Efficient expression of small RNA polymerase III genes from a novel Simian virus 40 vector and their effect on viral gene expression

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ABSTRACT

In the past, simian virus 40 (SV40) has been used as a cloning vehicle to clone foreign genes by substituting portions of the viral genome vital for viral replication. Propagation of these defective viruses required a helper virus and the recombinant viruses obtained could be grown only as a mixture. In this study, we describe a novel nondefective SV40 vector to clone small RNA polymerase III genes. Two small RNA polymerase III genes, an amber suppressor human serine tRNA gene and the adenovirus (Ad) VAI RNA gene, were cloned in the intron region of the large-T antigen gene of SV40 after deleting DNA sequences coding for the small-t polypeptide. The recombinant viruses grew to wild type levels and showed no growth defects. When CV-1p cells were infected with these viruses, the cloned RNA polymerase III genes were expressed at high levels at late times. Interestingly, large amounts VAI RNA in CV-1p cells infected with SV40-VA recombinant virus, did not enhance translation of viral mRNAs significantly but did lead to a 3 to 4 fold increase in the steady state levels of large-T mRNA suggesting a novel function for VAI RNA in SV40 infected monkey cells. Furthermore, VAI mutants which fail to function in Ad infected human cells also failed to enhance the levels of large-T mRNAs in monkey cells infected with SV40.

The simple SV40 vector described here may be useful to study the structure and function of small RNA polymerase III genes in the context of a eucaryotic chromosome. In addition, the nondefective recombinant SV40 which expresses the suppressor tRNA gene at high levels may provide a useful helper system to propagate animal viruses with amber mutations in essential genes.

INTRODUCTION

The simple genome structure and the ease with which it can be manipulated *in vitro* and propagated in tissue culture cells have made Simian virus 40 (SV40) a useful vector for cloning genes transcribed by both RNA polymerase II and III (reviewed in 1). In all these studies however, foreign DNA was cloned into the virus after deleting portions of the genome vital for viral replication. Foreign genes were cloned into the late region and expressed either from their own promoter or the SV40 late promoter. These recombinants were propagated with a helper virus having a ts lesion in the early gene (2-4). Similarly, SV40 recombinants with genes cloned into the early region were propagated with a helper virus which contained a ts lesion in the gene encoding the major capsid protein VP1 (5,6).

Structure, function and regulation of tRNAs and other host and viral encoded small RNA polymerase III genes are of general interest and are being investigated intensely in several laboratories. *In vitro* analyses of these genes have provided valuable information regarding the *cis* acting promoter elements (reviewed in 7 and 8) and host transcription factors that bind to these sequences (9), and the effect of virus encoded nuclear oncogenes on these transcription factors (10,11). To analyse the structure and

regulation of these genes *in vivo*, microinjection into amphibian oocytes and transfection approaches have been used. In a few cases, these genes have been cloned into SV40 based vectors in the late region (12-14). Although RNA polymerase III genes are smaller in size, the strategy for cloning and expressing these genes involved the deletion of an essential portion of the viral early or late regions and necessitated the propagation of these defective recombinants with appropriate thermosensitive helper viruses. Thus, a major drawback in the SV40 vector based cloning approach has been the propagation of pure populations of recombinant viruses; recombinants could only be propagated as defective viruses and the virus stocks invariably contained a considerable population of helper viruses. Consequently, obtaining recombinant viruses with high titers was also difficult.

In this study, we describe a novel nondefective SV40 cloning vehicle to clone small RNA polymerase III genes. Taking advantage of the fact that the small-t polypeptide is nonessential for viral growth in tissue culture cells (15,16), we cloned the amber suppressor (Su^+) human serine tRNA gene or the adenovirus (Ad) virus associated I (VAI) RNA gene in the intron region of the large-T antigen gene. In addition to providing good model systems to study the mechanism of RNA polymerase III transcription *in vivo* and *in vitro*, these two genes are of considerable biological interest. A well characterized suppressor tRNA gene is potentially useful in genetic analysis of nonsense mutations in mammalian and viral genomes. The Ad VAI RNA has been shown to be required for the efficient translation of viral mRNAs at late times after virus infection (17-19). The VAI RNA prevents the activation of host encoded eIF-2 α kinase which phosphorylates and thereby inactivates the protein synthesis initiation factor eIF-2 (20-22). Thus the presence of large amounts of VAI RNA in SV40 infected cells provided us with an opportunity to study the effect of VAI RNA on SV40 gene expression without the influence of other Ad genes. The SV40 recombinants which carry the two RNA polymerase III genes grow to wild type (WT) levels without a helper virus and express the cloned genes efficiently at late times. When CV-1p cells are infected with the SV40 recombinant which expresses large amounts of VAI RNA (SV-VA), the VAI RNA did not enhance translation of viral mRNAs significantly but did increase the steady state levels of large-T mRNA at late times suggesting a novel function for the VAI RNA in this system.

The simple nondefective SV40 vector described in this report is useful to study the structure, function and regulation of small RNA polymerase III genes in the context of a eucaryotic chromosome. In addition, the nondefective recombinant SV40 described here which synthesizes high levels of Su^+ (amber) human serine tRNA is potentially useful as a helper virus to propagate animal viruses with amber mutations in functionally important genes.

MATERIALS AND METHODS

Cells and viruses:

CV-1p (African green monkey kidney cell line) and HeLa cells were maintained in Dulbecco's modified Eagles medium containing 10% calf serum. The WT SV40 is a plaque purified derivative of the SVS strain (23). Mutant *d1704* is a phenotypically WT variant of Ad5 mutant which lacks the minor VAII species (24).

Cloning strategy

WT SV40 was cloned into pBR322 using the unique Bam HI sites of the two DNA molecules (pSVWT). The plasmid pSVWT was then cut with Bst XI at nucleotide SV4759 (SV numbers are from reference 25). The linear molecules were treated with Bal 31 and the end points were modified into Xba I sites using synthetic Xba I linkers following the established recombinant DNA protocols (26). The DNA sequences deleted in these mutants were determined by DNA sequence analysis (27). An SV40 recombinant plasmid (pSV2800) which retained 42 basepairs (bp) from the 5' border (splice donor site) and 47 bp from the 3' border (splice acceptor site) of the large-T intron was chosen to clone the VAI RNA gene. The Ad5 VAI gene is located at 29.0 map units (m.u.) on the viral chromosome. This gene is flanked at the 5' end by an Xba I site (at -30 position relative to RNA start site; Ad 10579) (Ad numbers are from reference 28) and a Bal I site at the 3' end (46 bp downstream from the transcription termination site; Ad 10812). Bal I site of the Ad5 DNA sequence at Ad10579 was modified into Xba I site in a plasmid containing the VAI RNA sequences. The 231 bp DNA fragment (from Ad10579 corresponding to Xba I site at 29.0 m.u. to Ad10812 corresponding to the Bal I site) containing the VAI RNA coding sequences and flanking sequences of 31 bp at the 5' end and 46 bp at the 3' end was cloned into pSV2800 taking advantage of the unique Xba I site of this plasmid. This virus is designated SV-VA. The two other SV40 variants, SV-VA/709 and SV-VA/719 contain 6 (between +43 and +53) and 13 (between +26 and +45) nucleotide substitutions respectively. These mutant VAI genes transcribe efficiently but fail to function in human cells (29,30, and Furtado *et al*, manuscript in preparation).

To clone the Su⁺ (amber) tRNA^{ser} gene, a 900 bp Sau 3A DNA fragment from an M13 clone which contains the Su⁺ human tRNA^{ser} sequences (kindly provided by Drs. U. RajBhandary and P.A. Sharp, M.I.T; see reference 31 for details) was subcloned into Bam HI site of pUC18. Large quantities of the above DNA fragment were then isolated from this plasmid, digested to completion with Sca I and then partially with Rsa I (Rsa I at -203 and Sca I site at +158 relative to the 5' end of the tRNA). Xba I linkers were added to this fragment and then cloned into Xba I site of pSV2800. The viral DNA portion of the pSV2800 which contains the VAI RNA or the tRNA gene was excised from the plasmid using the Bam HI sites and recircularised. The CV-1p cells were then transfected with the viral DNA using the DEAE dextran procedure (15,16). The recombinant viruses were propagated following the established protocols (15,16).

RNA analysis

Analysis of low molecular weight RNAs: CV-1p and HeLa cells were infected with SV40 variants and a phenotypically WT Ad5 (*dl704*) respectively at 20 PFU/cell and immediately labeled with ³²P inorganic phosphate. The cells were harvested at the times indicated in the legends to the figures and the ³²P-labelled total cytoplasmic RNA was isolated as described (24). The RNA samples were electrophoresed on 8% or 12% polyacrylamide-8M urea gels (0.4mm thick; 600 V in Tris-Borate buffer, pH 8.0) for 14 h and autoradiographed (24).

Northern blot hybridizations: Northern blot analysis of cytoplasmic RNA was performed as described by Rave *et al* (32) using a nitrocellulose membrane for RNA transfer. Cells were infected with SV40 vari-

ants at 20 PFU/cell for the time periods indicated in the legends to Fig. 2B and 4A and total cytoplasmic RNA was prepared as described (24). To analyse Su^+ tRNA^{ser}, total RNA was fractionated on a 2% agarose-formaldehyde gel and transferred to a nitrocellulose filter. The filter was then probed with a nicktranslated DNA probe which contains the Su^+ tRNA^{ser} sequences. To determine large-T mRNA levels, cells were infected at 10 PFU/cell, total cytoplasmic RNA was isolated 40 h post infection and fractionated on 1.2% agarose-formaldehyde gels (32). RNA samples were then transferred to a nitrocellulose filter and probed with an early (Hind III, SV4002 to SV5171) or late region (SV1782 to SV2533) specific DNA fragment.

Primer extension analysis: CV-1p cells were infected with SV40 variants for 36 h and total cytoplasmic RNA was prepared. Fifty μ g of RNA was annealed to 5×10^4 cpm of the 5' end labeled oligonucleotide primer complementary to SV40 early mRNA spanning from SV4526 to SV4557. Primer extension was carried out with 40 units of reverse transcriptase (Pharmacia) for 90 min at 42 °C in a buffer which contained 50.0 mM Tris-HCl, pH 8.0, 5.0 mM MgCl₂, 5.0 mM DTT, 50 mM KCl and 40 μ M of 4 dNTPs (26). The extended products were resolved on a 4% denaturing polyacrylamide gel.

Polypeptide analysis:

Cells were infected at a multiplicity of 10 PFU/cell and labeled with ³⁵S methionine (100 μ Ci/ml; specific activity 800 Ci/m mole, Amersham Corp.) for durations indicated in the legend to Fig. 4. The cells were then washed twice with phosphate buffered saline (pH 7.4) and lysed in cold RIPA buffer (0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1.0% sodium deoxycholate, 1.0% Triton X-100, 1.0 mM EDTA, 20 mM Tris, pH 7.4) at a concentration of 2.0×10^6 cells/ml as described by Cepko and Sharp (33). Cell lysates from equal number of cells were immunoprecipitated with a monoclonal antibody specific for large-T (pAB419, ref. 34). The immunoprecipitated proteins were analysed on 20% SDS-polyacrylamide gels (35).

Assay for the Su^+ (amber) tRNA^{ser} activity in virus infected cells:

To determine the Su^+ (amber) tRNA activity of the recombinant SV40 virus, CV-1p cells were transfected with the plasmid RSVCAT in which the chloramphenicol acetyl transferase coding sequences were fused with the Rous sarcoma virus long terminal repeat or RSVam27/CAT in which the serine codon at 27th position from the N-terminal end of the CAT coding sequences of the RSVCAT was changed to an amber chain termination codon (36). Six h later they were infected with SV40 variants at 10 PFU/cell. Forty two h after infection, cell lysates were prepared and the CAT activity in the cell lysates was determined as described by Gorman et al (37).

RESULTS

Two small RNA polymerase III genes were cloned in the intron region of the large-T antigen of SV40:

Two early polypeptides, large-T and small-t are encoded by two overlapping messages in SV40. Earlier reports have shown that deletion of DNA sequences coding for the unique region of small-t antigen does not affect the virus lytic cycle or the splicing of large-T mRNA (15,16). Therefore, we cloned the genes coding for the Su^+ (amber) human tRNA^{ser} and Ad VAI RNA in this region of the SV40 genome.

Figure 1. Structure of viral DNAs of the SV40 variants described in the text.

A. Structure of SV-tRNA which shows the position of the Su^+ (amber) $tRNA^{ser}$ gene on the SV40 genome. The arrow shows the direction of transcription. Spliced early messages coding for the large-T and small-t are shown as solid lines. Late messages are shown as a single broken line. The location of the replication origin (Ori) of the virus is shown by an open triangle. The shaded area represents the tRNA gene.

B. Details of deletion and substitution of DNA sequences in the intron region of the SV40 genome coding for the large-T mRNA. Open box represents deletion. Shaded areas in SV-tRNA and SV-VA represent substitution of tDNA and Ad sequences respectively. The arrows represent tRNA or VAI RNAs. The VAI RNAs are shown by two arrows representing A and G species (38). The SV numbers correspond to the numbering system shown in reference 25. The Ad numbers correspond to nucleotide sequence positions published by Gingeras (28).

Using the protocols described in Materials and Methods, the following five SV40 mutants were constructed: (i) *dl2800* which contained a 258 bp deletion in the intron region of the genome coding for the large-T, with the deletion terminating with an XbaI site, (ii) SV-tRNA in which a 359 bp DNA fragment which codes for the Su^+ (amber) human $tRNA^{ser}$ gene is cloned into the Xba I site of the *dl2800* and (iii), SV-VA in which a 231 bp Ad DNA segment containing the WT VAI RNA gene was cloned into *dl2800* in the large-T intron, (iv) SV-VA/709 and (v) SV-VA/719. Mutants SV-VA/709 and SV-VA/719 contain VAI genes in which 6 and 13 bp are substituted within the 5' half of the gene. These VAI mutants transcribe efficiently but fail to function in human cells (29,30 and Furtado et al in preparation). The direction of transcription of the VAI RNA or the tRNA gene is opposite to that of SV40 early transcription. Figure 1 shows the structure of the DNAs of these recombinant viruses.

Figure 2. Synthesis of Su^+ (amber) tRNA^{ser} by the SV-tRNA in CV-1p cells.

A. Analysis of low molecular weight RNAs synthesized in CV-1p cells by SV-tRNA, and the WT and *dl2800* controls. ^{32}P labeled total cytoplasmic RNAs isolated from mutant and WT infected cells were electrophoresed for 14 h on a 12% polyacrylamide-8M urea gel as described under Materials and Methods. The suppressor tRNA of SV-tRNA is shown by an arrow.

B. Northern blot analysis of total cytoplasmic RNA isolated from CV-1p cells infected with SV-tRNA. Cells were infected with SV-tRNA at 10 PFU/cell and total cytoplasmic RNAs were extracted. 2.5 μ g of total RNA for each time point was loaded on a 2% agarose-formaldehyde gel, blotted on to a nitrocellulose filter and probed with a nicktranslated 359 bp DNA fragment which contains the Su^+ tRNA^{ser} sequences.

The SV40 recombinants synthesize abundant quantities of polymerase III transcripts:

Polymerase III transcripts of the inserted genes were efficiently expressed from the SV40 recombinants. Figure 2A shows the analysis by denaturing polyacrylamide gel electrophoresis of ^{32}P -labelled low molecular weight RNA isolated from CV-1p cells infected with the SV-tRNA recombinant. A tRNA band with about 8 fold higher intensity compared to a host tRNA band of control lanes (WT or *dl2800*) was detected in the lane corresponding to the SV-tRNA infected CV-1p cells. Host serine tRNA was

Figure 3. Synthesis of VAI RNA by SV-VA in CV-1p cells.

A. A time course study. ^{32}P labelled total cytoplasmic RNA isolated from mutant infected cells was electrophoresed on an 8% polyacrylamide-8M urea gel for 14 h as described under Materials and Methods. Top and bottom 8 cm of the autoradiogram are not shown. HeLa cells infected with a phenotypically WT variant *dl704* was used as a standard. The numbers on the top represent the time (h) at which the cells were harvested after infection. VAI A and G refer to two VAI RNA species which are initiated with A and G residues respectively (38)

B. Electrophoretic analysis of the VAI RNAs synthesized by SV-VA, SV-VA/709 and SV-VA/719 in monkey cells and by *dl704* in HeLa cells. CV-1p cells were infected with SV40 variants for 40 h and HeLa cells were infected with *dl704* for 24 h, both at a M.O.I of 20, and total cytoplasmic RNAs were analysed as described above. To quantitate the VAI RNAs synthesised by these mutants, the bands corresponding VAI RNAs were excised and counted.

shown to comigrate in this position by previous workers (31). To confirm that this was the Su^+ tRNA^{ser}, the low molecular weight RNAs isolated from cells infected with SV-tRNA for different time periods were analysed by Northern blot hybridizations. As shown in Fig 2B, the DNA probe which contained the Su^+ tRNA^{ser} sequences hybridized to the low molecular weight RNA species at all time points tested. Extent of hybridization increased 8-10 fold between 36 and 48 h after infection, indicating the high level of expression of the cloned tRNA gene at late times.

Similarly, expression of the Ad VAI RNA gene from SV-VA was monitored at various times after infection by analysing the ^{32}P -labeled low molecular weight RNAs prepared from CV-1p cells infected with the recombinant virus (Fig. 3A). The VAI RNA was detected in the cytoplasm as early as 16 h after infection, increased steadily up to 40 h and decreased thereafter. This pattern is comparable to that seen in Ad infected human cells if an allowance is made for the difference in the time periods of the viral DNA replication of these two viruses (25). In the experiments shown in Fig. 3A, the *dl704* RNA was

used only as a marker. To accurately compare the quantity of VAI RNA synthesised by SV-VA with that of WT Ad5 control, an equal number of CV-1p and HeLa cells were infected with SV-VA recombinant and *dI704* respectively at 20 PFU/cell and total cytoplasmic RNAs were isolated at 40 h post infection in the case of SV-VA and 24 h in the case of *dI704*. The RNAs were analysed on an 8% denaturing polyacrylamide gel as described before (Fig. 3B). To quantitate the VAI RNA synthesized, the radioactive bands were excised from the gels and their radioactivity determined (data not shown). These results show that on a per cell basis, the quantity of VAI RNA synthesised in CV-1p cells infected with SV-VA is roughly comparable to that of *dI704* in HeLa cells. Figure 3B also shows the VAI RNAs synthesised by SV-VA/709 and SV-VA/719. The mutant VAI genes in these cases transcribe efficiently and show altered mobilities as reported earlier (29 and Furtado *et al*, in preparation)

Viral gene expression is normal in the SV-tRNA recombinant:

To determine whether cloning the polymerase III gene in the intron region of the early gene of SV40 affected viral gene expression, several events of the viral gene expression were monitored in the SV-tRNA recombinant virus.

Figure. 4A shows results of an experiment in which steady state levels of large-T mRNA were determined in CV-1p cells infected for 40 h with SV-tRNA, *dI2800* or WT SV40 by Northern blot analysis. The steady state levels of large-T mRNA were found to be similar in CV-1p cells infected with these variants.

Next, we determined the levels of the large-T polypeptide and VP1 in CV-1p cells infected with the SV40 variants. CV-1p cells were infected with SV-tRNA or the WT controls for 39 h and then labelled with ^{35}S -methionine for 1 h. Large-T polypeptide was immunoprecipitated from these cells using a monoclonal antibody specific for the large-T and immunoprecipitated polypeptides were analysed on SDS-polyacrylamide gels. As shown in Fig.4B similar levels of large-T were detected in cells infected with WT or SV-tRNA. Thus, an actively transcribing polymerase III gene in the intron region of the SV-tRNA did not affect the expression of the early gene. To determine the synthesis of viral late polypeptides, the ^{35}S labelled cell lysates were analysed directly on SDS polyacrylamide gels. As shown in Fig. 4C, synthesis of viral capsid protein VP1 was not affected in SV-tRNA infections.

As the size of the large-T polypeptide synthesized in SV-tRNA infections is identical to that of WT controls it was likely that the large-T mRNA is spliced appropriately in SV-tRNA infections. This was further examined by a primer extension experiment. Total cytoplasmic RNAs isolated from CV-1p cells infected with SV40 variants for 36 h were annealed to a synthetic oligonucleotide complementary to large-T mRNA sequences immediately downstream from the splice acceptor site (SV4526 to SV4557). The primer was then extended with reverse transcriptase as described (26) and the extended products were resolved on a 4% denaturing polyacrylamide gel (Fig. 5). Lane 3 shows an approximately 360 nucleotide long cDNA product for the WT sample, which is in agreement with the previously published reports for the transcription initiation site of the early promoter (39). Products of identical size were also obtained for *dI2800* and SV-tRNA viruses, indicating the correct splicing of the large-T mRNA of SV-tRNA.

Figure 4. Analysis of large-T mRNA and viral polypeptides.

A. Steady state levels of large-T mRNAs in CV-1p cells infected with SV40 variants WT, *dl2800* and SV-tRNA. Five μ g of total cytoplasmic RNAs isolated from CV-1p cells infected with the SV40 variants were electrophoresed on 1.2% agarose gels containing 6% formaldehyde (32), transferred to nitrocellulose filters and probed with an early region specific DNA fragment as described under Materials and Methods.

B. Electrophoretic analysis of large-T polypeptide synthesised in CV-1p cells after infection with WT SV40, and SV-tRNA. Cells were infected at 10 PFU per cell for 39 h and then labeled with ^{35}S methionine for 1 h and the cell lysates were prepared as described under Materials and Methods. Cell lysates corresponding to 2×10^9 cells were immunoprecipitated with a monoclonal antibody specific for large-T (pAB419, Ref. 34). Immunoprecipitated proteins were analysed on 20% SDS polyacrylamide gels. Accurate quantitation of the large-T was achieved by subjecting the supernatants after the first immunoprecipitation to another cycle (lanes b). The arrow marks the position of the large-T.

C. Analysis of late polypeptides synthesised by the SV40 variants in CV-1p cells at 40 h post infection. To analyse late polypeptides, the samples were loaded directly on to the gel. Each lane represents an extract derived from approximately 2.7×10^4 cells. The major capsid protein VP1 is shown by an arrow. To quantitate the labelled polypeptides, the protein bands were excised from the gels and the radioactivity present in these bands were determined.

Figure 6 shows the growth properties of the SV40 variants. Growth patterns of the SV-tRNA and SV-VA viruses which contain two different RNA polymerase III genes in the intron region of the large-T gene were similar to that of WT with comparable virus yield indicating no growth defects for the recombinant viruses. Mutants SV-VA/709 and SV-VA/719 also showed no growth defects (data not shown).

Su⁺ (amber) tRNA^{ser} gene cloned into SV40 virus is functional:

To determine if the Su⁺ tRNA of SV-tRNA is functional in virus infected cells, we tested the ef-

Figure 5. Primer extension analysis of the large-T mRNAs synthesised by the SV40 variants. Fifty ug of total cytoplasmic RNA isolated from CV-1p cells infected with SV40 variants was annealed to a 30 nucleotide long 5' end labeled synthetic primer spanning from SV4526 to SV4557 as described under Materials and Methods. The extended products were resolved on a 4% polyacrylamide-8M urea gel along with markers. Electrophoresis was carried out at 1000 V in 100.0 mM tris-borate buffer (pH 8.0) for 8 h. Lane A, 3' end labeled Hinf I digest of pBR322. Lane B, 3' end labeled 123 bp ladder (Bethesda Research Laboratories)

efficiency of suppression of an amber mutation in the *E. coli* Tn9 CAT gene which was fused into the Rous sarcoma virus long terminal repeat (RSVam27CAT) (36). The RSVam27CAT has a CAT gene which contains an amber codon at 27th position from the N-terminal end. When cells are transfected with this construct, CAT activity cannot be detected unless an Su^{+} (amber) tRNA is also introduced by infection or transfection (36). CV-1p cells were transfected with RSVamCAT and then infected with the SV40 variants after 6 h. CAT activity was evaluated in the cell lysates 42 h after infection. RSVCAT plasmid was used as a control. Forty eight hours after transfection, CAT activity was quantitated in the cell lysates

Figure 6. Growth Kinetics of SV40 mutants. To monitor the growth kinetics, CV-1p cells were infected with various mutants at a multiplicity of 3 PFU per cell. After incubation of 1 h, the dishes were washed three times with Tris-saline and refed with DME containing 2% fetal calf serum. At the indicated times, the virus yield was measured by a plaque assay on CV-1p cells.

as described (37). As shown in Fig. 7, the amber mutation in the CAT gene was not suppressed by WT SV40 whereas high levels of CAT activity (40% of control experiment using RSVCAT) were detected in cells infected with SV-tRNA. These results demonstrate that the suppressor tRNA of SV-tRNA is able to function efficiently.

The SV-VA recombinant synthesises 4 fold higher levels of large-T antigen at late times:

In human cells infected with Ad the VAI RNA is required for the efficient translation of viral as well as host mRNAs at late times (17-19). The VAI RNA appears to enhance translation by blocking the activation of the kinase (eIF-2 α kinase) which phosphorylates and thereby inactivates the protein synthesis initiation factor eIF-2 (20-22). To study the effect of VAI RNA on the translation of early and late viral mRNAs, CV-1p cells were infected with WT SV40, dl2800, SV-VA and the two defective VAI

Figure 7. Suppression of CAT activity by SV-tRNA. CV-1p cells were transfected with 5 ug of RSV CAT or RSV am27CAT (amber mutant) and, after 6 h, infected with WT SV40 or SV-tRNA. Forty two h after virus infection, cell extracts were prepared and CAT activity present in the lysates, corresponding to an equal number of cells, was determined as described (37).

mutants, SV-VA/709 and SV-VA/719 at 10 PFU per cell, pulse labelled for 1h with ^{35}S -methionine at times indicated in Fig. 8. An identical amount of the extract from each dish was immunoprecipitated using a monoclonal antisera (pAB419) (34) directed against the large-T antigen. The immunoprecipitated proteins were analysed on 20% SDS-polyacrylamide gels and the large-T was quantitated by direct determination of the radioactivity present in the protein bands. Data presented in Fig 8A showed that at 40 h postinfection, large-T was present in 4 fold excess in cells infected with SV-VA compared to the WT controls (WT or *dt2800*). Cells infected with two SV40 mutants which contain defective VAI genes (SV-VA/709 or SV-VA/719) synthesized large amounts of mutant VAI RNAs (Fig. 3B) without exhibiting increased levels of large-T polypeptide (Fig. 8A). These results were reproducible in several independent experiments. This differential synthesis was observed only at late times after infection. At early times, 14 h after infection, large-T was present in equal quantities in cells infected with any one of these mutants or the WT virus (Fig 8B).

Viral late polypeptides were analysed on gels directly by fractionating the ^{35}S labelled proteins isolated from an equal number of cells infected by each of these mutants (Fig. 8C). Quantitation of the radioactivity present in VP1 band showed that VP1 in SV-VA infected cells was present in about one and half fold increased quantities as compared to that of the control samples. This observation was independently confirmed by quantitating the VP1 in CV-1p cells infected with the variants by Western blot analysis (40) (data not shown).

The levels of large-T mRNA are also increased by 3 to 4 fold in SV-VA infections:

The increase in the synthesis of large-T protein in SV-VA infected cells may be due to translation enhancement mediated by the large quantities of VAI RNA. Alternatively, the increased synthesis of the

Figure 8. Electrophoretic analysis of large-T and the late polypeptides synthesized in CV-1p cells after infection with SV40 variants.

A, synthesis of large-T at late times (40 h) in CV-1p cells infected with SV40 variants. B, synthesis of large-T at early times (14 h) in CV-1p cells infected with SV40 variants. Experimental details are as in Fig. 4B. C, analysis of late polypeptides synthesized by the SV40 variants in CV-1p cells at 40 h post infection. Each lane represents an extract derived from approximately 2.7×10^4 cells. To quantitate the labelled polypeptides, the protein bands were excised from the gels and the radioactivity present in these bands was determined directly.

early gene product at late times may be due to increased amounts of large-T mRNA present in the cytoplasm. Abundant quantities of VAI RNA, by stabilization or other mechanism, may increase the concentration of the large-T message at late times. To distinguish between these possibilities, the levels of large-T mRNA present in the cytoplasm of cells infected with WT, SV-VA and the two recombinant SV40 viruses which contain defective VAI genes SV-VA/709 and SV-VA/719 were analysed by Northern blot hybridizations. Results of two independent experiments are shown in Fig. 9A and B. It is clear that the levels of large-T mRNA in cells infected with SV-VA are increased by about 3-4 fold compared to that of cells infected with WT or *dl2800*. This observation was confirmed in several independent experiments. The levels of large-T in cells infected with two SV40 variants which carry the VAI mutations (SV-VA/709 or SV-VA/719) did not increase indicating that (i) this effect is specific for the VAI RNA and (ii) this effect is not due to insertion of an actively transcribing polymerase III gene. This is also supported by the fact that the levels of large-T mRNA did not increase in CV-1p cells infected with SV-tRNA (Fig 4A).

Similar experiments were performed to determine if the levels of late mRNAs are affected by the large quantities of VAI RNA. Northern blot hybridizations did not detect any appreciable difference in

Figure 9. Northern blot analysis of early (A and B) and late (C-E) viral mRNAs.

Cytoplasmic RNA was prepared from CV-1p cells at 40 h after infection with SV40 variants at 10 PFU/cell. The indicated amounts (in ugs) were subjected to electrophoresis (100 V for 4 h in 1.2% agarose gels containing 6% formaldehyde (32), transferred to nitrocellulose filters and probed with an early region specific DNA fragment (Hind III B, SV4002 to SV5171) for experiments in A and B or a late region specific DNA fragment (from Eco RI to Bam HI site; SV1782 to SV2533) for experiments in C-D. 18S and 28S represent the positions of the two ribosomal RNA species.

A and B show the results of two independent experiments in which the levels of large-T mRNA were determined. C and D show the results of two independent experiments in which the levels of late mRNAs (19S and 16S; shown by arrows) were determined. E, a darker exposure of the gel shown in C. 5 ug of cytoplasmic RNA was used in experiments C and D.

the levels of mRNAs coding for VP1 (16S) or VP2 (19S) in cells infected with any of the SV40 variants. Results obtained in two independent experiments are shown in Fig. 9C and D (16S and 19S messages are shown by two arrows in Fig. 9C; E is the darker exposure of the gel shown in C). Therefore, the VAI RNA does not enhance the levels of all SV40 mRNAs.

Thus our studies show that a four fold increase in large-T antigen synthesis in SV-VA infections correlated with a 3 to 4 fold increase in the effective concentration of large-T message in the cytoplasm. Hence, we conclude that the VAI RNA does not significantly enhance the translation of SV40 messages in monkey cells but increases the steady state levels of large-T mRNA by an unknown mechanism.

DISCUSSION

This is the first time that SV40 has been used as a nondefective vector to clone and express a foreign gene. Several investigators in the past have cloned genes coding for tRNAs, Ad VAI RNA or polymerase II products in the late region, deleting portions of the viral genome essential for viral replication.

As stated earlier, the propagation of such viruses required helper viruses and obtaining pure populations of recombinant viruses was not possible. Our vector, therefore, provides an alternative for the cloning and expression of pure stocks of viruses harboring small RNA polymerase III genes. We have not at present determined the maximum limit for the size of the foreign gene that can be cloned in the intron region of the SV40 large-T antigen gene. The size of the DNA that can be cloned into SV40 chromosome is usually limited due to strict packaging constraints (41,42). Viral genomes up to 105% in size are packaged efficiently. We have, however, found that an SV40 recombinant in which two copies of Ad VAI RNA gene are cloned in tandem shows no growth defects (unpublished results). Thus, foreign sequences upto at least 460 bp can be cloned into the vector described in this report.

Construction and characterization of SV-VA recombinant virus described in this study also allowed us to examine whether or not large amounts of VAI RNA stimulated translation of SV40 messages the way it does adenoviral messages. Our results show that the VAI RNA does not effect translation of SV40 messages significantly. Recent data suggest that the mechanism by which the VAI RNA stimulates translation is by blocking the activation of the eIF-2 α kinase which phosphorylates the protein synthesis initiation factor eIF-2 (20,22). The eIF-2 α kinase is induced by interferon and activated by double stranded RNAs (43-45). In cells infected with an Ad5 mutant lacking the VAI RNA gene, kinase activation leads to phosphorylation of eIF-2 and adversely affecting the host translation apparatus. It is not yet clear if the eIF-2 α kinase is activated during SV40 infection of monkey cells. If the eIF-2 α kinase is activated during SV40 infections, it is conceivable that SV40 might have evolved its own mechanism to counteract this activation. Therefore abundant quantities of VAI RNA in SV-VA infections may not offer any additional advantage for the virus in its translation. We have recently observed that monkey cells infected with an Ad5 mutant lacking both VAI and VAI RNA genes (*dl-sub720*) shows a phenotype characteristic of that of a translational block. However if these cells are preinfected with WT SV40, the translation defect is suppressed raising the possibility that SV40 may encode or induce factors which can complement for the VAI RNA function (33). Further, in CV-1p cells doubly infected with SV40 and reovirus, interferon treatment inhibits the translation of reovirus mRNAs but not SV40 mRNAs (47) presumably due to some factors encoded or induced by SV40 which antagonize the eIF-2 α kinase activity.

The observation that the levels of large-T messages in SV-VA infections increased at late times is intriguing. This effect seems to be specific to the large-T message; the levels of SV40 late mRNAs are not affected. In addition, the VAI RNA does not affect the levels of Ad specific messages in human cells (17; Bhat and Thimmappaya, unpublished data). This effect is not due to positioning of an actively transcribing polymerase III gene in the intron region because SV-tRNA and the recombinants which carry defective VAI genes do not show increased large-T mRNA levels. Several explanations may be offered for this observation. For example, the large-T may have a role in stabilizing its own message by binding to it. The VAI RNA may bind to large-T facilitating its interaction with the message. Alternatively, the VAI RNA may act at some other step in the biosynthesis of the large-T message, such as rate of its transport or splicing. Further experiments are necessary to determine the molecular events which lead to an

increase in the cytoplasmic levels of the large-T message.

It is interesting to note that the VAI mutants which are defective for function in human cells are unable to increase the levels of large-T mRNA in monkey cells suggesting that the same domains are involved in these two different functions. It is possible that the VAI RNA may interact with two different host targets (proteins) in these two systems and the two host proteins may recognize similar structural features of the VAI RNA.

The viruses described in this paper should be useful in several studies. As shown by previous workers (31) and in this study, abundant quantities of Su^+ (amber) tRNA^{ser} do not affect viral replication. The SV-tRNA, therefore, can be used as a helper virus to generate defective animal viruses which contain amber mutations in several essential genes. Viruses which are permissive in monkey cells and which contain amber mutations in several essential genes can be constructed *in vitro* and propagated either by preinfection or coinfection with SV-tRNA. Pure populations of defective viruses can then be obtained by separating the two viruses by physical methods.

Another advantage of this vector is to study the promoter structure of an RNA polymerase III gene in the milieu of the nucleoprotein complex. Cis acting transcriptional control elements of the eucaryotic tRNA genes (7) and the Ad VA RNA genes (8) and the transcription factors which interact with the promoter elements of these genes *in vitro* (9) have been studied in several laboratories. However, many such observations have not been duplicated *in vivo* in the context of a eucaryotic chromosome. For example, regions of eucaryotic chromosomes which contain actively transcribing RNA polymerase II genes were shown to contain nuclease hypersensitive sites presumably due to nucleosome free regions (48). In the nuclei of SV40 virus infected cells, the viral DNA exists in a chromatin like structure called minichromosomes (49,50). A segment of the SV40 chromosome which contains the replication origin and early and late promoters is also known to contain nucleosome free region with hypersensitive sites (51). Chromatin structure of polymerase III genes and the associated hypersensitive sites and their relationship with gene activation have not been rigorously investigated (52,53) Because of the simpler structure of the SV40 genome, the vector described here should be useful in carrying out such studies.

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