Expression of the Late Gene of Simian Virus 40 under the Control of the Simian Virus 40 Early-Region Promoter in Monkey and Mouse Cells

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We constructed a recombinant plasmid (pVNR4) with the simian virus 40 (SV40) early promoter positioned 30 nucleotides upstream from the major SV40 late transcription initiation site at residue 325. After transfection of the recombinant plasmid DNA into COS and mouse L cells, the transcripts of the SV40 late region were analyzed by S1 nuclease and primer extension analysis. The following are the principal findings. (i) The 16S and 19S late RNAs used the characteristic wild-type splice; no detectable levels of 19S unspliced RNA were observed. (ii) The majority of the late RNAs were heterogeneous and initiated in the early region (upstream and downstream from the Hogness-Goldberg sequence), and a minor population initiated at residue 325, the principal 5' terminus of the wild-type late RNA. (iii) During SV40 lytic infection there was a shift in initiation sites used to transcribe the early region from sites that are downstream to sites which are upstream (up RNA) of the origin of DNA replication. We observed that unlike lytic infection, T antigen and viral DNA replication were not needed for the appearance of up RNA in mouse L cells. (iv) In mouse L cells late RNAs were made, and the residue 325 5' end was utilized in the absence of T antigen or DNA replication. (v) In COS cells we found down RNA and up RNA transcribed from the extrachromosomally replicating plasmid but only down RNA produced by the integrated SV40 genome.

Simian virus 40 (SV40) is a small DNA virus that will lytically infect or transform cells. It has been investigated as a model for cellular transformation by viruses, as a model for a regulated gene system functioning in the nucleus of animal cells, and as a vector for the expression of inserted DNA elements in lytically infected and transformed cells (13, 20, 22, 25, 26, 30, 36, 39, 43, 55). During the lytic cycle, SV40 mRNA is synthesized in two phases. In the early phase, which lasts until the beginning of viral DNA replication, there is synthesis of two classes of early mRNAs coding for the SV40 small t and large T antigens, respectively. Although the function of small t antigen remains obscure, large T antigen is known to be required for the initiation of viral DNA synthesis. In the late phase beginning with the onset of viral DNA replication, two classes of late mRNAs are synthesized. These are 16S mRNA which codes for the major structural protein VP1 and the late 19S mRNA which codes for the VP2 and VP3 structural proteins. The SV40 early-region promoter is contained within sequences adjacent to the origin of viral DNA replication (*ori*) (Fig. 1A). Three separate elements are involved in SV40 early transcription (3, 12). At least one copy of a short guanine-cytosine (GC)-rich repeated sequence is necessary for early transcription. In addition, a sequence (50) within the 72-base-pair (bp) repeat has been shown to be an essential element. Finally, a Goldberg-Hogness or TATA box sequence (17) within a larger 17-bp adenine-thymine (AT)-rich block is responsible for positioning the 5' ends of early RNA.

The SV40 early region is one of the best studied examples

of regulation mediated by a known protein. Large T antigen regulates its own synthesis by decreasing the level of early-region transcription (27, 32, 51, 54). T antigen binds to SV40 DNA at three adjacent sites overlapping *ori* and the early promoter (Fig. 1). Early in lytic infection, transcripts appear with 5' ends mapping downstream (down RNAs) from the center of the 27-bp inverted repeat at *ori*. As the lytic cycle progresses into the late phase, there is a decrease in the utilization of these 5' termini, and a new set of RNAs appear with 5' ends mapping upstream of the inverted repeat (up RNAs), which become the predominant species (3, 16). Later studies have suggested that the shift requires large T antigen (12).

Less is known about the transcriptional control signals of SV40 late genes. The late mRNAs have heterogeneous 5' ends with the major late initiation site located at residue 325 (18, 24, 33), and the late region lacks a typical RNA polymerase II promoter upstream from the mRNA cap sites. Recent *in vitro* and *in vivo* work on the late-promoter control region (6, 7, 11, 42, 44, 45) has suggested that there are three distinct elements which control the expression and utilization of the major start site at residue 325: (i) a surrogate TATA box-like sequence located 30 nucleotides upstream of residue 325; (ii) GC-rich motifs contained in the 21-bp repeat region; (iii) the sequence between -21 and +6 of the major start site. In addition the 72-bp repeats or DNA replication or both may play a role in augmenting late transcription (31).

The present work was undertaken to refine our understanding on aspects of SV40 late-gene expression. In this report we examine the properties of an SV40 hybrid that contains the SV40 early-promoter region positioned immediately upstream from the SV40 late-gene coding region, oriented so as to drive transcription of the late gene. There is

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FIG. 1. (A) Diagram of the SV40 segment containing the early-region promoter, the origin of replication, and the large-T-antigen binding sites. The rectangle with black and white diagonals marked ORI represent the 27-bp palindrome which is implicated in DNA replication (28). The region immediately adjacent, the 17-bp AT block, has the TATA box. The small black boxes to the left of the AT block represent six GC-rich repeats. The two rectangles with diagonal stripes represent the two 72-bp enhancer elements. The three DNA-binding sites for large T antigen are also shown. The 5' ends of two sets of early-region transcripts of down (do) RNA and up RNA are indicated by the arrows pointing right. The nucleotide numbers are those of the SV numbering system (55) which is used throughout. The wavy line represents the late gene of SV40, and the dashed line represents the PAT 153 sequence. (B) Sequence of the fusion junction of the SV40 early-region promoter with the late gene. The *Hind*III restriction site indicated has been destroyed because of the insertion, of the *Xho*I linker. The nucleotide numbers are those of the SV numbering system (55).

one earlier report in which an SV40 mutant was constructed with the T-antigen gene transposed to the late region of the viral genome and the VP1 gene to the early region, but the late-gene expression was not the focus of interest (35).

We observed that late mRNA was made when pVNR4 was transfected into COS cells but not CV1 cells by using a transient expression system. On the other hand, when this plasmid was cotransfected with the *tk* gene into Ltk⁻ mouse cells and the *tk*⁺ colonies were analyzed for late mRNA synthesis, we found that SV40 late mRNA was produced as efficiently as the COS cells. The late transcripts were analyzed by S1 nuclease mapping and primer extension techniques and were found to be similar to the wild-type SV40 late transcripts. Both the 19S and 16S RNAs were spliced normally. The majority of the 5' ends of the SV40 transcript were that of the wild-type early RNA, and the major initiation site of the SV40 late RNA at residue 325 was utilized inefficiently. In mouse L cells T antigen was not needed for the appearance of up RNA, late RNA was expressed in the absence of T antigen or DNA replication, and the utilization of residue 325 as the 5' end was independent of the presence of T antigen. Therefore, it appears that the local sequences around residue 325 and late-region genomic structures apart from the upstream control elements are sufficient to specify utilization of the residue 325 site.

MATERIALS AND METHODS

SV40 nucleotide sequence numbering. The SV40 nucleotide numbering system is that of the SV numbering system (55) which is used throughout.

Construction of recombinant plasmids. The general procedures for the separation and purification of plasmid DNA in *Escherichia coli* have been described previously (38). *E. coli* C-600 or DH-1 was used as the host for all plasmid DNAs.

(i) **Construction of pVNR1.** Plasmid pVBt2 (49) contains the SV40 early promoter from the *Eco*RI restriction site (nucleotide 270, originally a *Pvu*II site) to the *Hind*III restriction site (nucleotide 5171), the T-antigen intron, and a polyadenylation signal cloned into pBR322. Plasmid pVNR1 is a derivative of pVBt2 in which the *Hind*III restriction site (nucleotide 5171) in the SV40 early promoter has been converted to an *Xho*I restriction site by the insertion of a synthetic *Xho*I linker.

(ii) **Construction of pVNR2.** The SV40 DNA was digested with *Taq*I and cloned into the *Cla*I site of pBR322. This pBR-SV40 plasmid DNA was cleaved with *Kpn*I (nucleotide 294) and incubated with T4 DNA polymerase and the four deoxynucleoside triphosphates to create blunt ends. This DNA was ligated to kinased *Xho*I linkers with T4 DNA ligase, and the products were used to transform an *E. coli* Dam⁻ Dcm⁻ strain. Alternatively, *Xho*I linkers were also introduced into the *Kpn*I site of pBR-SV40 after S1 nuclease treatment. The transformants were picked, and their plasmid DNAs were analyzed by restriction enzyme digestion to identify the modified pVNR2 recombinants.

(iii) **Construction of pVNR3.** The pVNR1 plasmid DNA was digested with *Xho*I and *Bam*HI, and the pVNR2 plasmid DNA was cleaved with *Xho*I and *Bcl*II. Both of the above digestions were run on a low-melting-temperature agarose horizontal slab gel. The fragments of interest (4,300 bp and 2,500 bp) were purified from the gel and ligated with T4 DNA ligase, and the products were used to transform *E. coli* C-600 or DH-1. The transformants were picked, and their plasmid DNAs were analyzed by checking the insert size and also by colony hybridization with the SV40 late-region fragment from the *Hpa*II site (nucleotide 348) to the *Bam*HI restriction site (nucleotide 2533) as the probe. Plasmid pVNR3 now has the late gene of SV40 under the control of the SV40 early promoter.

(iv) **Construction of pVNR4.** Plasmid pVNR3 was digested with *Xmn*I and cloned into the *Xmn*I restriction site of PAT 153 (56). This plasmid pVNR4 (Fig. 2) with the late gene of SV40 under the control of the SV40 early promoter is in a vector which has the poison sequences deleted (37).

DNA sequencing analysis. DNA sequencing analysis was performed by the method of Maxam and Gilbert (40).

Cell growth and viral infection. CV1 cells are a continuous line of monkey kidney cells. COS cells are SV40-transformed monkey kidney cells obtained by transformation and stable integration with an origin-defective, replication-negative SV40 DNA (19). The integration pattern of the SV40 DNA allows the stable expression of both SV40 large T and small t antigens but not VP1. Most of the experimental procedures, including conditions for cell culture and infection with wild-type virus (776), were as described in detail previously (48). Mouse Ltk⁻ cells (lacking thymidine kinase activity) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.).

Transfection of animal cells with plasmid DNA. In transfections performed with plasmid DNA, semiconfluent

FIG. 2. Structure of plasmid pVNR4. The wavy segment represents PAT 153 sequences. The thin segment represents sequences derived from pBR322. The hatched segments are derived from the SV40 early-region promoter which extends from the *PvuII* site at nucleotide 270 to the *HindIII* site at nucleotide 5171. The shaded segment represents sequences derived from the SV40 late region extending from the *KpnI* site at nucleotide 294 to the *BclI* site at nucleotide 2770. Tet^R, Tetracycline resistance.

monolayers of CV1 and COS cells were treated with 10 μ g of pVNR4 DNA (per 100-mm plate), using the calcium phosphate precipitation method (21). Where indicated, cytosine arabinoside (ara-C) was added to the culture media at 30 μ g/ml. Ltk⁻ cells (10^6) were transfected with 0.5 μ g of tk DNA (a plasmid with a herpes simplex virus *tk* gene in pBR322) (9) and 5 μ g of salmon sperm DNA or with 0.5 μ g of tk DNA, 5 μ g of pVNR4 DNA, and 5 μ g of salmon sperm DNA by the method of Wigler et al. (61). *tk*⁺ transformants were selected in a culture medium supplemented with hypoxanthine (15 μ g/ml), aminopterin (1 μ g/ml), and thymidine (5 μ g/ml), and isolated colonies were grown. Cells transfected to *tk*⁺ with the tk plasmid served as negative controls. The stably transfected mouse L cell lines producing the SV40 late RNA were called MSL cells.

DNA replication. Subconfluent monolayers of COS cells were transfected with 100 ng of the form 1 recombinant plasmid and the low-molecular-weight DNAs were extracted 48 h later by the Hirt method (29) and analyzed for replication by the method of Bergsma et al. (4). Either SV40 or PAT

153 DNA (100 ng per dish) was used as the positive or negative control, respectively, in this experiment.

Isolation of RNA. Cells were harvested 48 to 50 h after transfection (transient expression), and in the case of *tk*⁺ cell lines they were harvested after they reached confluency. Immediately after harvesting, cells were fractionated into nuclei and cytoplasm. Cytoplasmic RNA was isolated by the Nonidet P-40 method from 10^6 cells by the method of Maniatis et al. (38). Poly(A)⁺ RNA was isolated as described earlier (1).

We used two different methods for determining the splices and 5' termini of late 16S and 19S RNAs, primer extension and S1 mapping.

(i) **Primer extension analysis of SV40-specific late RNA.** 5' ³²P primer extension analysis of SV40-specific late RNA was done as described in detail previously (46). The primer for 16S RNA was a synthetic oligonucleotide primer extending from residues 1464 through 1497; the synthetic primer for 19S RNA extended from residues 561 through 591. The extended products were subjected to nucleotide sequence analysis by the method of Maxam and Gilbert (40).

(ii) **S1 mapping analysis of 5' termini of SV40 mRNA.** The positions of the 5' ends of SV40 late RNA were determined by the Weaver and Weissman modification (59) of the Berk and Sharp S1 nuclease procedure (5). For every RNA sample, a DNA probe was prepared from the plasmid DNA that was used to transfect the cells. The 5' ends of plasmid-derived poly(A)⁺ RNAs were determined, using as a probe appropriate restriction fragments that were labeled with ³²P at one or both 5' ends. Cytoplasmic poly(A)⁺ RNA was mixed with the ³²P-labeled (10^6 cpm/ μ g) DNA probe and precipitated with ethanol. The precipitate was dissolved in 40 μ l of buffer containing 80% formamide, 400 mM sodium chloride, 40 mM PIPES (piperazine-*N,N'*-bis[ethanesulfonic acid]) (pH 6.4), and 1 mM EDTA, heated at 70°C for 15 min, and then incubated at 50°C for 16 h. The hybridized samples were diluted with 360 μ l of cold buffer (0°C) containing 250 mM sodium chloride, 30 mM sodium acetate, 1 mM zinc acetate, 10 μ g of denatured salmon sperm DNA per ml, and 2,000 U of S1 nuclease per ml. After incubation at 30°C for 2 h, 10 μ g of tRNA was added, and the samples were precipitated by the addition of 2 volumes of ethanol. The products of the S1 nuclease reactions were separated by

FIG. 3. Structures of wild-type (WT) SV40 late RNAs. Three classes of 16S RNAs and four of 19S RNAs, distinguished on the basis of splice and leader structure (18, 47), are shown. Regions of mRNAs that are spliced out are indicated by peaked lines (A). Vertical arrows localize 5' termini of RNAs, thick arrows denote abundant termini, and thin arrows show less abundant termini. Nucleotide numbering is that of the SV system (55).

FIG. 4. Mapping of the 3' splice sites of late mRNAs formed in transfection of COS cells, CV1 cells, and mouse cells with pVNR4 in the presence or absence of ara-C. The DNA probe, isolated as described in the text, was a DNA fragment from wild-type SV40 extending from the *Eco*RI restriction site (nucleotide 1786) in the late region to the *Bgl*II restriction site (nucleotide 5235). This fragment was 5' ³²P-labeled at the *Eco*RI site. The S1 nuclease analysis was performed as poly(A)⁺ RNA from 5 × 10⁶ COS cells and 5 × 10⁵ mouse Ltk⁺ cells. COS cells were transfected with pVNR4 for 48 h with or without ara-C treatment (30 μg/ml). Lanes: a, Ltk⁺ cells (MSL); b, control Ltk⁺ cells; c, COS cells transfected with pVNR4 in the presence of ara-C; d, COS cells transfected with pVNR4; e, COS cells; f, CV1 cells transfected with pVNR4; g, CV1 cells; m, DNA size markers. The numbers on the left margin correspond to the size of the protected fragments obtained from the 16S spliced RNA (324 nucleotides) and 19S spliced RNA (1,230 nucleotides). The numbers on the right margin indicate DNA size markers.

electrophoresis in 8 M urea—4 to 8% polyacrylamide gels. The gels were dried, and autoradiographs were prepared with Kodak X-OMAT film for 1 to 4 days at -70°C with an intensifying screen. By using these electrophoretic condi-

tions, the fragments differing by only a few nucleotides that result from nibbling by S1 nuclease are not resolved.

RESULTS

Structure of the chimeric plasmid pVNR4. The chimeric plasmid pVNR4 was constructed as shown in Fig. 2. The late-gene coding fragment was in an orientation opposite to its natural orientation in the SV40 genome. The structure of the fusion junction of the SV40 early-region promoter with the late gene is shown in Fig. 1A. The sequence around the junction (*Kpn*-*Hind*III) was determined by the method of Maxam and Gilbert (40), and it was found that the late-gene fragment retained residue 298 and all sequences downstream but lacked residues 274 to 297. Residue 298 reads into a synthetic *Xho*I linker (octamer) to residue 5172 (*Hind*III site) in the SV40 early promoter (Fig. 1B). The plasmid pVNR4 replicated in COS cells (data not shown).

Splicing of late RNAs of pVNR4-transfected COS cells and MSL cells (mouse tk⁺ simian virus late). For reference, Fig. 3 shows the structures of wild-type SV40 late 16S and 19S RNAs. The splice sites of late 16S and 19S RNAs in COS or CV1 cells transiently transfected and in mouse L cells stably transfected with pVNR4 were determined independently by primer extension and S1 nuclease mapping. There was a significant amount of 16S and 19S spliced RNAs in both pVNR4-transfected COS cells and mouse L cells (Fig. 4), but none could be seen in pVNR4-transfected CV1 cells (Fig. 4, lane f). The molar ratio of 16S to 19S RNA in COS and L cells appeared to be less than that found in wild-type SV40. Figure 5 shows the spliced 16S and 19S late mRNAs from MSL cell lines that expressed different levels of late RNA. The gel electrophoresis patterns of the cDNAs synthesized from the 5' ends of cytoplasmic poly(A)⁺ late 16S and 19S RNAs obtained from CV1 cells infected with wild-type SV40 virus or COS cells transfected with plasmid pVNR4 are shown in Fig. 6 and Fig. 7A, respectively. As with wild-type virus, a multiplicity of cDNAs with different 5' termini were also synthesized on the 16S and 19S RNAs of plasmid pVNR4. Figure 7B shows the gel electrophoresis

FIG. 5. Mapping the 3' splice sites of late RNA formed in various mouse L cells (MSL cell lines) which were transfected with plasmid pVNR4. All analysis was done with total cytoplasmic RNA isolated from 10⁶ cells. The probe used for S1 nuclease analysis was the same as that described in the legend to Fig. 4. The products of the S1 nuclease reactions were separated by electrophoresis in an 8 M urea—6% polyacrylamide gel. Lanes: m, DNA size markers (numbers on left in nucleotides); a, wild-type SV40-infected CV1 cells; b, c, d, e, g, h, i, j, k, different pVNR4-transfected mouse L cells; f, tk⁺-transfected mouse L cells. The numbers in the right margin indicate the size (in nucleotides) of the S1 nuclease-protected fragments from the late 16S and 19S RNAs.

patterns of the cDNAs synthesized from the 5' ends of poly(A)⁺ cytoplasmic late 19S RNA obtained from COS cells transfected with plasmid pVNR4. The sequences of the low-abundance cDNA bands could not be read clearly. Primer extension and sequence analysis showed that the late mRNA produced by pVNR4 used the same 19S major splice site as in wild-type SV40, but the 5' end usage was quantitatively different from wild type. First, the residue 373-to-558 19S splice was the only splice detected. Second, there was no detectable 19S unspliced RNA, and no new splice sites were used. Although pVNR4 had the donor splice site at residue 526, it differed as compared with the wild-type SV40 in that it made no 19S RNA containing the residue 526-to-558 splice, whereas it used the 5' splice site at residue 526 to make 16S mRNA. These results are similar to observations made earlier by Ghosh et al. (15) using several late-leader deletion mutants of SV40.

5' ends of late RNAs of pVNR4-transfected COS cells and MSL cells. In previous studies (18, 48), the primary structures of the 5'-terminal regions of wild-type SV40 late RNAs were determined by the method of primer extension. We used this procedure and the S1 nuclease analysis technique to analyze the 5'-terminal primary structures of the late RNAs produced by plasmid pVNR4 when transfected in COS and mouse cells. RNA appearing early in wild-type virus infection had 5' ends that were clustered at nucleotides 5232 through 5237 (down RNAs), whereas at later times the majority of the RNAs appeared to be initiated upstream at nucleotides 30 through 33 (up RNAs). In SV40-transformed cells we also found RNAs initiated at nucleotides 70 through 73 (further up RNAs) (16). In COS cells transfected with plasmid pVNR4 there was slightly more up RNA and further up RNA than down RNA (Fig. 8A, lane a), unlike wild-type early infection in which we found only down RNA. In MSL cell lines we found relatively more down RNAs compared with up RNAs (Fig. 8C, lane b). The analysis of early RNA produced in COS cells, from the integrated template, showed only the down RNA start sites and not the up start sites (data not shown). We found that ara-C decreased the formation of both up and down RNA (Fig. 8B, lane b); ara-C treatment also reduced the total production of SV40 late RNA about 20-fold (data not shown).

Residue 325 is the most abundant 5' terminus of the late wild-type RNAs (18, 23, 48). We found that the late 16S RNA made by pVNR4 in COS and MSL cells utilized the residue 325 start site, with reduced efficiency (Fig. 9, lanes b, d, and f), and the 19S RNA did not use it at all, as determined by primer extension and cDNA sequence analysis. The relative amounts of 16S RNA initiating at residue 325 were roughly the same in the presence and/or absence of T antigen (Fig. 9, lanes b, d, and f). Our preliminary results obtained with the late RNA made from the endogenous template in COS cells show that residue 325 is utilized as efficiently as in the wild type (Fig. 10, lane a), even in the absence of the 16S major splice or extrachromosomal DNA replication. Ernoult Lange et al. (11) previously showed that initiation at residue 325 occurs irrespective of the structural gene inserted downstream. In pVNR4, the same template utilized the residue 325 5' start site and the early up and down RNA start sites. Nandi et al. (44) have studied mutants with base substitutions at nucleotide positions 295, 296, and 297 and have found decreased utilization of the start site at residue 325. Coincidentally, in plasmid pVNR4, all the above three residues were missing, and we also found reduced utilization of residue 325 for 16S and none at all for 19S RNA.

These results show that in mouse L cells the SV40 late

FIG. 6. cDNAs transcribed from 5' ends of wild-type and pVNR4 16S RNAs. Methods for the synthesis and separation of the cDNAs are given in the text. Lanes: a, wild-type SV40-infected CV1 cells; b, COS cells; c, pVNR4-transfected COS cells; d, DNA size markers (same as used in Fig. 4); e, COS cells transfected with wild-type SV40 DNA.

genes can be transcribed from the SV40 early-region promoter in the absence of T antigen and viral DNA replication and that in these cells T antigen is not needed for up RNA production.

We, like Piatak et al. (45), observed that sequences proximal to the major RNA start site at residue 325 seem to be most critical in selecting a site, although the level to which that site is utilized may be modulated by downstream late genomic sequences and the upstream control elements.

DISCUSSION

In this paper we reported the results obtained with a chimeric transposition plasmid in which the control elements

FIG. 7. (A) cDNAs transcribed from 5' ends of wild-type and pVNR4 19S RNAs. Methods for the synthesis and separation of the cDNAs are given in the text. Lanes: a, COS cells; b, wild-type SV40-transfected COS cells; c, wild-type SV40-infected CV1 cells; d, pVNR4-transfected COS cells; m, DNA size markers (numbers on left in nucleotides). (B) cDNAs transcribed from the 5' ends of the late 19S RNAs obtained after transfection of COS cells with pVNR4. The numbers given in the right margin indicate the bands which were sequenced by the Maxam and Gilbert method (40) as described in the text.

of the SV40 late promoter are substituted by the SV40 early-region promoter that comprises the TATA box sequences, the 21-bp repeats, and the two 72-bp repeats. This chimeric plasmid, pVNR4, when transfected into both permissive COS and nonpermissive mouse cells produced significant amounts of SV40 late RNA. Results presented in this paper show efficient expression of the transcripts of the late region of SV40 in mouse cells in the absence of T antigen or template DNA replication, consistent with reports that large T antigen may not be required for the synthesis of late mRNA in SV40-transformed mouse cells (10, 34).

The ratio of 16S to 19S RNA produced by wild-type SV40 is about 3:1 to 10:1 (60). Villarreal et al. (57), Ghosh et al. (15), and Barkan and Mertz (2) have reported that some late-leader mutants produce late RNAs with variably reduced 16S-to-19S ratios. The late transcripts made by pVNR4 in COS and mouse cells also showed a 16S-to-19S ratio of approximately 1. This suggests that the structure of

the 5' ends of late RNA or the efficiency of translation of late peptides influences the ratio of 16S to 19S RNA.

In SV40 wild-type virus, late transcription resulted in the production of three spliced species of 19S RNA as well as unspliced 19S RNA and a major 16S RNA with a single splice. In plasmid pVNR4 the deletion removed the 19S 5' splice site at residue 294 but retained the 5' splice sites at residues 373 and 558. pVNR4 late viral 19S RNA utilized the residue 373-to-558 splice but not the residue 526-to-558 splice, and there was not detectable level of unspliced 19S RNA. The 16S RNA retained the characteristic wild-type splice. Thus, unlike wild-type virus, the 5' splice site at residue 526 was not used to make 19S RNA.

For wild-type virus, there is an internal splice within the leader of some 16S RNAs such that residue 294 is spliced to residue 334. Most 16S mRNAs with 5' ends lying upstream of residue 290 use this intraleader splice. Conversely, the intraleader splice has never been detected in 19S mRNA with 5' ends located at or upstream from residue 290, so that there is a functional coupling between the intraleader splice and the downstream 16S splice. We, like Piatak et al. (45), observed that the intraleader splice is not an obligatory precursor to the formation of the downstream major 16S mRNA splice.

Whereas eucaryotic cellular genes commonly give rise to mRNAs with a single 5' terminus, certain viral mRNAs such as SV40 early and late wild-type mRNAs have heterogeneous 5' ends. With pVNR4, the majority of the 5' ends of the cytoplasmic RNAs lie in the early region at residues between 5229 and 5237 (down RNA) and between 30 and 34 (up RNA). Two minor species were identified only in pVNR4-transfected COS cells, at residues 70 through 74 (further up RNA).

In cells infected with wild-type SV40, early mRNAs synthesized during the initial lytic phase contained down RNA but not up RNA. With the onset of the late lytic phase, there was a progressive decrease in the amount of down RNA and a progressive increase in the amount of up RNA. Recent studies have suggested that SV40 large T antigen mediates the shift in transcription in two ways: first, large T antigen represses transcription at the downstream sites late in infection by binding to the region where these RNAs are initiated; second, T antigen promotes transcription from sites on the upstream side by initiating replication or amplification of the template DNA (8, 16). In pVNR4-transfected COS cells there was sufficient T antigen to initiate DNA replication, and we found more up RNA than down RNA. In mouse cells transfected with plasmid pVNR4, up RNA was also seen, although the down RNA appeared to be more abundant. In this case there was no T antigen, so down RNAs were not repressed and up RNA was made in the absence of autonomous replication of viral DNA. The above observation is in contrast to the results obtained earlier with SV40-transformed mouse cells in which there was no up RNA. In MSL cells, it appeared that T antigen and a functioning *ori* or template amplification were not needed for the upstream shift in the sites used for RNA initiation. Recently, Wasylyk et al. (58) have also reported using an SV40 β -globin recombinant in which up early RNA is formed *in vivo* in HeLa cells even in the absence of DNA replication.

The early RNA produced in COS cells transcribed from the integrated SV40 template showed no up RNA. Thus, within the same cell we found roughly equal amounts of down RNA and up RNA transcribed from the extra-chromosomally replicating plasmid pVNR4 but only down

FIG. 8. Mapping the 5' ends of RNA formed in transfection of mouse L cells and COS cells with pVNR4 in the presence or absence of ara-C. Cytoplasmic poly(A)⁺ RNA was isolated from 5×10^5 COS cells 48 h after transfection, from 2×10^6 mouse L cells, and from 5×10^6 COS cells transfected for 48 h with or without ara-C treatment (30 μ g/ml) and subjected to S1 nuclease analysis. The DNA fragment extending from the *Eco*RI restriction site (nucleotide 270) to the *Hpa*I restriction site (nucleotide 501) obtained from pVNR4 and labeled at the 5' end of *Hpa*I restriction site was used as the probe for S1 nuclease analysis. The S1 nuclease-protected fragments were run on an 8 M urea-6% or 8% polyacrylamide gel. The labels up, down, and further up in the margins indicate the late RNA species initiated at nucleotides 5232 to 5237 and 73, respectively. (A) Lanes: a, COS cells transfected with pVNR4; b, COS cells; m, DNA size markers (numbers on right in nucleotides). (B) Lanes: a, COS cells transfected with pVNR4 DNA; b, COS cells transfected with pVNR4 DNA in the presence of ara-C; c, COS cells; d, COS cells in the presence of ara-C. In both panels A and B the S1 nuclease-protected fragments obtained from integrated SV40 template are not shown. (C) Lanes: a, control mouse Ltk⁺ cells; b, mouse Ltk⁻ cells transfected with pVNR4 DNA (MSL cell lines); m, DNA size markers. A dark band appearing in lanes a and b below the up and down RNA bands is from the double-stranded protected probe.

RNA produced by the integrated SV40 genome. Buchman et al. (8) argued that only amplification of the template, a condition that requires functional T antigen and an intact functional *ori* sequence, appears to be needed for production of up RNA; moreover, replication does not have to be initiated at an *ori* sequence that overlaps the promoter to increase up RNA production. Our results suggest that the

shift from down to up RNA is not due to a limiting availability of a diffusible cellular factor(s).

Earlier reports have suggested that the production of late transcripts is stimulated by T antigen. The late transcript of the endogenous SV40 template in COS cells shows that residue 325 is utilized as efficiently as in the wild type even in the absence of viral DNA replication. Also, unlike wild-

FIG. 9. S1 nuclease mapping of the 5' ends of late mRNA produced in pVNR4-transfected COS cells and mouse L cells. Cytoplasmic poly(A)⁺ RNA was isolated from COS cells (10^4) and COS cells (10^4) transfected with plasmid pVNR4 in the presence or absence of ara-C (30 μ g/ml). Poly(A)⁺ RNA was also isolated from mouse Ltk⁺ and MSL cells and from CV1 cells and wild-type SV40-infected CV1 cells. The RNAs were subjected to S1 nuclease mapping. The probe for S1 nuclease mapping was derived from pSV1-16SIVS, a cloned mutant of SV40 that lacks nucleotides 526 to 1463 which encode the intervening sequence of the SV40 major late 16S RNA (generously supplied by J. Sklar and J. Mertz). The 1,353-bp *Eco*RI-to-*Taq*I restriction fragment (SV40 nucleotide 1786 to 4739) of pSV1-16SIVS was labeled at both ends with ³²P, hybridized to RNA, and treated with S1 nuclease as described in the text. Lanes: m, DNA size markers; a, COS cells; b, COS transfected with pVNR4; c, COS transfected with pVNR4 in presence of ara-C; d, same as lane b; e, Ltk⁺ cells; f, MSL cells; g, CV1 cells; h, wild-type SV40-infected CV1 cells. The numbers on the left margin indicate the size markers and on the right the size of S1 nuclease-protected fragments. In lane a the 532-bp fragment is obtained from the endogenous early-region 5' end at residue 73. In lanes b and d the 324-bp fragment is derived from the 19S late RNA, the 525-bp fragment is from the 16S late RNA whose 5' end is initiated at residue 325, and the 556-bp fragment is again the late 16S RNA with the 5' ends mapping in the early region. In lane h, except for the 538-bp fragment which is derived from the early 5' end, the rest are all from late 19S and 16S RNAs.

FIG. 10. S1 nuclease analysis of transcripts initiated at the late initiation sites in COS cells and pVNR4-transfected COS cells in the presence or absence of ara-C. Cytoplasmic poly(A)⁺ RNA was isolated from 10⁶ COS cells in all cases and subjected to S1 nuclease analysis with a ³²P-labeled DNA probe singly labeled at the *Hpa*I site (nucleotide 501) and extending up to the *Xho*I site (fusion of nucleotide 270 and 5171) from pVNR4. After S1 nuclease treatment, late RNA species protected by S1 nuclease were separated by electrophoresis on an 8 M urea–8% polyacrylamide gel. The analyses were performed with RNA transfected for 48 h with or without ara-C treatment (30 µg/ml). Lanes: a, COS cells; b, COS cells in the presence of ara-C; c, pVNR4-transfected COS cells; d, same as in lane c but in the presence of ara-C. The numbers in the left margin are the DNA size markers (lane m), and the ones on the right indicate the size of the S1 nuclease-protected fragments.

type virus, with pVNR4, RNA with a 5' end at residue 325 was found in 16S RNA but not at all in 19S RNA. Our results with the late transcripts obtained from MSL cell lines also show equivalent usage of residue 325 in the absence of T antigen. This shows that the utilization of residue 325 is not necessarily dependent on T antigen or DNA replication.

As noted, residue 325 is the most abundant 5' terminus of the late wild-type RNAs (18, 23, 48). In fact that mutants lacking this site are viable (2, 24, 41, 52, 53) indicates that other sites can also serve as the 5' ends of functional late mRNAs. In COS and mouse cells transfected with pVNR4, initiation took place at residue 325, although all late sequences upstream of residue 298 were deleted and replaced by the SV40 early-promoter sequences. An apparent preference of residue 325 5' ends for 16S RNA over 19S RNA was also observed early by Piatak et al. (46) with wild-type and late-leader-region deletion mutants. Ghosh et al. (14, 15) pointed out the role of the downstream sequences in determining the location of late RNA 5' ends. Mutants of SV40 with the late promoter sequence positioned upstream from the SV40 earlier-gene coding region are able to synthesize T antigen (11), and the transcripts initiate at the major late RNA initiation site (residue 325), although late sequences downstream from residue 332 are deleted and replaced by the coding sequence of T antigen. We showed that residue 325 is utilized in COS cells despite the absence of the major residue 526-to-1463 wild-type 16S splice and downstream SV40 late sequences. This result supports the early result obtained by Piatak et al. (46) that sequences around residue 325 are most critical in locating RNA initiation at this residue.

In pVNR4, the same template uses the residue 325 and early up and down start sites, and the relative abundance of

the 5' ends at these initiation sites may depend on the availability and relative affinity of RNA polymerase for these sites. Possibly, most of the RNA polymerase molecules are trapped by the early promoter(s) that is located near the enhancer.

Nandi et al. (44) have recently presented additional evidence for a TATA box sequence located 30 nucleotides upstream of residue 325 (7). Mutants with base substitutions at nucleotide positions 295, 296, and 297 resulted in decreased utilization of residue 325. Contrary to Nandi et al., Piatak et al. (46) reported that mutants with deletions at the *Kpn*I restriction site (nucleotide 294) resulted in efficient use of residue 325. In plasmid pVNR4, all this sequence is missing, and this could be an alternative or additional explanation for the decreased utilization of this site. We can summarize that the local sequences around residue 325 may control the utilization of residue 325 and that late-region downstream sequences as well as upstream control elements may regulate the efficiency of utilization of this site.

The results obtained by Piatak et al. (46) confirmed that the initiation codon used for translation of the major viral capsid protein, VP1, functions effectively even if it is preceded by two or more initiation codons present in the late leader sequence (36). We are now in the process of studying the synthesis of late viral structural proteins in both COS and mouse cells.

We made the following observations and conclusions from the present study. Both the 16S and 19S RNAs are expressed in significant amounts in COS and mouse L cells in the absence of T antigen; the molar ratio of 16S to 19S RNA is less than that of wild-type SV40; in mouse L cells neither T antigen nor viral DNA replication are needed for the appearance of early up RNAs; residue 325, the principal 5' terminus of the wild-type late RNAs, was used in 16S RNA and not at all in 19S RNA from our construct; this site was utilized independently of T antigen and viral DNA replication; local sequences around residue 325 seem to be most critical in selecting this site; within the same cell (COS cell) we found down RNA and up RNA transcribed from the extra-chromosomally replicating plasmid but only down RNA produced by the integrated SV40 template.

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