the four major spots corresponding to unmodified 3' monophosphates (Ap, Cp, Up and Gp) several minor spots were observed. These were identified on the basis of their mobility as: pseudouridine 3'-phosphate (ψp), dihydrouridine 3'-phosphate (Dp), ribothymidine 3'-phosphate (Tp), 1-methylguanosine 3'-phosphate (m¹Gp), N²-methylguanosine 3'-phosphate (m²Gp), N²-dimethylguanosine 3'-phosphate (m²₂Gp), 5-methylcytidine 3'-phosphate (m⁵Cp), 1-methyladenosine 3'-phosphate (m¹Ap) and pCp. The spot corresponding to pCp which is derived from the 5' terminus of the tRNA^{Tyr} was also identified by its conversion to pC following treatment with nuclease P1 (which has a 3'-phosphatase activity) and chromatography under other conditions. The modified nucleotide acp³Up reported to be present in mammalian tRNA^{Tyr} (12,22) migrates similarly to m¹Gp and probably forms one part of the doublet in Figure 4. The modified nucleotide N⁶-Isopentenyl-adenosine (i⁶Ap), which is present on the 3'-side of the anticodon in yeast tRNA^{Tyr} was not found nor was N-[N-(9-D-ribofuranoslypurin-6-yl)carboxyl]

Figure 4. Modified nucleotide composition analysis of ^{32}P -labeled tRNA^{Tyr} . tRNA^{Tyr} purified from SV-tT/SV-rINS-7 infected CV-1 cells by electrophoresis (RNA from lane SV-tT/SV-rINS-7 in panel B or Fig.3) was digested with RNase T2 and the products were separated by two dimensional thin-layer chromatography.

threonine 3'-phosphate (t^6Ap) which has been reported to be a component of rat liver tRNA^{Tyr} (23).

Oligonucleotides present in T1 RNase digests. The homogeneity and sequence of the overproduced tRNA can be partially determined by digestion with a more specific RNase such as T1. A fingerprint of a T1 RNase digestion of the ^{32}P -labeled RNA purified by electrophoresis in two different percentage acrylamide gels is shown in Figure 5A. First, the complexity of the pattern is that expected for a single tRNA species. Second, further digestion of the oligonucleotides with T2-RNase and analysis of the nucleotides produced by thin layer chromatography have allowed assignment of most of the oligonucleotides on the basis of the expected sequence (Fig.5B; see Table I for products

Figure 5. Fingerprints of oligonucleotides present in T1-RNase digests of ^{32}P -labeled tRNA^{Tyr} . ^{32}P -labeled *X. laevis* tRNA^{Tyr} was eluted from the prominent band marked in Fig. 3, panel B (lane SV-tT/SV-rINS-7) and digested with RNase T1. The oligonucleotide products were resolved by two dimensional homochromatography (20) and are shown in panel A. Panel B is a schematic representation of the oligonucleotide sequences. RNase T1 digestion products of the equivalent band from the mock lane of panel B (Fig. 3) are shown in panel C. Panel D shows the RNase T1 products of ^{32}P -labeled tRNA^{Tyr} purified by hybridization selection of RNA from uninfected CV-1 cells and subsequent electrophoresis in acrylamide gel (panel C of Fig. 3, lane pSV-tT). T1 oligonucleotides were resolved by electrophoresis on cellulose acetate at pH 3.5 in the first dimension and homochromatography on DEAE cellulose at 65°C in the second dimension.

of RNase T2 digestion) and their location on the original fingerprint (20). T1-fingerprint analysis of the overproduced tRNA^{Tyr} suggests that the 5' and 3' termini are correctly processed. The 5' terminus was released in the T1 oligonucleotide pCCUUCGp. A low molar yield of the T1 oligonucleotide ACCA corresponding to the 3' terminus was obtained. This is, however, most likely due to inefficient transfer and/or retention of ACCA on the DEAE-plate during two-dimensional homochromatography. A similar low yield was observed in the analysis of endogenous monkey cell tRNA^{Tyr} . Further evidence that the mat-

TABLE 1

Relative Amounts of Nucleotides
Released by T2 Digestion of
Oligonucleotides

Oligonucleotide		
Number		Identity
1	Ap=Cp=Up> ψ p=Gp	A ψ CCUUAGp
2	Cp=Up>pCp* (Gp)	pCCUUCGp
2.1	Cp>Ap=Up= ψ p	
3	Cp>Ap=Up (Gp)	CUCAGp
4	Ap>Up>m ² Gp	AUAm ² Gp
5	Ap=Cp=Up=Gp	ACUGp
6	Cp=Up>m ¹ Ap (GP)	m ¹ AUUCCGp
7	Cp>Up>Gp	CUCGp
8	Ap>Gp	AAGp
9	Cp=Up= ψ p (Gp)	T ψ CGp
10	Ap= ψ p=m ¹ Gp	ψ Am ¹ Gp
11	Cp>Up=Gp>Dp	CDGp+CUGp
12	N.I.	
13	Ap (Gp)	AGp
14	N.I.	
15	Cp=m ² Gp	Cm ² Gp
16	Gp	Gp
17	-	ACCA

*pCp contained equivalent amounts of ³²P as Cp and Up; therefore, pCp is present in 1/2 the molar yield as Cp and Up.

(Gp) means that Gp was streaked in the fingerprint and therefore its relative intensity could not be judged.

N.I., not identified.

ure *X. laevis* tRNA is processed at its 3'-terminus is derived from the finding that the oligonucleotide ACGp which spans the CCA addition site in the precursor is absent from the fingerprint of the mature tRNA.

In experiments to be reported elsewhere, fingerprints of T1 RNase cleavage products of tRNA transcribed *in vitro* from pSV-

tT DNA have been compared with those from *in vivo* labeled *X. laevis* tRNA^{Tyr} (Fig.5A). The only differences observed are due to incomplete modification and processing of the *in vitro* product. The combination of *in vitro* labeling with each $\alpha^{32}\text{P}$ nucleoside triphosphate and secondary digestions of T1-RNase oligonucleotides by T2-RNase yielded the sequence of most of the assigned oligonucleotides in Fig.5B (manuscript in preparation). Thus the overproduced tRNA species in SV-tT/SV-rINS-7 infected cells is the anticipated *X. laevis* tRNA^{Tyr} species.

Analysis of endogenous tRNA^{Tyr}. The T1 RNase fingerprint of the endogenous monkey tRNA^{Tyr} species is shown in Figures 5C and D. The ^{32}P -labeled RNA resolved in Figure 5C was purified from uninfected monkey cells in parallel with the *X. laevis* tRNA^{Tyr} sample by sequential electrophoresis in two different percentage acrylamide gels (see Fig.3A and B). In this sample, the tRNA^{Tyr} species is contaminated by other RNAs that yield prominent spots here but minor spots in the overproduced tRNA^{Tyr} analyzed in Figure 5A. A T1 RNase fingerprint of the ^{32}P -labeled RNA prepared by hybridization selection with pSV-tT DNA from uninfected monkey cell RNA is shown in Fig.5D. The hybridization-selected RNA was also purified by electrophoresis in acrylamide-urea gels (see Fig.3C). The T1 fingerprint of the hybridization selected RNA from uninfected monkey cells is identical to that of the overproduced *X. laevis* tRNA^{Tyr}, suggesting that the *X. laevis* tRNA^{Tyr} species is very similar if not identical in sequence to the endogenous monkey tRNA^{Tyr} species.

DISCUSSION

Nonsense suppressors have long been sought in mammalian cells but not yet identified. An attractive approach for creation of a mammalian cell nonsense suppressor would be to use site-specific mutagenesis to convert a tRNA gene to a suppressor. The results reported in this paper show that the tRNA^{Tyr} gene of *X. laevis* is an excellent candidate for such site specific mutagenesis. When cloned into the late region of SV40, this gene produces high levels of mature tRNA^{Tyr} after infection of monkey cells. The mature tRNA^{Tyr} is apparently completely processed. This includes the removal of a 13bp intervening se-

quence and appropriate modification of some of the nucleotides.

Based on DNA sequence studies, Muller and Clarkson (10) concluded that the *X. laevis* tRNA^{Tyr} gene contained a 13b intervening sequence and that this intervening sequence was located adjacent to the 3' side of the anticodon (6). Their assignment on the exact location of the intervening sequence was influenced by a report that rat liver tRNA^{Tyr} contained t⁶A (23). Since t⁶A has so far only been found immediately adjacent to the 3' side of the anticodon, assignment of the splice site by Muller and Clarkson assumed that an A residue was present next to the anticodon. The *X. laevis* tRNA^{Tyr} species that is overproduced after infection with the recombinant SV40 virus, however, does not contain t⁶A and has instead m¹G in this position. Kuchino and Nishimura (12) also failed to find t⁶A in tRNA^{Tyr} of rat liver. T1 RNase fingerprint analysis of the *X. laevis* tRNA^{Tyr} expressed in mammalian cells, unambiguously places the 13b intervening sequence in the tRNA^{Tyr} gene one base to the 3' side of the anticodon (Fig.6), in the same position as in all tRNA genes of yeast (24).

It is not surprising that the *X. laevis* tRNA^{Tyr} gene is efficiently expressed in mammalian cells. The T1 RNase fingerprint of the endogenous monkey tRNA^{Tyr} species was identical to that of the overproduced *X. laevis* tRNA^{Tyr} species. Recently, Nishimura and colleagues have completed the sequence of tRNA^{Tyr} from rat liver (12). Their sequence agrees with that deduced from the overproduced tRNA^{Tyr} species with one possible difference. They suggest the rat tRNA^{Tyr} has an A at position 27 instead of the G found by Muller and Clarkson (10) and confirmed in this analysis. Given the similarities of the endogenous monkey and the *X. laevis* tRNA^{Tyr} species it is quite likely that the overproduced *X. laevis* tRNA^{Tyr} will be active in protein synthesis in mammalian cells.

Little is known about the regulation of synthesis of tRNAs in mammalian cells. The finding that a mature tRNA^{Tyr} can be overproduced by transcription of high copy numbers of an SV40-tRNA^{Tyr} recombinant suggests that tRNA synthesis is not auto-regulated. Indeed, not only were high levels of tRNA^{Tyr} transcribed, this RNA was also fully processed and modified.

Figure 6. Sequence and secondary structure of *X. laevis* tRNA^{Tyr}. The DNA sequence data of Muller and Clarkson (10) was combined with results from Kuchino and Nishimura (12) on rat liver tRNA^{Tyr} and on RNA fingerprint data from this paper to predict the sequence of the *X. laevis* tRNA^{Tyr} expressed in CV-1 cells. This tRNA is very similar, if not identical, to the endogenous tRNA^{Tyr} of CV-1 cells. The hypermodified guanosine derivative queuosine is frequently found in the first position of the anticodon of tRNA^{Tyr}. Kuchino and Nishimura (12) have reported this modified base in rat liver tRNA^{Tyr}. Neither the *X. laevis* tRNA^{Tyr} species synthesized in CV-1 cells nor the endogenous CV-1 tRNA^{Tyr} species contain appreciable levels of queuosine.

These observations might suggest that the pool of tRNA present in mammalian cells represents constitutive levels of transcription of endogenous genes. However, these results should be interpreted with caution as transcription of endogenous genes might be regulated under normal physiological conditions and this regulation affected by either virus infection or amplification of template. For the purpose of these studies, however, it is clear that a tRNA^{Tyr} gene linked to replicating SV40 DNA is efficiently expressed. Consequently, linkage of a suppressor tRNA to SV40 DNA should yield reasonably high levels of the

suppressor tRNA.

Among eukaryotic organisms, suppression of nonsense mutations due to the expression of a mutant tRNA gene has been demonstrated only in yeast (1,2) and recently in *Caenorhabditis elegans* (25). In mammalian cells, amber suppressors may be less deleterious than UAA or UGA suppressors, because of the scarcity of amber codons as termination signals in cellular and viral genes and among eukaryotes in general. For example, none of the SV40 or adenovirus genes terminate in UAG codons. Thus, it might be possible to establish an amber suppressor in a mammalian cell line or at least to transiently establish a suppressor state by infection with an SV40-tRNA suppressor recombinant. The *X.laevis* tRNA^{Tyr} gene is a good candidate for conversion to an amber suppressor by *in vitro* site-specific mutagenesis as a single-point mutation converting the anticodon 5' GUA to 5' CUA should be sufficient.

Finally, a number of amber mutants in viruses of mammalian cells have been isolated and characterized. These have been in nonessential viral gene products such as the SV40 sequences in defective Ad2⁺ND1 hybrid viruses (26) and the thymidine kinase gene of Herpes Simplex virus (27). In addition, an amber codon has been found to separate the gag and pol genes of moloney leukemia virus (28) and in a SV40 mutant defective for synthesis of the A gene product (29). These viruses and cell lines can be used to establish the suppressor efficiency of a suppressor tRNA gene. The availability of suppressors for the isolation of mutants of animal viruses with nonsense mutations in essential genes should greatly advance understanding of animal virology.

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ABBREVIATIONS

HeBS: Hepes Buffered Saline
 TE: 10mM Tris, 1mM EDTA
 CPE: Cytopathic effect
 DME: Dulbecco's modified Eagle's media
 PBS: Phosphate Buffered Saline
 m.o.i.: Multiplicity of infection

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NOTE ADDED IN PROOF

Recently, Temple *et al.*, (Nature 296, 537-540, 1982) have described the construction of an amber suppressor derived from human tRNA^{lys} gene and its activity following microinjection into *Xenopus* oocytes.