The EMBO Journal vol.4 no.1 pp.213 – 221, 1985

Amber, ochre and opal suppressor tRNA genes derived from a human serine tRNA gene

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Communicated by H. J. Gross

Amber, ochre and opal suppressor tRNA genes have been generated by using oligonucleotide directed site-specific mutagenesis to change one or two nucleotides in a human serine tRNA gene. The amber and ochre suppressor (Su^+) tRNA genes are efficiently expressed in CV-1 cells when introduced as part of a SV40 recombinant. The expressed amber and ochre Su^+ tRNAs are functional as suppressors as demonstrated by readthrough of the amber codon which terminates the NS1 gene of an influenza virus or the ochre codon which terminates the hexon gene of adenovirus, respectively. Interestingly, several attempts to obtain the equivalent virus stock of an SV40 recombinant containing the opal suppressor tRNA gene yielded virus lacking the opal suppressor tRNA gene. This suggests that expression of an efficient opal suppressor derived from a human serine tRNA gene is highly detrimental to either cellular or viral processes.

Key words: tRNA genes/ambre, ochre and opal suppressor/serine tRNA

Introduction

The availability of *Escherichia coli* strains with a variety of nonsense suppressor mutations was crucial for much of the early work on genetic analysis of chain terminating mutations in bacterial and bacterial viral genomes (Steege and Soll, 1979). Among eukaryotes, nonsense suppressors have been well characterized only in yeast (Sherman, 1982) and more recently, an amber suppressor, in *Caenorhabditis elegans* (Wills *et al.*, 1983). In mammalian cells, in spite of several attempts, classical genetic selections have so far not yielded any cell lines carrying nonsense suppressors.

Recently, we described the use of site-specific mutagenesis to generate amber and ochre suppressors derived from a *Xenopus laevis* tyrosine tRNA gene. The suppressor tRNA genes were shown to be functional in mammalian cells and cell lines carrying the suppressors established (Laski *et al.*, 1982b, 1984; Hudziak *et al.*, 1982). Temple and co-workers similarly generated amber suppressor tRNA genes from human lysine and glutamine tRNA genes (Temple *et al.*, 1982), although definitive evidence for their function as suppressors in mammalian cells is lacking.

Suppression of nonsense mutants by particular tRNAs may not in all cases restore a normal phenotype since the amino acid which is inserted at the position of the nonsense codon may be incompatible with structure, function or stability of the gene product. It is thus important to have a variety of suppressors which insert different amino acids. In this paper we describe the generation of amber (UAG), ochre (UAA) and opal (UGA) suppressor tRNA genes, all derived from a human serine tRNA gene. Using replicating SV40 recombinant vectors, we show that the amber and ochre suppressor tRNA genes are functional and produce tRNAs which suppress amber and ochre codons, respectively. Interestingly, several attempts to obtain the equivalent SV40 virus stocks containing the opal suppressor tRNA gene have, so far, been unsuccessful.

Results

Oligonucleotide-directed site-specific mutagenesis of human $tRNA^{Ser}$ gene to generate amber, ochre and opal Su^+ genes

The recombinant plasmid p17Sau3A (obtained from Dr. N. Martin) contains a 900-bp *Sau*3A fragment, encoding a single copy of a human serine tRNA gene, cloned into the *Bam*HI site of pBR322. The structure of the tRNA, as deduced from the gene sequence is shown in Figure 1a. The only difference between the human $tRNA^{Ser}$ and the rat liver $tRNA^{Ser}$, previously sequenced, is the presence of U (or possibly a modified U), in the first position of the anticodon in place of I in the latter (Ginsberg *et al.*, 1971; Randerath *et al.*, 1981; Seung Yoo and Martin, unpublished).

Single-stranded DNA template used for mutagenesis was obtained by subcloning the *Sau*3A fragment into the *Bam*HI site of M13mp9. Site-specific mutagenesis was carried out as described in Materials and methods using the pentadecamer deoxyribooligonucleotides shown in Figure 1b as primers for the synthesis of covalently closed circular double-stranded DNA. In the case of the ochre suppressor, the desired mutation would change the 5'TGA anticodon to 5'TTA and, in the process, create a new *Dra*I site. The primer synthesized to generate an opal suppressor contains a C-C mismatch, which would change the 5'TGA anticodon to 5'TCA. An oligonucleotide primer containing a two base mismatch was used to generate an amber suppressor, changing the 5'TGA anticodon to 5'CTA. This mutation introduces a new *Hinf*I site.

The desired mutants were, in each case, identified by screening individual plaques by hybridization with the respective primer oligonucleotides under conditions of increasing stringency (Wallace *et al.*, 1978; Zoller and Smith, 1983). The frequency of mutagenesis for generating the ochre and opal suppressor tRNA genes was found to be ~10%. The mutagenic efficiency in generating the amber suppressor tRNA gene was significantly lower (one positive out of 72 phage plaques screened). This lower mutagenic frequency is presumably due to the fact that the oligonucleotide used contained a two base mismatch (starred nucleotides in Figure 1b). The presence of the desired change in each mutant type was confirmed by DNA sequence analysis of the M13 recombinant virion DNAs using the dideoxy method (Sanger *et al.*, 1977).

Production of SV40 (Su^+) recombinants

The genes encoding wild-type, ochre, opal and amber tRNA suppressors were excised from their respective M13 recombinants by digestion with *Sau*3A and cloned into the unique *Bam*HI site of pSVIgt3, yielding constructs designated pSV-

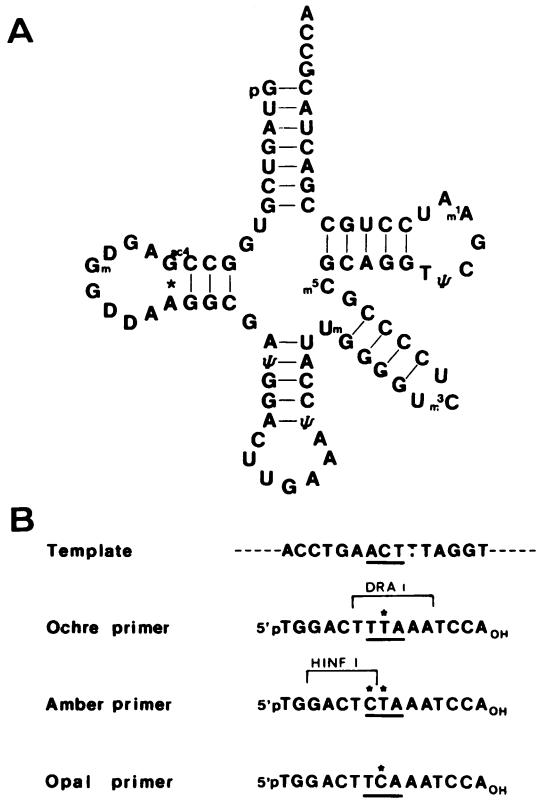

Fig. 1. (A) Sequence and structure of human $tRNA^{Ser}$. The DNA sequence from the human serine tRNA gene was combined with the results of Ginsberg *et al.* (1971) on rat liver $tRNA^{Ser}$ and the RNA fingerprint data reported in the present work to predict the structure of human serine tRNA. The human serine tRNA is identical in sequence to the rat liver serine tRNA with the exception of a substitution of U for I in the first position of the anticodon in the latter. The modified base i^6A reported to be present on the 3′ side of the anticodon in the rat liver tRNA was not detected in human serine tRNA expressed in CV-1 cells. **(B)** Sequence of oligonucleotide primers used in mutagenesis. The template DNA sequence shown is from M13-tS, a derivative of M13mp9 which contains the coding strand of the human serine tRNA gene. The oligonucleotide primers are complementary to the template sequence except that they have either one base mismatch (for generation of ochre and opal suppressors) or two base mismatch (for generation of amber suppressor). Asterisks indicate the nucleotides which are not complementary to the template DNA.

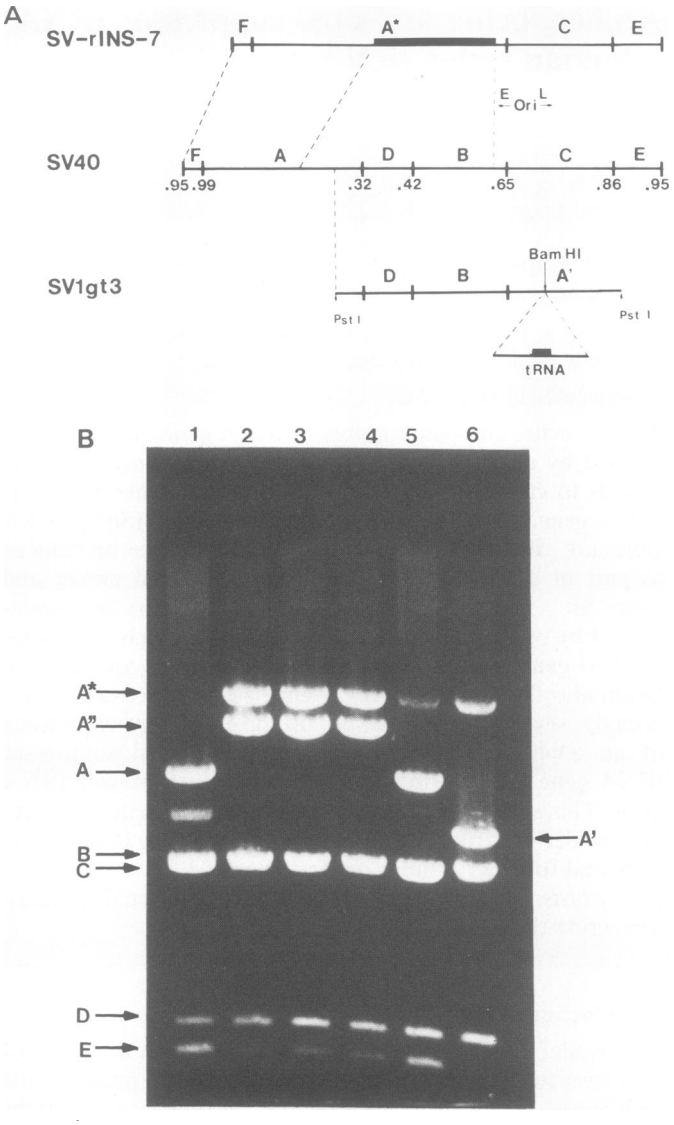

Fig. 2. (A) *Hind*III restriction map of SV-rINS-7, SV40 and SV1gt3. Solid vertical lines represent *Hind*III sites. SV-rINS-7 has an insertion substitution in the early region, creating a new *Hind*III restriction fragment A*. This construct has been described previously (Horowitz *et al.*, 1983). SV1gt3 contains a deletion of the late region of SV40, creating a new restriction fragment A′ which contains the unique *Bam*HI cloning site (Drabkin and RajBhandary, in preparation). pSV-tS Su$^-$, pSV-tS Su$^+$ (ochre), pSV-tS Su$^+$ (amber) and pSV-tS Su$^+$ (opal) contain the serine tRNA gene as a 900-bp fragment cloned into the unique *Bam*HI site of pSV1gt3, creating a new *Hind*III restriction fragment A′′. These latter recombinants are identical to each other except for point mutations in the sequence corresponding to anticodon of the tRNA. **(B)** Agarose gel electrophoretic analysis of viral DNA isolated from CV-1 cells infected with: **(1)** SV40; **(2)** SV-tS Su$^-$; **(3)** SV-tS Su$^+$ (ochre);**(4)** SV-tS Su$^+$ (amber); **(5)** SV-tS Su$^+$ (opal); and **(6)** SV1gt3. Viral DNA was digested with *Hind*III and the fragments were separated by electrophoresis on a 1% agarose gel. The bands are labeled as indicated in **A**.

tS Su$^-$, pSV-tS Su$^+$ (ochre), pSV-tS Su$^+$ (opal), and pSV-tS Su$^+$ (amber), respectively. pSVIgt3 is a SV40/pBR322 hybrid containing part of SV40 sequences, including the origin of replication and early functions (see Figure 2a). Virus stocks were prepared by co-transfecting each of the above recombinants into CV-1 cells along with the helper virus DNA SV-rINS-7 after removing the pBR322 sequences: in the case of pSVIgt3 by digestion with *Pst*I or, in the case of SV-rINS-7 by digestion with *Eco*RI. SV-rINS-7 is a SV40 derivative that has a deletion substitution in the early region (Figure 2a; Horowitz *et al.*, 1983; Laski *et al.*, 1982a). Virus stocks were harvested when cytopathic degeneration of the cells was evident (~14 days post-transfection). These viral stocks are designated SV-tS Su$^-$, SV-tS Su$^+$ (ochre), SV-tS Su$^+$ (amber), and SV-tS Su$^+$ (opal).

For analysis of the virus stocks, CV-1 cells were infected with the different recombinant stocks or with wild-type SV40 and replicating viral DNA was purified by Hirt extraction 48 h later. Analysis by agarose gel electrophoresis after digestion with *Hind*III demonstrated that SV-tS Su$^-$, SV-tS Su$^+$ (ochre), and SV-tS Su$^+$ (amber) recombinant virus stocks yielded the pattern of fragments expected from the two complementing genomes (Figure 2b, lanes 2–4). The recombinant virus stocks and wild-type SV40 (used at a m.o.i. of 10) produced comparable levels of DNA indicating that the recombinant virus stocks had a high titre. This result is in agreement with that previously obtained with *X. laevis* ochre and amber Su$^+$ $tRNA^{Tyr}$ genes, indicating that the presence of genes encoding ochre or amber suppressors on high copy

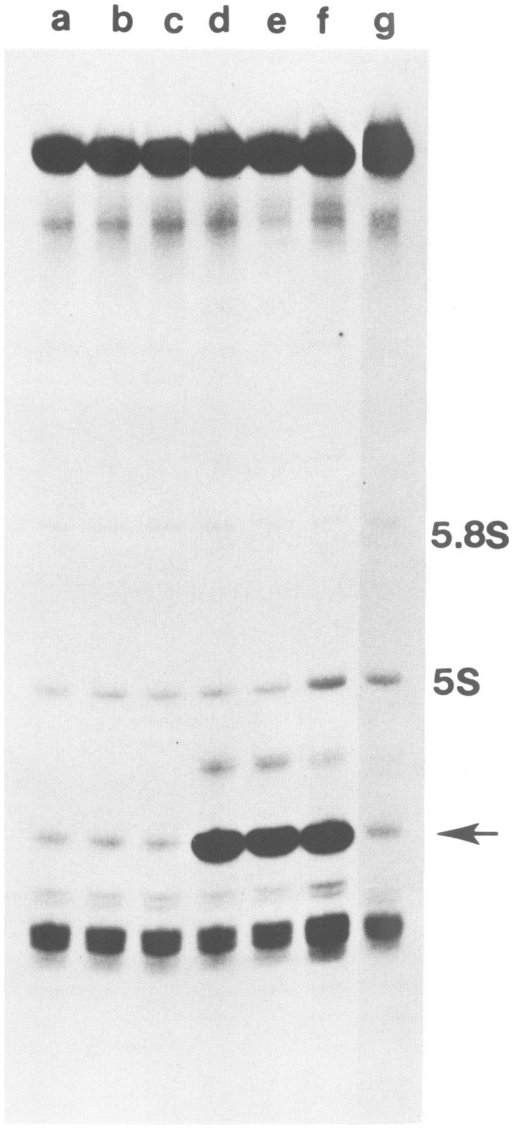

Fig. 3. Autoradiogram of ^{32}P-labeled RNA extracted from CV-1 cells and analyzed by electrophoresis on a 10% polyacrylamide/8 M urea gel. CV-1 cells (3 x 10^6) were: **(a)** mock-infected; **(b)** infected with 0.5 ml SV40 (10^8 p.f.u./ml); or infected with 1 ml of recombinant virus stocks from **(c)** SVgt3; **(d)** SV-tS Su$^-$; **(e)** SV-tS Su$^+$ (ochre); **(f)** SV-tS Su$^+$ (amber; and **(g)** SV-tS Su$^+$ (opal), respectively. The arrow indicates the position of mature human tRNA^{Ser}.

SV40 vectors does not inhibit SV40 replication to a significant extent (Laski *et al.*, 1982b, 1984).

The virus stocks obtained using pSV-tS Su$^+$ (opal) DNA generated a *Hind*III digestion pattern which was indistinguishable from that of wild-type SV40 (cf. Figure 2b, lanes 1 and 5). In five independent transfections we have been unable to obtain SV40 recombinant virus stocks containing the Su$^+$ opal tRNA gene. The possible reasons for this are discussed more fully below.

Expression of tRNA^{Ser} Su$^+$ ochre and amber genes in mammalian cells

Expression of the tRNA^{Ser} wild-type and Su$^+$ genes was examined by infecting CV-1 cells with SV-tS Su$^-$, SV-tS Su$^+$ (ochre), SV-tS Su$^+$ (amber), SV-tS Su$^+$ (opal) or SV40 and labeling with $^{32}PO_4$ 46 h later. Following a 6 h labeling period, total RNA was extracted from the cells and analyzed by electrophoresis on a 10% polyacrylamide/8 M urea gel. As demonstrated in Figure 3, infection with SV-tS Su$^-$, SV-tS Su$^+$ (ochre) or SV-tS Su$^+$ (amber) resulted in a significant over-expression of an RNA species with an electrophoretic mobility expected for the mature 85 nucleotide long human tRNA^{Ser} (Figure 3, lanes d–f). The intensity of the corresponding band seen in the mock-infected control was unaltered by infection with wild-type SV40 or with virus stock prepared from SVIgt3/SV-rINS-7 (Figure 3, lanes a–c). As compared with the levels of expression obtained with the wild-type tRNA gene, no significant differences were observed with either SV-tS Su$^+$ (ochre) or SV-tS Su$^+$ (amber) (cf. Figure 3, lanes d, e and f).

In contrast to the above results, virus stocks obtained by transfection with DNA containing the opal Su$^+$ tRNA gene did not overproduce a corresponding serine tRNA (Figure 3, lane g).

Modified base composition of tRNA^{Ser} isolated from CV-1 cells

Wild-type and mutant radioactive tRNAs made in CV-1 cells were purified by electrophoresis on 10% polyacrylamide/8 M urea gels and eluted. Base composition was determined by complete RNase T2 digestion of tRNAs followed by two-dimensional t.l.c. In addition to the four major spots corresponding to the unmodified 3′-monophosphates (Ap, Gp, Cp and Up), spots corresponding to pseudouridine 3′-phosphate (ψp), dihydrouridine 3′-phosphate (Dp), ribothymidine 3′-phosphate (Tp), 5 methylcytidine 3′-phosphate (m^5Cp) and 3 methylcytidine 3′-phosphate (m^3Cp) were observed (data not shown). The detection of 1-methyladenosine 3′-phosphate (m^1Ap) which is present in rat liver tRNA^{Ser} was obscured by its co-migration with Ap. The modified nucleotide 2′O methyl Gp also present in rat liver tRNA^{Ser} was detected as the dinucleotide GmpGp. Um is present at UmpGp which co-migrates with Gp. Of the several modified bases found in rat liver tRNA^{Ser}, only N-6 isopentenyl adenosine (i^6Ap), present on the 3′ side of the anticodon in rat liver tRNA^{Ser}, was not found in the human tRNA^{Ser} expressed in CV-1 cells.

Fingerprint analysis of tRNA^{Ser} synthesized in CV-1 cells

For comparative analysis of Su$^-$ and Su$^+$ tRNAs made in CV-1 cells, the radioactive tRNAs were digested with RNase T1 and analyzed by two-dimensional homochromatography or by electrophoresis on 20% polyacrylamide/8 M urea gels (Figure 4).

The changes introduced at the tRNA gene level to generate ochre and amber suppressors result in the replacement of the G residue with U in the anticodon of both the ochre and amber suppressor tRNAs. Thus two T1-resistant oligonucleotides found in the Su$^-$ tRNA corresponding to ACUUGp and AAAψCCAUUmGp should disappear in the mutants and be replaced by the pentadecanucleotide ACUUUAAAψ-CCAUUmGp in the ochre Su$^+$ and ACUCUAAAψCCAU-UmGp in the amber Su$^+$ tRNAs. The T1 RNase fingerprint patterns (Figure 4) show this to be the case. The longest oligonucleotide present in the Su$^-$ tRNA (a 10 mer, Figure 4 panel A and panel D, lane 1) is absent in both ochre (Figure 4 panel B and panel D, lane 2) and amber (Figure 4 panel C and panel D, lane 3) Su$^+$ tRNAs and is replaced by the expected pentadecanucleotide. The loss of ACUUGp in the Su$^+$ tRNAs is indicated by the reduction in intensity of the pentanucleotide region which in the Su$^-$ tRNA consists of a mixture of DDA-AGp and ACUUGp (Figure 4, panel D).

Psuedouridine is present in the anticodon of yeast tRNA^{Tyr}

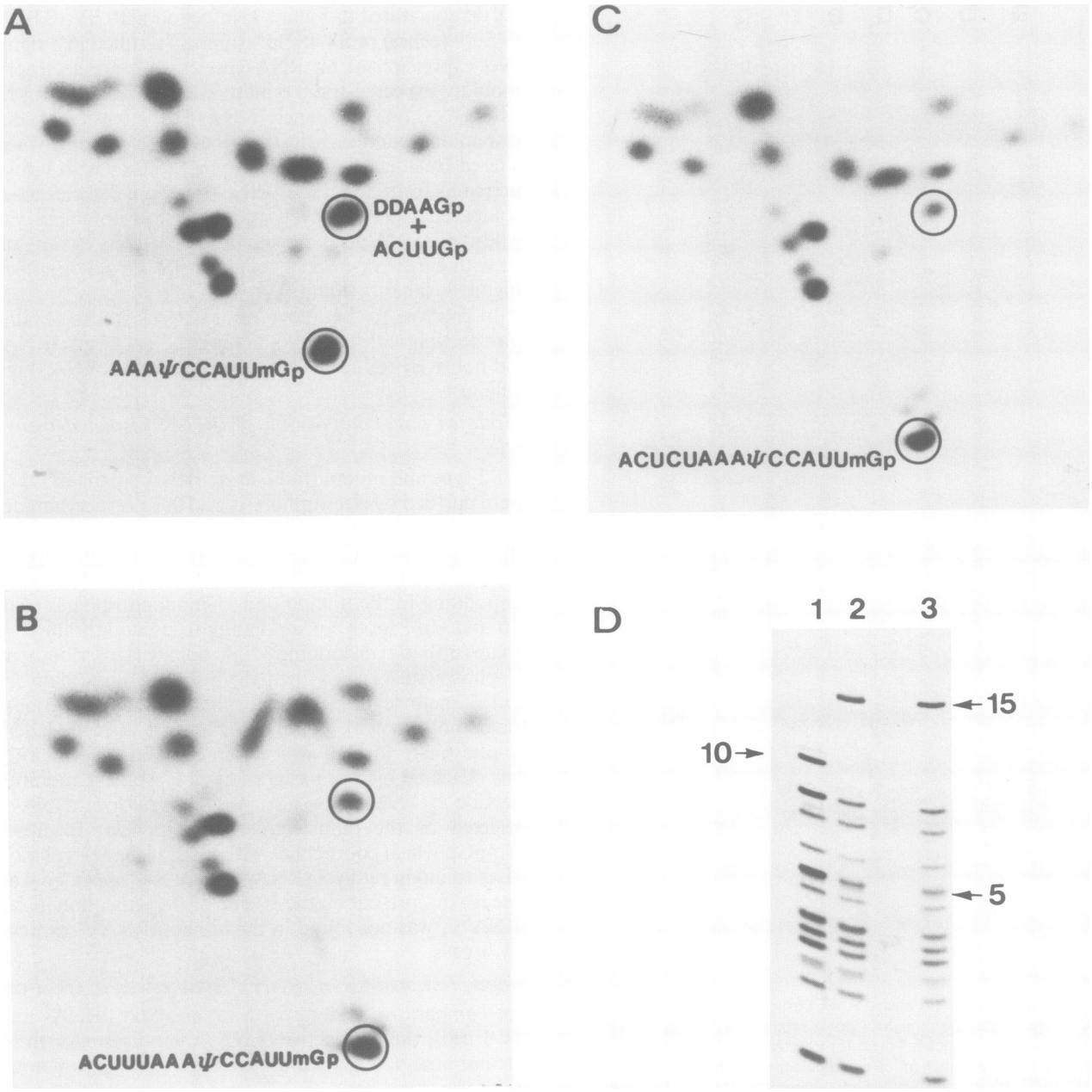

Fig. 4. RNase T1 analysis of ^{32}P-labeled tRNA^{Ser} purified from cells infected with: SV-tS Su$^-$ **(panel A)**; SV-tS Su$^+$ (ochre) **(panel B)**; and SV-tS Su$^+$ (amber) **(panel C)**. Radioactive tRNA was eluted from bands indicated by the arrow in Figure 3, digested with RNase T1, and analyzed by two-dimensional homochromatography **(panel A – C)** or by electrophoresis on a 20% polyacrylamide/8 M urea gel **(panel D). Lanes 1, 2** and **3** in **panel D** refer to tRNA obtained from SV-tS Su$^-$, SV-tS Su$^+$ (ochre), and SV-tS Su$^+$ (amber) infected cells, respectively.

ochre and amber suppressors and is required for efficient suppressor activity (Johnson and Abelson, 1983). To examine whether the tRNA^{Ser} amber and ochre suppressors synthesized in CV-1 cells contain ψ in the anticodon, the RNase T1-resistant pentadecanucleotides from the ochre and amber Su$^+$ tRNAs were digested with pancreatic RNase. The data for the oligonucleotide derived from the amber suppressor is shown in Figure 5. RNase A digestion failed to release ψp, indicating that there is no ψ modification in the anticodon of the Su$^+$ tRNAs (Figure 5, panel D). Digestion of these 15 mers as well as the RNase T1-resistant 10 mer from the Su$^-$ tRNA with RNase T2 released ψp. This indicates that ψ is present at position 39 on the 3′ side of the anticodon stem as for rat liver tRNA^{Ser} (Figure 5, panels A and C).

In vivo suppression of UAA codons by tRNA^{Ser} Su$^+$ (ochre)

The 120 000 dalton hexon protein of adenovirus is encoded by sequences which terminate in a single UAA codon. The UAA is followed by 648 bases of open reading frame which is thought to encode a 23 000 dalton protease that is found in the virion (Akusjarvi *et al.*, 1981; Weber, 1976). Readthrough of this ochre termination codon would lead to the synthesis of a 140 000 dalton fusion protein. This system was previously used to test the suppressor activity of the *X. laevis* tRNA^{Tyr} Su$^+$ (ochre) gene (Laski *et al.*, 1984).

To examine the function of the tRNA^{Ser} Su$^+$ (ochre), CV-1 cells were infected with SV-tS Su$^-$, SV-tS Su$^+$ (ochre), SV-tS Su$^+$ (amber), or SV40 and incubated at 37°C for 24 h to allow synthesis of viral-encoded products including tRNA.

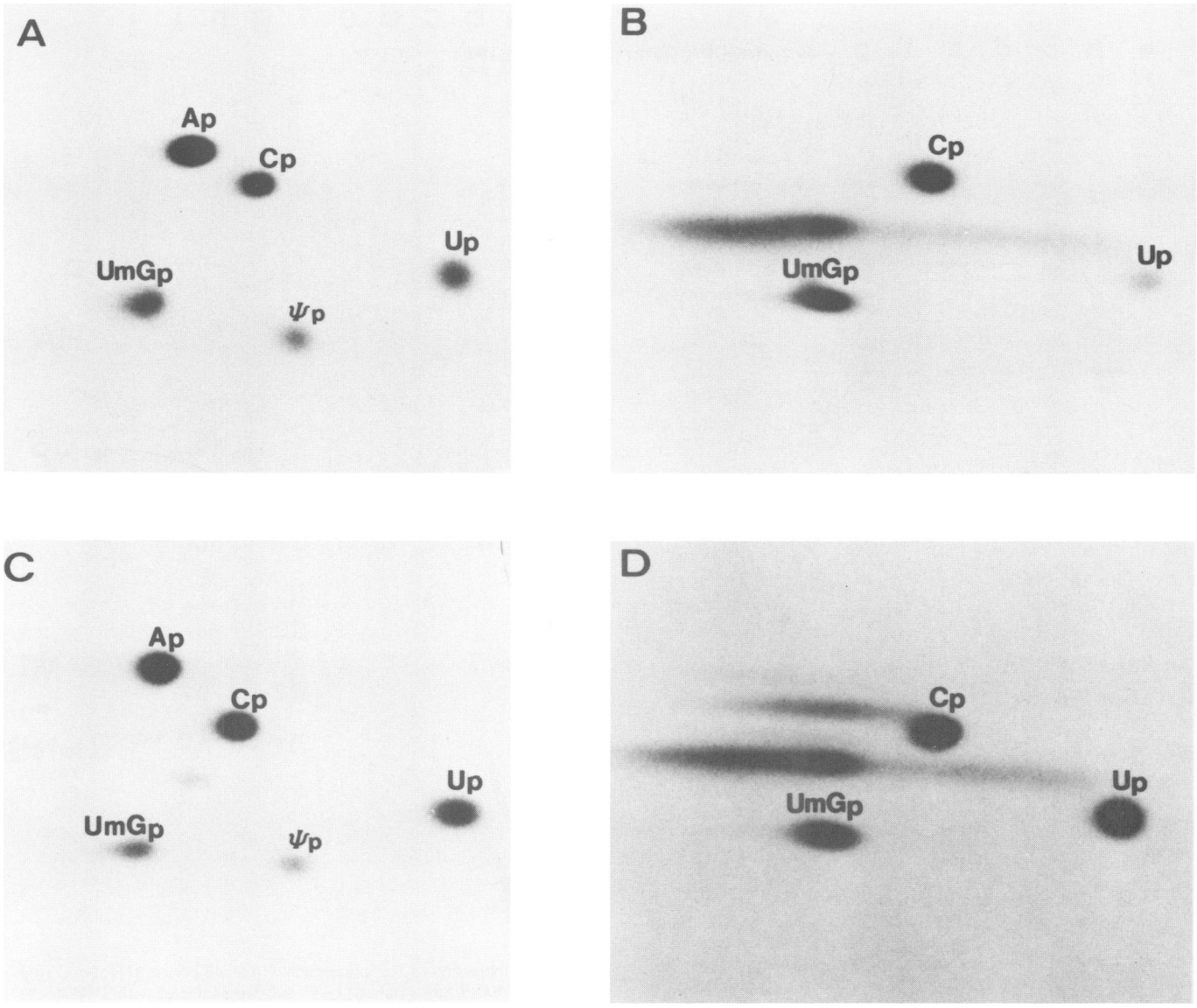

Fig. 5. Nucleotide composition analysis of the longest oligonucleotide in the RNase T1 digests derived from the anticodon region from ^{32}P-labeled tRNA^{Ser} (Su$^-$) and tRNA^{Ser} Su$^+$ (amber). The oligonucleotide designated AAAψCCAUUmGp in Figure 4A and ACUCUAAAψCCAUUmGp in Figure 4C were digested with RNase T2 (**panels A** and **C**) or RNase A (**panels B** and **D**), respectively, and the products were separated by two-dimensional t.l.c. (Silberklang *et al.*, 1979).

The cells were subsequently superinfected with adenovirus 2 and, 24 h later were pulse-labeled for 1 h with [^{35}S]-methionine. Whole cell lysates were prepared and analysed by electrophoresis on a 10% polyacrylamide gel. As shown in Figure 6a, lane i, only infection with SV-tS Su$^+$ (ochre) followed by adenovirus 2 resulted in the synthesis of a slower migrating species corresponding to the anticipated 140 000 dalton fusion protein (Laski *et al.*, 1984). The readthrough product was not observed in mock-infected cells or in cells infected with SV40, SV-tS Su$^-$, or SV-tS Su$^+$ (amber) followed by superinfection by adenovirus 2 (Figure 6a, lanes f–h and j).

The efficiency of suppression was estimated to be ~15%. This is a 1.5-fold higher level of suppressor activity than that found with the tRNA^{Tyr} Su$^+$ (ochre) gene (Laski *et al.*, 1984).

In vivo suppression of UAG codons by tRNA^{Ser} Su$^+$ (amber)

We have previously used influenza virus A/CAM/46 to examine and quantitate the levels of suppressor activity of tRNA^{Tyr} Su$^+$ (amber) (Young *et al.*, 1983). The NS1 mRNA of influenza virus A/CAM/46 encodes a 216 amino acid long protein which terminates in a UAG codon. At the mRNA level, this UAG is followed downstream by 20 triplets ending in an opal (UGA) codon. Thus suppression of the UAG would lead to the synthesis of an NS1 polypeptide which contains an additional 20 amino acids at the COOH terminus (Parvin *et al.*, 1983).

To examine the suppressor activity of SV-tS Su$^+$ (amber), CV-1 cells were infected with the different SV40 recombinants and superinfected with influenza virus A/CAM/46 36 h later. The cultures were labeled with [^{35}S]methionine 6 h later. After 1 h, cell extracts were prepared and analysed by electrophoresis on a polyacrylamide gel (Young *et al.*, 1983). A readthrough protein of the anticipated mol. wt. was observed only in cells infected with SV-tS Su$^+$ (amber) followed by the influenza virus (Figure 7, lane j). The efficiency of sup-

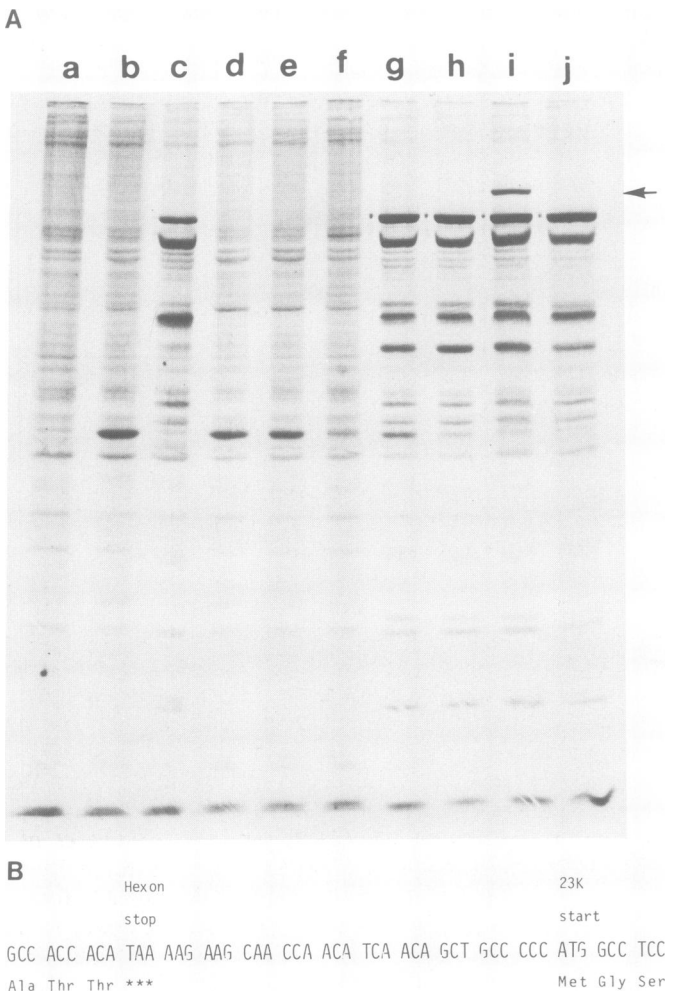

Fig. 6. Suppression of the adenovirus hexon mRNA UAA stop codon by SV-tS Su^+ (ochre). **(A)** CV-1 cells (3 x 10^6) were infected with 0.2 ml of SV40 (10^8 p.f.u./ml) or 0.5 ml of the recombinant SV40 stocks SV-tS Su^-, SV-tS Su^+ (ochre) or SV-tS Su^+ (amber). 24 h later the cells were superinfected with adenovirus 2 (m.o.i. 100). After a further 24 h incubation, the cells were labeled for 1 h with 100 μCi of [^{35}S]methionine. The cells were lysed with RIPA buffer (Cepko *et al.*, 1981) and aliquots were analyzed by electrophoresis on a 10% polyacrylamide gel (Laemmli, 1970). **Lane a**, mock infected; **lane b**, SV40 infected; **lane c**, mock infected followed by Ad 2; **lane d**, SV-tS Su^- infected; **lane e**, SV-tS Su^+ (ochre) infected; **lane f**, SV-tS Su^+ (amber) infected; **lane g**, SV40 followed by Ad 2; **lane h**, SV-tS Su^- followed by Ad 2; **lane i**, SV-tS Su^+ (ochre) followed by Ad 2; **lane j**, SV-tS Su^+ (amber) followed by Ad 2. The arrow indicates the species generated by readthrough of the UAA termination codon of the hexon mRNA (Laski *et al.*, 1984). **(B)** Sequence surrounding the Ad 2 hexon stop codon and the methionine initiation site of the 23-K polypeptide (Akusjarvi *et al.*, 1981).

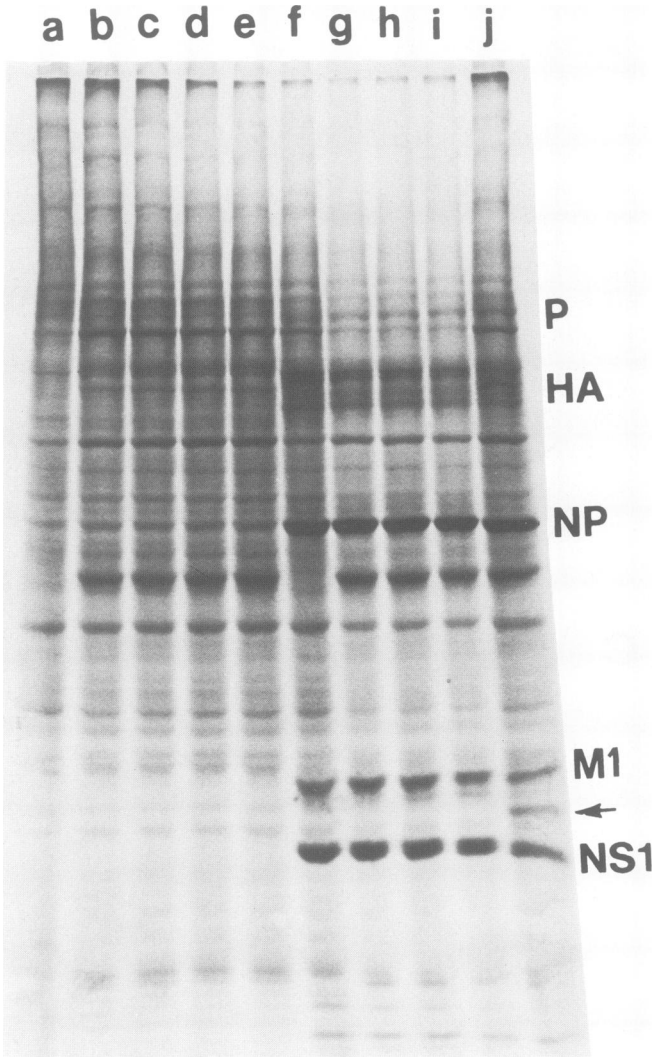

Fig. 7. Suppression of the influenza A/CAM/46 NS1 mRNA UAG stop codon by SV-tS Su^+ (amber). CV-1 cells were infected with SV40 or the recombinant virus stocks. 36 h later the cells were superinfected with influenza A/CAM/46. After 6 h the cells were labeled with [^{35}S]methionine for 1 h. Cell extracts were prepared and analyzed by electrophoresis on a 7–14% gradient polyacrylamide gel as described previously (Young *et al.*, 1983). **Lane a**, mock infected; **lane b**, SV40 infected; **lane c**, SV-tS Su^- infected; **lane d**, SV-tS Su^+ (ochre) infected; **lane e**, SV-tS Su^+ (amber) infected; **lane f**, mock infected followed by A/CAM/46; **lane g**, SV40 followed by A/CAM/46; **lane h**, SV-tS Su^- followed by A/CAM/46; **lane i**, SV-tS Su^+ (ochre) followed by A/CAM/46; **lane j**, SV-tS Su^+ (amber) followed by A/CAM/46. The arrow indicates the position of the NS1 readthrough product (Young *et al.*, 1983).

pression of this UAG termination codon was estimated to be 25–30%. This value is similar to that obtained with the $tRNA^{Tyr}$ Su^+ (amber) (Young *et al.*, 1983).

As seen in Figure 7, lane i, SV-tS Su^+ (ochre) was unable to suppress the UAG termination codon. In bacterial systems, ochre suppressors are able to recognize both amber and ochre codons (Brenner and Beckwith, 1965). The results presented here as well as those obtained with the *X. laevis* $tRNA^{Tyr}$ Su^+ (ochre) demonstrate that mammalian ochre suppressors do not recognize amber codons.

Attempts to isolate a SV40 recombinant virus stock containing the Su^+ (opal) gene

Attempts to generate an equivalent SV40 recombinant virus stock containing the opal (Su^+) tRNA gene were unsuccessful. When the replicating SV40 recombinant molecules were isolated from cells infected with virus stocks prepared from pSV-tS Su^+ (opal) and analyzed by agarose gel electrophoresis after digestion with *Hind*III, it was found that the *Hind*III digestion pattern was indistinguishable from wild-type SV40 (Figure 2b, lane 5). Significantly, there was a total absence of the *Hind*III fragment (A′′) which contains the serine tRNA gene and almost total absence of fragment A* which is derived from the helper virus SV-rINS-7. The transfection experiment was independently repeated a total of five times with different preparations of the SV40 recombinant and helper virus DNA and yielded the same result; the gene encoding the opal suppressor tRNA was absent from the

virus stocks which were produced.

DNA sequence analysis of the mutated tRNA gene and its flanking sequences indicated that there were no changes introduced by the mutagenesis other than the desired G→C change in the anticodon. Furthermore, the pSV-tS Su^+ (opal) construct was identical to the other tRNA gene containing recombinants as determined by digestion with several restriction enzymes.

We conclude that expression of the opal Su^+ gene, at least from a high copy SV40 vector, either affects SV40 propagation and/or is lethal to CV-1 cells. The virus stocks consisting of wild-type SV40 which were produced most likely arose from low level recombination between the SV40 recombinant and the helper virus DNA. This conclusion is supported by the fact that onset of cytopathic effect with these transfected molecules took, on average, 5–6 days longer than was the case with the other tRNA gene-containing recombinants.

Discussion

The availability of a variety of mammalian cell lines carrying defined nonsense suppressors, which insert different amino acids, would be a major advance towards the isolation and characterization of nonsense mutants in cellular and viral genes. As part of this objective, we have reported here the generation of amber, ochre and opal suppressor tRNA genes derived from a human serine tRNA gene. Using replicating SV40 recombinant vectors to introduce the tRNA genes into CV-1 cells, we have shown that the amber and ochre tRNA genes are expressed to yield efficient suppressors of amber and ochre codons, respectively. Interestingly, several attempts to obtain SV40 virus stocks containing the opal suppressor tRNA gene only yielded SV40 recombinants which were essentially wild-type SV40 and lacked the opal suppressor tRNA gene.

Expression of human serine tRNA genes in CV-1 cells

The human serine tRNA gene is expressed very efficiently in CV-1 cells to produce greater than 20 times the level of serine tRNA over a 6 h labeling period as compared with the control (cf. Figure 3 lanes d–f *versus* lanes a–c). Interestingly, the amounts of wild-type (Su^-), and the amber and ochre suppressor tRNAs produced are almost the same (Figure 3 lanes d–f). This finding is somewhat different from a previous result (Laski *et al.*, 1982b, 1984), in which amber and ochre suppressor tRNAs derived from a *X. laevis* tyrosine tRNA gene were present at 20% of the level of the wild-type tyrosine tRNA. The lower levels of *X. laevis* amber and ochre tyrosine suppressor tRNAs in CV-1 cells was previously attributed to a lower rate of splicing of the intron in the *X. laevis* tyrosine tRNA precursor. This explanation is consistent with the current result with the human serine tRNAs, since in contrast to the *X. laevis* tRNA gene, the human serine tRNA gene does not contain an intron.

Analysis for modifications in the serine tRNAs produced in CV-1 cells showed that neither the amber nor the ochre suppressor contained ψ in the middle position of the anticodon. Using yeast amber and ochre tyrosine suppressor tRNA genes, either lacking or containing an intron, Johnson and Abelson (1983) have demonstrated that modification of U to ψ in the middle position of the anticodon occurs only when the tRNA gene contains an intron. Our finding that the human amber and ochre serine suppressor tRNAs, derived from a gene lacking an intron, does not contain ψ is consistent with a similar requirement for modificaiton of U to ψ in a mammalian system. In contrast to the results obtained with the human $tRNA^{Ser}$ gene, wild-type (Su^-), Su^+ amber, and Su^+ ochre tRNAs derived from a *X. laevis* tyrosine tRNA gene containing an intron all had ψ in the middle position of the anticodon (Laski *et al.*, 1982b, 1984). This result also rules out the possibility that lack of U to ψ modification in the human serine tRNAs is due to the absence of the appropriate modifying enzyme in CV-1 cells.

Evidence for in vivo suppression in CV-1 cells

Both the $tRNA^{Ser}$ (Su^+ amber) and the $tRNA^{Ser}$ (Su^+ ochre) were shown to be active as suppressors in CV-1 cells.

Readthrough of the ochre (UAA) codon at the end of the adenovirus hexon mRNA was used to demonstrate the suppressor activity of the $tRNA^{Ser}$ (Su^+ ochre). Infection of cells with SV-tS Su^+ (ochre) followed by adenovirus 2 led to the synthesis of a 140 000 dalton fussion protein (Figure 6A, lane i). This results from the readthrough of the single UAA codon present between the reading frames encoding the hexon mRNA (120 000 daltons) and the 23 000 dalton protease polypeptide (Laski *et al.*, 1984; Figure 6B). The efficiency of suppression is ~15%. Under identical conditions, the $tRNA^{Tyr}$ Su^+ (ochre) suppressed this codon with an efficiency of 10% (Laski *et al.*, 1984). This difference may be due to altered levels of expression of these two tRNA genes rather than to differences in intrinsic efficiencies of suppression by these two tRNAs.

Previously, we used an influenza virus strain in which the NS1 mRNA contains an amber codon to examine the suppressor activity of $tRNA^{Tyr}$ Su^+ (amber) (Young *et al.*, 1983). Using the same virus strain to examine the activity of $tRNA^{Ser}$ Su^+ (amber), the readthrough efficiency was found to be ~25–30% (Figure 7, lane j). This level is comparable with that observed using the *X. laevis* $tRNA^{Tyr}$ Su^+ (amber). The lack of suppression of the amber codon in NS1 mRNA by the $tRNA^{Ser}$ Su^+ (ochre) (Figure 7, lane i) shows that as in the case of the *Xenopus* $tRNA^{Tyr}$ Su^+ (ochre), the human $tRNA^{Ser}$ Su^+ (ochre) is also ochre specific. This result supports the growing consensus that unlike *E. coli* in which ochre suppressors also suppress amber codons (Brenner and Beckwith, 1965), in eukaryotes such as yeast (Sherman, 1982) and mammalian cells (Laski *et al.*, 1984) ochre suppressors are ochre specific.

Attempts at isolation of SV40 virus stocks carrying the opal suppressor gene

The inability to generate virus stocks containing the opal suppressor tRNA gene indicates that the over-expression of a UGA suppressor is lethal to CV-1 cells and/or affects SV40 propagation. Our results so far do not allow a distinction among these possibilities. In mammalian cells, the three nonsense codons are more or less equally used (Kohli and Grosjean, 1981). Given this fact, if the problem in generating SV40 virus stocks carrying the opal suppressors is due to a deleterious effect on cellular metabolism, this raises the question of why the opal suppressor should behave differently from the amber or the ochre suppressors.

If the deleterious effect of opal suppressors is on the propagation of SV40, the most likely site of action is on VP1, the major capsid protein. Of the proteins coded by SV40 DNA, VP1 mRNA is the only one which used UGA as a termination codon and mutants in VP1 are known to be defective in virus assembly (see Tooze, 1981). The sequence of SV40 DNA predicts that readthrough of the UGA in VP1 mRNA would yield a VP1 which is at least 40 amino acids longer. It is,

therefore, possible that readthrough of UGA in VP1 mRNA interferes with viral assembly. Alternatively, since SV40 contains 360 copies of VP1, another possibility is that even a low level readthrough of UGA in VP1 mRNA could have a serious effect on SV40 propagation in that it yields a protein which can assemble but produces defective virions. The remaining proteins coded by SV40 are all terminated by UAA including the minor capsid proteins VP2 and VP3, yet overproduction of ochre suppressors in CV-1 cells has no apparent effect on production of SV40 recombinants. There are several possible explanations for this; (i) UAA termination codons in mRNA for VP2 and VP3, which share the same C-terminal sequence, are much less susceptible to readthrough compared with the UGA codon in VP1 because of context effects (Bossi *et al.*, 1983; Engleberg-Kulka, 1981; Caskey and Campbell, 1979; Miller and Albertini, 1983); (ii) The UAA codon in VP2 and VP3 is followed by another termination codon, UGA, seven amino acids downstream. Thus, even if the UAA codon in VP2 and VP3 mRNAs is read through at a reasonably high efficiency, this may not effect assembly or infectivity of the virions.

The inability to obtain SV40 virus stocks carrying the opal suppressor tRNA gene does not rule out the possibility of establishing mammalian cell lines carrying a low copy number of such tRNA genes. It is likely that our inability to obtain such a virus stock is due more to the presence of the opal suppressor tRNA gene as part of a high copy replicative recombinant vector, resulting in deleterious levels of suppressor activities. The presence of an opal suppressor tRNA gene at low copy number may not be too deleterious.

Finally, the *X. laevis* $tRNA^{Tyr}$ amber and ochre suppressor tRNA genes derived by site-specific mutagenesis were previously used to establish mammalian cell lines which exhibit levels of suppressor activity sufficient for most genetic studies (Hudziak *et al.*, 1982; Laski *et al.*, 1984). The availability of human serine tRNA genes encoding ochre, amber and opal suppressors described in this report should facilitate the generation of different cell lines carrying well defined suppressor tRNAs including possibly, one carrying an opal suppressor tRNA gene for the first time.

Materials and methods

Subcloning of the human serine tRNA gene into M13mp9

The 900-bp *Sau*3A fragment of p17Sau3A, which contains a single copy of a human tRNA gene, was purified by agarose gel electrophoresis and cloned into the *Bam*HI site of M13mp9. The recombinant M13 virions which contained the coding strand of the tRNA gene in viral DNA were identified by hybridization with 5′ ^{32}P-labeled human placental serine tRNA (kindly provided by Dr. B. Roe). Viral DNA from one such isolate, designated M13-tS, was prepared on a large scale by phenol chloroform extraction of polyethylene glycol precipitated virions. The DNA was further purified by alkaline sucrose gradient sedimentation prior to its use as a template for site-specific mutagenesis (Kudo *et al.*, 1981).

Oligonucleotides used as primers

The pentadeca-deoxyriboligonucleotides 5′TGGACTTTAAATCCA, 5′TGGACTTCAAATCCA, and 5′TGGACTCTAAATCCA were synthesized on an Applied Biosystems 380A DNA synthesizer. Oligonucleotides were purified first by absorption to Sep-Pak cartridges (Waters Asssociates) and then by electrophoresis on 20% polyacrylamide/8 M urea gels as described in detail by Lo *et al.*, (1984).

Purified, fully deprotected oligonucleotides were labeled at their 5′ ends with ^{32}P and characterized by sequencing (Silberklang *et al.*, 1979).

Oligonucleotide-directed site-specific mutagenesis

Mutagenesis was carried out as described by Zoller and Smith (1983) and as modified by Drabkin and RajBhandary (in preparation). Briefly, 1 pmol of M13-tS virion DNA was annealed with 40 pmol of phosphorylated primer in a final volume of 10 μl of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol (DTT). The mixture was heated at 100°C for 3 min, quickly cooled to 25°C and incubated at this temperature for a further 60 min. An equal volume of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM each of dATP, dCTP, dTTP, and 1 mM ATP was added along with 10 units of the large fragment of DNA polymerase I, and 5 units of T4 DNA ligase. Incubation was continued at 25°C for 4 h. Single-stranded DNA and incompletely elongated DNA molecules were cleaved by treatment with S1 nuclease and covalently closed heteroduplex DNA was purified by electrophoresis on agarose gels as described (Drabkin and RajBhandary, in preparation). The recovered DNA was used to transform *E. coli* JM103.

Individual phage plaques were screened for the desired mutations by hybridization to the respective oligonucleotide primers (Wallace *et al.*, 1978; Zoller and Smith, 1983). Autoradiography of the filter after washings at sequentially higher temperatures allowed phage containing the mutant DNA to be identified. Phage showing strong positive hybridization were plaque purified and the presence of the desired mutations was confirmed by DNA sequence analysis (Sanger *et al.*, 1977) and, where appropriate, by digestion of the M13 recombinant RF DNAs with restriciton enzymes.

Cloning of the wild-type (Su^-) and mutant (Su^+) $tRNA^{Ser}$ genes into SV40

The 900-bp *Sau*3A fragment was excised from RF DNA of the M13mp9 clones containing the Su^- or the Su^+ human serine tRNA genes and separately cloned into the unique *Bam*HI site of the SV40-pBR322 hybrid vector pSV1gt3. pSV1gt3 has a deletion of the SV40 late region and contains the SV40 origin of replication, the early region, a duplication of the region from 0.14 to 0.32 map units, and a single *Bam*HI site in place of *Pvu*II at 0.71 map units on SV40, and pBR322 (R. Mulligan, personal communication). pSV1gt3 was digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase prior to ligation with the individual tRNA genes. The constructs which were produced are designated pSV-tS Su^-, pSV-tS Su^+ (ochre), pSV-tS Su^+ (amber), and pSV-tS Su^+ (opal). These clones are identical to each other except for the point mutations in the tRNA genes. Prior to transfection, the pBR322 sequences are released with *Pst*I.

Preparation of virus stocks

The late region defects in the SV40 recombinants described above are complemented by pSV-rINS-7, a SV40-pBR322 recombinant containing an insertion-substitution in the early region (Horowitz *et al.*, 1983). The pBR322 sequences from pSV-rINS-7 are removed by digestion with *Eco*RI.

The complementing recombinants pSV-tS (Su^-, Su^+) and pSV-rINS-7 were released from their pBR322 sequences by complete digestion with *Pst*I or *Eco*RI, respectively, and recircularized as described previously (Laski *et al.*, 1982a). The complementing vectors were co-transfected into CV-1 cells by the DEAE dextran procedure (Sompayrac and Danna, 1981). Virus stocks were harvested as described (Laski *et al.*, 1982a) when CPE was observed. The virus stocks so prepared are designated SV-tS Su^-, SV-tS Su^+ (ochre), SV-tS Su^+ (amber), and SV-tS Su^+ (opal).

Infection of CV-1 cells with SV40 recombinants

Infection of CV-1 cells with the SV40 recombinants and preparation and purification of DNA and ^{32}P-labeled RNA was as described previously (Laski *et al.*, 1982a; Drabkin and RajBhandary, in preparation).

Fingerprint analysis of ^{32}P-labeled tRNA by two-dimensional homochromatography after digestion with RNase T1 was carried out as described (Silberklang *et al.*, 1979; Laski *et al.*, 1983). Digestion of tRNA with RNase T2 and pancreatic RNase A was done according to Silberklang *et al.*, (1979) and the products were analyzed by two-dimensional t.l.c. (Nishimura, 1972).

Other viruses

Influenza virus A/CAM/46 and adenovirus 2 was prepared and used as described previously (Young *et al.*, 1983; Laski *et al.*, 1984).

Acknowledgements

We are grateful to Drs. Nancy Martin and Seung Yoo for generously providing the plasmid p17Sau3A and sequence of the serine tRNA gene. We thank Dr. P. Palese Jr. for providing us with a supply of influenza A/CAM/46, Drs. S. Jones and H. G. Khorana for advice in oligonucleotide synthesis and in making the DNA synthesizer available for our use and Dr. B. Roe for human placental serine tRNA. The expert help and advice of Drs. H. Drabkin and R. Reilly and the technical assistance of M. Esteve is gratefully acknowledged. This work was supported by National Institutes of Health Grant GM17151 and American Cancer Society Grant NP114 to ULR; National Institutes of Health Grant GM32467 and National Science Foundation Grant PCM8200309 to PAS, and partially by National Institutes of Health Core Grant CA14051 to the Center for Cancer Research at MIT. JPC is a recipient of a fellowship from the Medical Research Council of Canada.

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Received on 9 October 1984