Nucleic Acids Research

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

Frank A.Laski, Birgit Alzner-DeWeerd, Uttam L.RajBhandary and Phillip A.Sharp

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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 utiliz-
ed 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 selec-
tion of nonsense suppressor tRNAs (1,2). Selection of equiva-
lent 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 applica-
tion 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. Synthe-
sis of large amounts of the suppressor-tRNA from the replicat-
ing SV40 vector would ensure efficient suppression. In addi-
tion, 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 Dagert 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
(10µl) 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 200µl TE ph 8.0 (10mM Tris pH 8.0, 1mM EDTA) and diluted to
2ml with ligation buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 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 re-suspended in 10µl TE pH 8.0 and 0.5ml 2x HeBS (Hepes 10g/l;
NaCl 15g/l; KCl 0.74g/l; $Na_2HPO_4.H_2O$ 0.25g/l; dextrose 2g/l;
pH 7.05) was added. 0.5ml of 0.25M $CaCl_2$ 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 $CaCl_2$ 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 $CaCl_2$. 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, 100µg/ml penicillin
and 10µg/ml streptomycin; the cells were incubated at 37°C.
Twenty-four hours later the medium was changed to DME containing 2% calf serum, 100µg/ml penicillin and 10µg/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(-PO$_4$) and labeled with 500µCi of carrier free ^{32}PO$_4$ (New England Nuclear) in 3ml of DME(-PO$_4$) 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 MgCl$_2$). 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 ^{32}P-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. ^{32}P-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 2-3μl H_2O and subjected to two-dimensional homochromatography finger-printing (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% pyridium 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 H_2O at 65°C and then chromatographed in homomix (7M urea, 30% yeast tRNA hydrolyzed in 50mM KOH over-night at 65°C, then neutralized to pH 4.5) at 65°C. The plate was dried and autoradiographed.

The ^{32}P-labeled oligonucleotides were recovered as describ-ed previously (20).

Analysis of Nucleotide Composition by Digestion with T2-RNase

[32]P-labeled tRNA or oligonucleotide was mixed with 0.5 A_{260} unit of carrier yeast tRNA and digested with 1 unit RNase T2 for 6 hours at 37°C in 20mM NH_4 acetate pH 4.5. The sample was lyophilized five times, then resuspended in 1-2μl H_2O and chromatographed in two dimensions on a pre-coated cellulose TLC plate (Merck 5757). Solvent for the first dimension was isobutyric acid: $NH_4OH:H_2O$, 48:3.2:32 vol/vol and for the sec-ond dimension, t-butyl alcohol: Conc.$HCl:H_2O$, 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.laevis 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 ex-cised 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] re-combinant DNA was linearized by digestion with BamHI and then

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HhaI          -50        -40        -30        -20        -10            tRNA Tyr→  10
GCGCCACCCTGTAGCAGCTGAACAATTGAAGTCCACCAAGTCACGGGCGATGGGTGCCCCACCGGCCTTCGATAGCTCAG
CGCGGTGGGACATCGTCGACTTGTTAACTTCAGGTGGTTCAGTGCCCGCTACCCACGGGGTGGCCGGAAGCTATCGAGTC

     20        30        37          38  40        50        60          70  73
CTGGTAGAGCGGAGGACTGTAGGTGTGATCGAGCAATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGACGCTTTGTT
GACCATCTCGCCTCCTGACATCCACACTAGCTCGTTAGGAATCCAGCGACCAAGCTAAGGCCGAGCTTCCTGCGAAACAA

+10      +20        +30        +40        +50        +60        +70        +80
TTCCAATGCGAGCCTAAGAAGGAGATGAGTGGCATTTCAGGAAAGCTCTTTCTGCACCATGTGGCTGAGGACGAATGCTG
AAGGTTACGCTCGGATTCTTCCTCTACTCACCGTAAAGTCCTTTCGAGAAAGACGTGGTACACCGACTCCTGCTTACGAC

+90      +100                HaeII
AGGACACCACACCCCAGTCAAAGCGCY-3'
TCCTGTGGTGTGGGGTCAGTTTCGCGR-5'
                            HhaI
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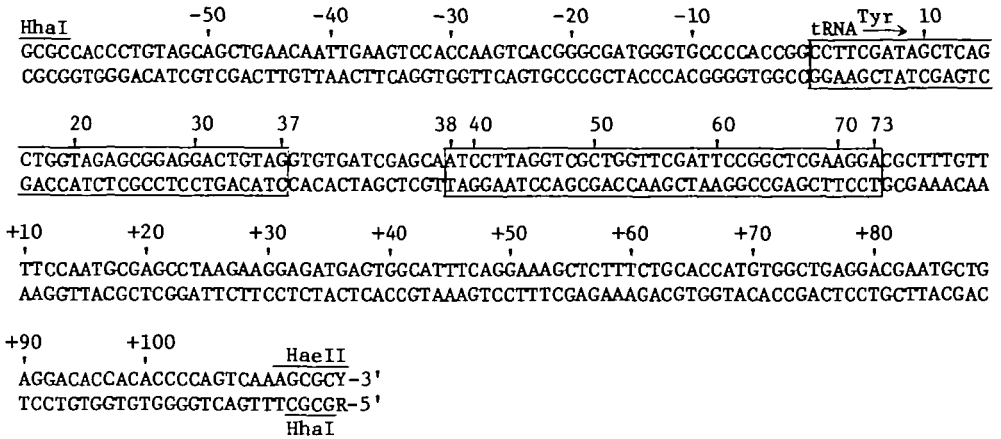
Figure 1. DNA sequence of the HhaI-HaeII restriction fragment of pt201 con-
taining the gene for tRNA^Tyr of *X. laevis*. This fragment was inserted into
the late region of SV40 in the construction of pSV-tT. The boxed sequence
is the coding region for the mature tRNA^Tyr. The two coding regions are
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 colon-
ies 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/dele-
tion 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 Meth-
ods). The approximate titer and homogeneity of the virus
stocks were determined by characterization of viral DNA extrac-
ted 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 con-
tains less than 1% w.t. recombinant virus. In addition no aber-
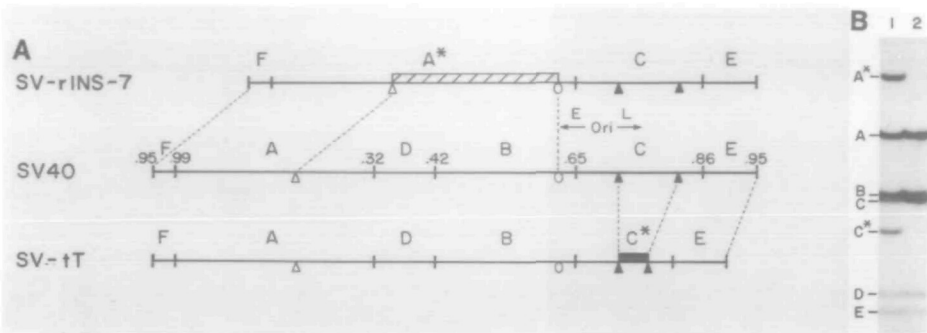rant viral DNA forms appear in the recombinant stock.

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; ▲ = TaqI site; ○ = 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
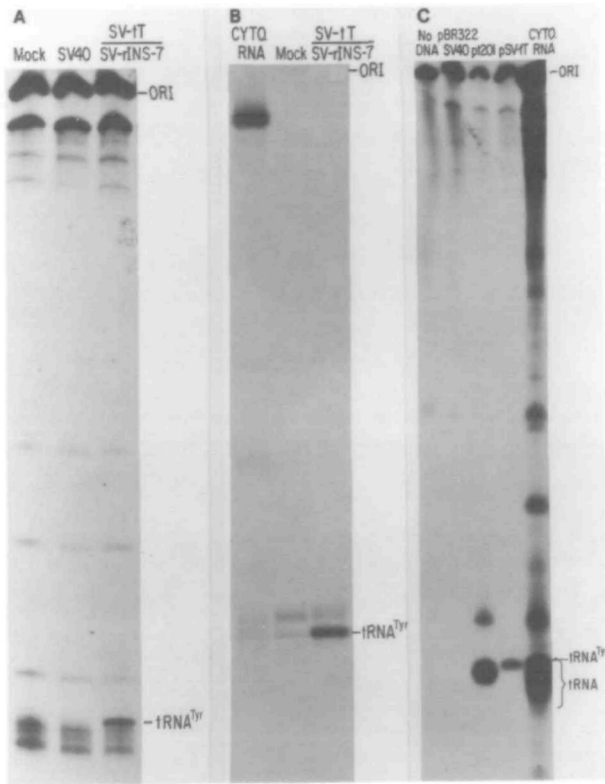
Figure 3A. Autoradiogram of ^{32}P–RNA purified from CV-1 cells and analyzed on a 7.5% polyacrylamide 8.3M urea gel. Cells were either mock infected, infected with SV40 or infected with the SV-tT/SV-rINS-7 virus stock. 46 hours post infection cells were labeled with ^{32}P- and 6 hours later cytoplasmic 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 20μg of denatured pSV-tT DNA, pt201 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 puri-
fied 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 pur-
ified by hybridization. ^{32}P-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 hy-
bridization, RNA was released and resolved by gel electrophore-
sis (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
^{32}P-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 corres-
ponding 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 (m^{1}Gp), N^2-methylguanosine 3'-
phosphate (m^{2}Gp), N^2-dimethylguanosine 3'-phosphate (m$_2^2$Gp), 5-
methylcytidine 3'-phosphate (m^{5}Cp), 1-methyladenosine 3'-phos-
phate (m^{1}Ap) 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 acp^{3}Up reported to
be present in mammalian tRNA^{Tyr} (12,22) migrates similarly to
m^{1}Gp and probably forms one part of the doublet in Figure 4.
The modified nucleotide N^6-Isopentenyl-adenosine (i^{6}Ap), which
is present on the 3'-side of the anticodon in yeast tRNA^{Tyr} was
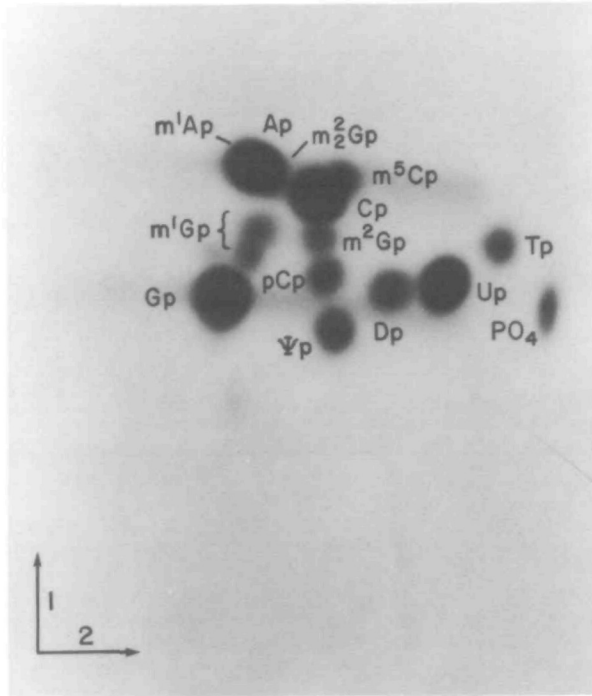not found nor was N-[N-(9-D-ribofuranoslypurin-6-yl)carboxyl]

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^{6}Ap) which has been reported to be a component of rat liver tRNA^{Tyr} (23).

Oligonucleotides present in Tl RNase digests. The homogeneity and sequence of the overproduced tRNA can be partially deter- mined by digestion with a more specific RNase such as Tl. A fingerprint of a Tl RNase digestion of the ^{32}P-labeled RNA pur- ified by electrophoresis in two different percentage acrylamide gels is shown in Figure 5A. First, the complexity of the pat- tern is that expected for a single tRNA species. Second, fur- ther digestion of the oligonucleotides with T2-RNase and analy- sis 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
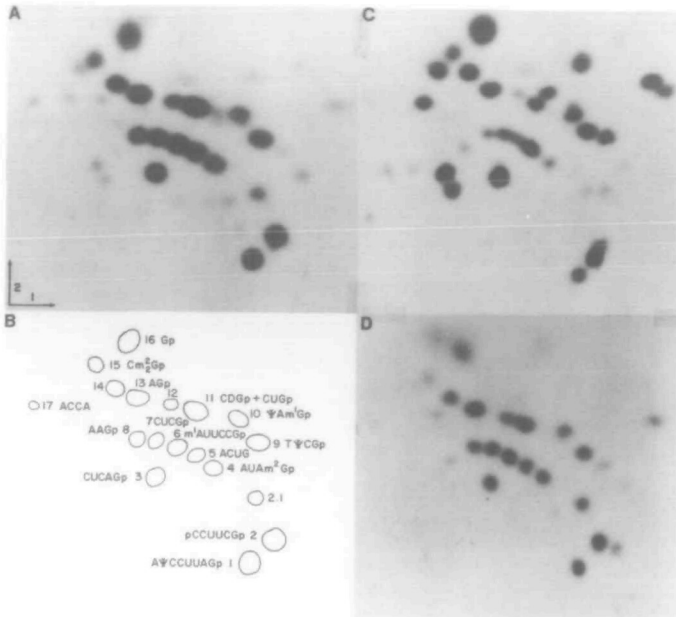
Figure 5. Fingerprints of oligonucleotides present in Tl-RNase digests of
^{32}P-labeled tRNA^{Tyr}. ^{32}P-labeled *X. laevis* tRNA^{Tyr} was eluted from the prom-
inent band marked in Fig.3, panel B (lane SV-tT/SV-rINS-7) and digested with
RNase Tl. The oligonucleotide products were resolved by two dimensional
homochromatography (20) and are shown in panel A. Panel B is a schematic
representation of the oligonucleotide sequences. RNase Tl 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 Tl 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). Tl oligo-
nucleotides 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 fin-
gerprint (20). Tl-fingerprint analysis of the overproduced
tRNA^{Tyr} suggests that the 5' and 3' termini are correctly pro-
cessed. The 5' terminus was released in the Tl oligonucleotide
pCCUUCGp. A low molar yield of the Tl oligonucleotide ACCA cor-
responding 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 homochromato-
graphy. 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>ψp=Gp	AψCCUUAGp
2	Cp=Up>pCp* (Gp)	pCCUUCGp
2.1	Cp>Ap=Up=ψp	
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≈Tp=ψp (Gp)	TψCGp
10	Ap=ψp=m^{1}Gp	ψAm^{1}Gp
11	Cp>Up>Gp>Dp	CDGp+CUGp
12	N.I.	
13	Ap (Gp)	AGp
14	N.I.	
15	Cp=m$^2_2$Gp	Cm$^2_2$Gp
16	Gp	Gp
17	–	ACCA

*pCp contained equivalent amounts of ^{32}P 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 finger-
print 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 $\alpha^{32}P$
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 prepara-
tion). Thus the overproduced tRNA species in SV-tT/SV-rINS-7
infected cells is the anticipated *X. laevis* tRNA^{Tyr} species.
<u>Analysis of endogenous tRNA^{Tyr}</u>. 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-lab-
eled RNA prepared by hybridization selection with pSV-tT DNA
from uninfected monkey cell RNA is shown in Fig.5D. The hy-
bridization-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}, suggest-
ing 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 crea-
tion of a mammalian cell nonsense suppressor would be to use
site-specific mutagenesis to convert a tRNA gene to a suppres-
sor. The results reported in this paper show that the tRNA^{Tyr}
gene of *X. laevis* is an excellent candidate for such site speci-
fic 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) con-
cluded that the *X. laevis* tRNA[Tyr] gene contained a 13b interven-
ing sequence and that this intervening sequence was located ad-
jacent to the 3' side of the anticodon (6). Their assignment
on the exact location of the intervening sequence was influenc-
ed by a report that rat liver tRNA[Tyr] contained t[6]A (23).
Since t[6]A 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 overpro-
duced after infection with the recombinant SV40 virus, however,
does not contain t[6]A and has instead m[1]G in this position.
Kuchino and Nishimura (12) also failed to find t[6]A in tRNA[Tyr]
of rat liver. Tl 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 ef-
ficiently expressed in mammalian cells. The Tl RNase finger-
print 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 differ-
ence. They suggest the rat tRNA[Tyr] has an A at position 27 in-
stead of the G found by Muller and Clarkson (10) and confirmed
in this analysis. Given the similarities of the endogenous mon-
key and the *X. laevis* tRNA[Tyr] species it is quite likely that
the overproduced *X. laevis* tRNA[Tyr] will be active in protein syn-
thesis 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 muta-
tions due to the expression of a mutant tRNA gene has been de-
monstrated 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 mam-
malian cell line or at least to transiently establish a suppres-
sor 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 synthe-
sis of the A gene product (29). These viruses and cell lines
can be used to establish the suppressor efficiency of a suppres-
sor tRNA gene. The availability of suppressors for the isola-
tion of mutants of animal viruses with nonsense mutations in es-
sential genes should greatly advance understanding of animal
virology.

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.