19S Late mRNAs of Simian Virus 40 Have an Internal Ribosome Entry Site Upstream of the Virion Structural Protein 3 Coding Sequence

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The late mRNAs of simian virus 40 (SV40) are polycistronic. The 19S mRNAs encode primarily the virion structural proteins VP2 and VP3. The VP2 and VP3 coding sequences are located in the same reading frame, and the VP3 AUG is an internal AUG for VP2. We tested whether an internal ribosome entry site (IRES) might be located upstream of the VP3 AUG that would facilitate its utilization, especially late in infection when cap-dependent translation is reduced (19). Using dicistronic reporter systems for IRES detection, we detected IRES activity within SV40 nucleotides (nts) 565 to 916, the region between the VP2 and VP3 AUGs. Nuclease protection analysis and primer extension analysis indicate no aberrant transcription or splicing that could account for false prediction of an IRES. Deletion analysis of the region indicates the presence of two functional IRESs, one within nts 661 to 830 and the other within nts 771 to 915. These two regions, each containing one IRES, have essentially the same IRES activity as the entire region spanning nts 616 to 915, which contains both IRESs. This suggests that potential secondary structures in the overlapping regions spanning nts 661 to 830 and nts 771 to 915 may be in dynamic equilibrium, such that there is only one functional IRES at any one time. These data strongly suggest that an IRES can be utilized for VP3 translation from the SV40 19S late mRNAs.

Eukaryotic translation can be initiated by either cap-dependent or cap-independent mechanisms. In some mRNAs, cap-independent translation is facilitated by internal ribosome entry sites (IRESs), first discovered in the uncapped picornavirus RNA genome. These sites enable ribosomes to initiate on highly structured regions located within untranslated regions 5' of the initiator AUG (9, 12, 13). Subsequently, many IRESs were identified in both RNA and DNA viral genomes (1, 6, 7, 9, 12, 13, 16) and in a broad range of cellular mRNAs from mammals, insects, and *Saccharomyces cerevisiae* (references 2, 8, and 17 and references therein).

The late coding region of the simian virus 40 (SV40) genome encodes four proteins, the virion structural proteins VP1, VP2, VP3 and the agnoprotein. The differential splicing of late transcripts produces two classes of mRNA, 16S and 19S late mRNAs (Fig. 1). Each class has a number of members due to heterogeneity in start sites and utilization of splice sites (15). However, the 5' end that is most utilized, the major cap site (Fig. 1), is at SV40 nucleotide (nt) 325, and the majority of transcripts display the splicing patterns shown in Fig. 1. The 16S mRNAs can encode the agnoprotein and the major virion structural protein VP1, and the start codons for VP2 and VP3 are spliced out. A ribosome entering at the cap would scan to the agnoprotein AUG (nt 335) and initiate and translate to the stop codon at nt 523, where it would terminate. We have predicted that ribosomes can then scan on for about 40 nucleotides and reinitiate at the VP1 AUG (nt 1499) (4). The 19S mRNA can potentially encode all of the SV40 late proteins: agnoprotein, VP2, VP3, and VP1 (Fig. 1). However, while it is

the primary source of VP2 and VP3, little, if any, VP1 is made from this messenger (4, 5). VP2 and VP3 are encoded in the same reading frame, with VP3 corresponding to the carboxy-terminal two-thirds of VP2 (3). Thus, the VP3 AUG is more than 350 nts downstream of the VP2 AUG, and there is no translational stop codon that might allow ribosomal termination and reinitiation at the VP3 AUG (4).

Despite this apparent poor location for VP3 AUG utilization, VP3 is produced at a higher rate than VP2 during late infection (14). In part, this might be due to the poor context of the VP2 AUG for ribosomal initiation (10). Previous studies have suggested that ribosomes could miss the VP2 AUG and scan to the VP3 AUG, which is in a good context for initiation (4). The potential use of IRESs to enhance VP3 AUG utilization was not considered in these early studies; few IRESs had been identified, and their prevalence was not yet known.

The possibility of the use of an IRES for VP3 translation became more significant with our recent observation that SV40 small t antigen induces hypophosphorylation of the eIF4E binding protein 1, 4E-BP1, late in SV40 infection (19). Although the hypophosphorylation of 4E-BP1 resulted in the expected decrease in cap-dependent translation, the synthesis of SV40 late proteins continued (19). Under these conditions, the continued synthesis of late proteins cannot be easily explained by ribosome scanning since scanning is cap dependent; however, IRES utilization is not (13). Thus, under the conditions that prevail late in SV40 infection, a strong case can be made for the use of an IRES for VP3 translation. It is conceivable that the inhibition of cap-dependent translation may favor the translation of VP3 if an IRES is used. In the following studies, the sequences between the VP2 AUG and the VP3 AUG, SV40 nts 565 and 915 (Fig. 1), were examined for an IRES that might affect VP3 AUG utilization. Deletion analysis of the region indicates the presence of two functional IRESs,

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FIG. 1. The late coding region of SV40. The black and gray bars at the top indicate the coding region for the agnoprotein (Agno) (AUG at SV40 nt 335 and stop codon at nt 523), VP2 (AUG at nt 562 and stop codon at nt 1620), VP3 (AUG at nt 916 and stop codon at nt 1620), and VP1 (AUG at nt 1499 and stop codon at nt 2591). The 19S and 16S classes of late mRNAs are shown. The late mRNAs have varied 5' ends beginning as far back as SV40 nt 120 (15). In addition, the splicing can be variable such that some mRNAs lack part or all of the agnoprotein coding region. However, the major species begin at nt 325, the major cap site (MC), and use the splicing shown in the figure (15). The region analyzed in these studies, between the VP2 and VP3 AUGs (SV40 nts 565 to 915), is shown.

one within nts 661 to 830 and the other within nts 771 to 915. These regions, each containing one IRES, have essentially the same IRES activity as the entire region spanning nts 616 to 915, containing both IRESs. This suggests that potential secondary structures in the overlapping regions spanning nts 661 to 830 and nts 771 to 915 may be in dynamic equilibrium, such that there is only one functional IRES at any one time. Overall, the data strongly suggest that an IRES can be utilized for VP3 translation from the SV40 19S late mRNAs.

MATERIALS AND METHODS

Cell line and transfection. CV-1 cells were propagated and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM GlutaMAX (all reagents obtained from Invitrogen, Carlsbad, CA). All transfections were performed using FuGene6 (Roche Applied Science, Indianapolis, IN) based on the manufacturer's instructions.

Plasmids. All SV40 fragments were amplified by PCR from SV40 genomic DNA, cloned into the p4xUAS reporter plasmid (20), and sequenced. pDL/565-915 was constructed by subcloning the fragment of nts 565 to 915 into the dual-luciferase plasmid pDL/N (18).

Luciferase assays. All luciferase assays were performed using either the *Renilla* luciferase assay system or the Dual-Luciferase reporter assay system from Promega.

Nuclease protection assays. Nuclease protection assays were performed according to a modified protocol for the RNase protection kit from Roche Applied Science. A ³²P-labeled antisense RNA probe for the dicistronic mRNA of p4xUAS/565-915 was made by *in vitro* transcription, using T7 polymerase with the corresponding pCDNA3 plasmid. Total RNA (5.0 µg) from transfected CV-1 cells was added to labeled RNA probe in 30 µl of hybridization buffer, heated for 5 min at 90°C, and immediately transferred to 45°C overnight. Then the hybridization mixtures were digested with RNase A and T1 for 30 min at 37°C. The reaction mixtures were further treated with sodium dodecyl sulfate, proteinase K, and phenol extraction and then coprecipitated with 2.0 µg yeast tRNA. Finally, the digestion products were separated by electrophoresis in a 4% polyacrylamide-7 M urea gel. The gels were dried and visualized by autoradiography.

Primer extension analysis. To map 5' ends of dicistronic mRNAs, primer extension analysis was done using the primer extension system from Promega. Primer 5'-GCG GGT ACC GTG CCC TAG TCA GCG GAG ACC-3', complementary to the Gal4VP16 N-terminal coding region in p4xUAS, was labeled with [γ -³²P]ATP. The labeled primer was hybridized with total RNA. cDNAs were synthesized with avian myeloblastosis virus reverse transcriptase at 42°C for 30 min. Products were separated on denaturing polyacrylamide gels and detected by autoradiography.

RESULTS

A potential IRES in the sequences between the VP2 and VP3

AUGs. To test SV40 sequences for IRES activity, we used p4xUAS (Fig. 2A) (20). This plasmid encodes a dicistronic mRNA with the *Renilla* luciferase gene as the first cistron, followed by an intracistronic sequence (ICS), and the gene for the Gal4VP16 fusion protein as the second cistron. Transcription is driven by a minimal promoter containing four copies of the Gal4 upstream activation sequence and a TATA box. This nonmammalian promoter produces very low levels of the dicistronic mRNA and the luciferase reporter protein in mammalian cells. This is due to the absence of an IRES in the ICS, resulting in the inability to translate the second cistron encoding the Gal4VP16 transcriptional activator. However, an IRES inserted into the ICS facilitates the translation of Gal4VP16. This triggers a positive feedback loop as the increased levels of Gal4VP16 bind to UAS sequences in the promoter and increase the transcription of this mRNA, resulting in more luciferase and Gal4VP16 (20).

A PCR fragment of the sequences between the VP2 AUG and the VP3 AUG (SV40 nts 565 to 915; p4xUAS/565-915) (Fig. 1) and a smaller region (SV40 nts 616 to 915; p4xUAS/616-915) were cloned into the ICS of p4xUAS (Fig. 2A). The plasmids were transfected into CV-1 cells, and *Renilla* luciferase activity was measured in cell lysates at 60 h posttransfection. The results (Fig. 2B) show that both of the SV40 inserts caused an 18- to 20-fold increase in luciferase, indicating the presence of a potential IRES within the insert.

The sequence containing the potential IRES has no apparent promoter activity or aberrant splicing. It is essential to show that the sequences spanning nts 565 to 915 and nts 616 to 915 inserted into the ICS have no inherent promoter activity that would result in transcription initiation at this internal site, resulting in a transcript containing only the second cistron (11). This would produce the Gal4VP16 fusion protein, resulting in false prediction of an IRES. In addition, it is essential to show the integrity of the transcript through the inserted region up to the AUG for the Gal4VP16 fusion protein, to assure that no

FIG. 2. (A) Diagram of the dicistronic gene on p4xUAS vector containing the ICSs into which SV40 nts 565 to 915 (4xUAS/565-915), nts 616 to 915 (4xUAS/616-915), and the human CMV promoter (4xUAS/CMV) were inserted. Also indicated are the probe used for the nuclease protection assays and the regions of the RNAs from each plasmid that would be protected by the probe. (B) Relative *Renilla* luciferase activity generated in CV-1 cells transfected with p4xUAS vector, p4xUAS/565-915, and p4xUAS/616-915. (C) Nuclease protection analysis of mRNA from CV-1 cells transfected with p4xUAS vector, p4xUAS/565-915, and p4xUAS/616-915, using the probe shown in panel A.

anomalous splicing occurred, resulting in a transcript that could allow translation of the second cistron in an IRES-independent manner (11).

To test these possibilities, we examined the transcripts from the plasmids by nuclease protection, using total RNA isolated from transfected CV-1 cells. The 648-nt RNA probe for the assay was complementary to 619 continuous bases from p4xUAS/565-915, including the 3' region of the *Renilla* luciferase gene, the entire ICS insert (including SV40 nts 565 to 915), and the 5' region of the Gal4VP16 gene. The same probe would protect 102- and 467-nt fragments from transcripts of p4xUAS/616-915 and 102- and 166-nt fragments from transcripts of the vector p4xUAS (Fig. 2A). If transcription initiated within the region spanning nts 565 to 915 or nts 616 to 915 or if there was aberrant splicing prior to the AUG for Gal4VP16, we would expect to detect protected fragments fewer than 619 nucleotides using p4xUAS/565-915 and fewer than 467 nucleotides using p4xUAS/616-915.

Figure 2C clearly shows the expected 619- and 467-nt protected fragments, indicating full-length transcription from p4xUAS/565-915 and p4xUAS/616-915, respectively. No other

protected fragment was detected that was not also seen in the controls. To clearly indicate where the 166- and 102-nt fragments migrate, we inserted the human cytomegalovirus (CMV) major immediate-early promoter (Fig. 2A) into the ICS; this provides strong transcription of the second cistron, which would lead to increased transcription from the 4xUAS promoter. As shown in Fig. 2C, the 166- and 102-nucleotide bands are clearly detected in the transcripts generated from p4xUAS/CMV. Examination of the protected fragments for p4xUAS/616-915 shows the expected 102-nt fragment; in addition, the assay of the vector transcripts shows a very small amount of the expected 166-nt fragment. In summary, these data suggest that the only transcripts resulting from p4xUAS/565-915 and p4xUAS/616-915 were full-length transcripts of the dicistronic gene. These results, combined with those of the luciferase assays shown in Fig. 2B, support the prediction of an IRES within the sequences spanning nts 565 to 915 and nts 616 to 519.

To further test for aberrant transcription initiation within sequences inserted into the ICS, we did primer extension assays using total RNA from transfected CV-1 cells (Fig. 3). We

FIG. 3. Primer extension analysis of plasmid mRNAs from CV-1 cells transfected with various plasmids used in this study, including mRNAs p4xUAS/565-915 and p4xUAS/616-915. The location of the primer is shown on the diagram of p4xUAS/565-915 at the bottom. See text for details.

looked for extension products that would have initiated within the ICS insert using a 5' ^{32}P -labeled primer complementary to sequences 160 nts downstream of the Gal4VP16 AUG (Fig. 3). For example, using RNA from cells transfected with p4xUAS/565-915, we would look for fragments between 160 and 567 nts in length, which would represent starts within the ICS insert (Fig. 3). In agreement with the nuclease protection analysis, we found no evidence for transcriptional start sites within the insert spanning nts 565 to 915. The gel was also highly overexposed (data not shown) and still failed to show aberrant starts. This was also true for transcripts from cells transfected with p4xUAS/616-915 and two other constructs containing inserts from nts 700 to 915 and nts 771 to 915 which are discussed

below. The positive control, p4xUAS/CMV, shows a 242-nucleotide band which represents the expected extension product for transcripts initiating at the start site in the CMV promoter. We detected very few, if any, full-length 1,503-nt transcripts that would migrate at the top of the gel; however, it is not unusual for primer extension assays to prematurely terminate prior to a length of 1,500 nucleotides.

The combination of the data in Fig. 2 and 3 strongly suggests that only full-length transcripts are produced from the plasmids containing the SV40 insert from the region between SV40 nucleotides 565 and 915.

The region spanning nts 565 to 915 functions as an IRES in a dual-luciferase reporter vector. In the analysis of an IRES, it

FIG. 4. Analysis of the IRES activity of the region spanning nts 565 to 915 in a dual-luciferase reporter. See text for details.

is essential that the expression of the second cistron is not due to ribosomes that read through the first cistron and initiate at the second or that terminate at the end of the first cistron and scan on to the second cistron. To rule out these possibilities, we inserted the region spanning nts 565 to 915 into the dicistronic vector pDL/N (Fig. 4) (18), which has *Renilla* luciferase as the first cistron and firefly luciferase as the second. In between is a sequence capable of forming a large stable hairpin in the mRNA, followed by a multiple cloning site into which the SV40 sequences were inserted. The purpose of the hairpin is to prevent scanning ribosomes from scanning through the first cistron and initiating in the second. The region spanning nts 565 to 916 was inserted into the multiple cloning site, and the two plasmids were transfected into CV-1 cells. At 60 h post-transfection, the two luciferase activities were assayed and their firefly/*Renilla* luciferase ratios determined. For Fig. 4, the ratio for the control plasmid pDL/N was normalized to 1; thus, insertion of SV40 nts 565 to 915 increased the ratio nearly

fourfold. These results confirm the presence of IRES activity in a second reporter system in which ribosome scanning is inhibited, thus preventing ribosomal read-through from the upstream cistron.

Deletion mapping suggests two IRES elements in the region spanning nts 565 to 915. We next used deletion analysis of the region spanning nts 565 to 915 to better define the boundaries of the IRES. These experiments were done with the p4xUAS vector. In Fig. 5, the activity of each insert is shown relative to the activity of the insert spanning nts 565 to 915, which was set at 100%. 3' deletion from nts 915 to 830 had little effect, while deletion to nts 750 and 670 significantly reduced IRES activity to 60% and 27%, respectively. These results suggest that the sequences between nts 670 and 830 are important for IRES function and establish nt 830 as the 3' boundary of the IRES region. We then defined the 5' boundary, beginning with deletion to nt 616, which showed 136% activity. 5' deletion to nts 661 (87%) and 700 (42%) defined the 5' border and confirmed

		% 565-915 IRES activity
565	915	100
565	830	106 ± 24
565	750	60 ± 10
565	670	27 ± 2
616	830	136 ± 11
661	830	87 ± 13
700	830	42 ± 2
661	770	19 ± 1
771	830	14 ± 3
616	915	108 ± 9
700	915	87 ± 8
771	915	136 ± 11

FIG. 5. Deletion analysis of the region spanning nts 565 to 915 assayed in the p4xUAS vector. All activities are shown as the percent activities of the p4xUAS/565-915 vector, which was set at 100%.

that the 160-nt region between nts 661 and 830 contains IRES activity. Separation of the region into nts 661 to 770 and nts 771 to 830 eliminated IRES activity, indicating that the entire region is essential.

We also tested the region from nts 616 to 915, which showed 108% activity relative to that of the full-length region. 5' deletion to nts 700 and 771 did not diminish IRES activity. We explained above that the region spanning nts 771 to 830 had no IRES activity; however, extension of this region to nt 915 restored activity. This suggests the presence of a second functional IRES in the 115-nucleotide region between nts 771 and 915.

DISCUSSION

In the studies presented above, we showed that the SV40 19S late mRNA contains IRESs in the region upstream of the VP3 AUG. The data suggest the possibility of two IRESs, one between nucleotides 661 and 830 and the other between nucleotides 771 and 915. IRESs are often found to be regions with high degrees of secondary structure in the RNA (13). Indeed, modeling of the regions spanning nts 661 to 830 and nts 771 to 915 indicates the ability to form extensive secondary structure (data not shown). However, defining any physiologically relevant structures will require significant fine-structure mutational analysis to map the IRES exactly and to define relevant structures using compensatory mutagenesis.

It is interesting that there may be two functional IRESs upstream of the VP3 AUG. The redundancy may assure VP3 AUG utilization. However, it is also possible that the IRES's potential secondary structures in the overlapping regions spanning nts 661 to 830 and nts 771 to 915 are in a dynamic equilibrium such that there is only one functional structure at any one time. This may account for the fact that the regions spanning nts 661 to 830 and nts 771 to 915, each containing one IRES, have essentially the same IRES activity as the region spanning nts 616 to 915, which contains both IRESs (Fig. 5).

The VP3 AUG is an interior AUG in the VP2 coding region; this location was one reason we predicted that it may utilize an IRES. Previous data, based on transfection and reporter gene studies, suggested that a scanning mechanism could account for utilization of the VP3 AUG (4). Indeed, the two translational mechanisms, scanning and IRES utilization, are not mutually exclusive. However, the discovery that cap-dependent translation is inhibited late in SV40 lytic infection (19) suggests that scanning, which is cap dependent, would be minimized. Thus, the use of an IRES for VP3 translation seemed even more likely. In fact, the freeing of ribosomes from cap-dependent translation may facilitate VP3 production via the IRES. This may also apply to the translation of VP1 and VP2. Although utilization of the VP2 AUG in the 19S message and the VP1 AUG in the 16S message can be accounted for by scanning mechanisms (Fig. 1) (4), the reduction of scanning ribosomes due to the inhibition of cap-dependent translation late

in infection suggests that IRESs may facilitate the translation of VP1 and VP2.

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