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Downstream Sequences Affect Transcription Initiation from the Adenovirus Major Late Promoter

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We analyzed a set of adenovirus-simian virus 40 (SV40) hybrids in which the SV40 T antigen coding sequences are inserted downstream from the adenovirus major late promoter within the first, second, and third segments of the tripartite leader. In infected cells, these viruses give rise to a matched set of hybrid SV40 mRNAs that differ only in the number of tripartite leader segments attached to the complete SV40 T antigen coding region. We found that the number of tripartite leader segments present at the 5′ end of the hybrid SV40 mRNAs had little effect on the efficiency of T antigen translation. Surprisingly, insertion of SV40 sequences within the first leader segment, at +33 relative to the start of transcription, significantly reduced the frequency of transcription initiation from the major late promoter. The 3′ boundary of this downstream transcriptional control element was mapped between +33 and +190 by showing that insertion of SV40 sequences within the intron after the first leader segment at +190 had very little effect on transcription initiation from the late promoter. A transient expression assay was used to show that the effect of downstream sequences on transcription initiation from the major late promoter is dependent on a *trans*-acting factor encoded or induced by adenovirus.

In a permissive host, the human adenoviruses, which have a double-stranded DNA genome of 36,000 base pairs, undergo a regulated program of gene expression that is divided into early and late phases, demarcated by the initiation of viral DNA replication. During the late phase of infection, viral transcription initiates primarily from the major late promoter, giving rise to primary transcripts that are processed by differential splicing and polyadenylation into more than 30 mRNA species. Each of the late mRNAs carries at its 5′ end a common 203-nucleotide untranslated sequence that is spliced to a protein-coding sequence. This 5′ region is called the tripartite leader because it is coded by three noncontiguous viral DNA segments that are located between the major start site for late transcription and the late protein-coding regions (for a review, see reference 59). Concomitant with the induction of late transcription, host cell protein synthesis is repressed, and late viral mRNAs are preferentially translated (16, 46). The function of the tripartite leader is not fully understood, but there are data which suggest that the leader is a signal that identifies late viral mRNAs for selective translation (4, 30, 57).

It is not practical to map the *cis*-acting sequences that control adenovirus late gene transcription and translation by directly mutagenizing viral DNA because the genome is too large and there is no convenient selection for conditional viral mutants. A partial solution to this problem has been to construct recombinant adenoviruses containing hybrid transcription units that are substituted for the normal early 1a (E1a) region. These defective recombinant viruses are complemented and propagated in human 293 cells which constitutively express adenovirus E1a and early region 1b proteins (18). For example, the adenovirus major late promoter and various portions of the tripartite leader, derived from a cDNA clone, were positioned upstream of a marker gene and inserted into the adenovirus E1a region (4, 30). Analysis of the transcription and translation of the marker genes indicated that a nearly complete tripartite leader is necessary for optimal levels of hybrid mRNA translation during the late phase of infection. However, it was not possible to use these recombinant viruses to assess systematically the contribution of *cis* control sequences to late promoter activity because the E1a enhancer is located upstream of the hybrid transcription unit and is likely to affect its activity. Moreover, the hybrid transcription units are missing the intron sequences between the leader segments, and thus potential effects of these sequences on late gene transcription would not have been detected.

A different method for the analysis of adenovirus late gene expression was developed that retains the normal spatial arrangement of the late promoter and leader sequences (56, 57). A combination of in vitro and in vivo recombination was used to precisely position a convenient marker gene, encoding simian virus 40 (SV40) T antigen, at four sites downstream from the adenovirus major late promoter within the region coding for the tripartite leader. Recombinant viruses that expressed SV40 T antigen were selected by virtue of the helper function activity of T antigen that allows adenovirus to grow on otherwise nonpermissive monkey cells (14, 20, 27, 42, 58). Defects in late gene expression caused by deletion of adenovirus DNA and insertion of SV40 sequences were complemented with wild-type helper virus. These hybrid viruses allowed us to examine the effects of progressively deleting DNA sequences 3′ to the authentic late promoter in the context of a normal viral infection by assaying the transcription and translation of SV40 T antigen sequences.

Three of the hybrid adenoviruses contain the SV40 T antigen coding region inserted within the intron sequences after the first, second, and third leader segments (56, 57). Each virus produces hybrid SV40 mRNAs that carry different amounts of the tripartite leader, depending upon the location of the SV40 insert. In each case, the leader sequences are joined to SV40 sequences by splicing from an unpaired 5′ leader splice site to a cryptic 3′ splice site in the

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T antigen coding region. As a result, 48 nucleotides of SV40 sequences are removed, including the normal AUG initiation codon, and thus translation begins at a downstream AUG codon in-frame with the correct coding region. Another hybrid virus has T antigen coding sequences inserted directly into the third leader segment, leaving no unpaired 5′ splice sites. This virus, designated Ad-SVR284, encodes hybrid SV40 mRNA that carries a nearly complete copy of the tripartite leader fused to the entire T antigen coding region such that translation initiates from the normal T antigen initiation codon.

All four of these viruses produce identical amounts of hybrid SV40 mRNAs, indicating that none of the SV40 inserts impinges upon DNA sequences that affect adenovirus late transcription in *cis*. The hybrid SV40 mRNA produced by Ad-SVR284 is translated 10 times more efficiently than that produced by the other hybrid viruses. Unfortunately, it was not possible to determine whether the efficient translation of Ad-SVR284 SV40 mRNA is caused by its 5′ leader structure or by the presence of the normal SV40 T antigen initiation codon and its surrounding sequences.

To distinguish between these possibilities, we report the construction of two new adenovirus-SV40 hybrid viruses in which the T antigen coding region is fused directly to either the first or second leader segment. In each case, the SV40 insert eliminates the leader splice donor and thereby precludes splicing to the cryptic SV40 acceptor. Together with Ad-SVR284, these new viruses form a matched set encoding hybrid SV40 mRNAs that differ only in the number of tripartite leader segments attached to their 5′ ends. Moreover, one of these hybrid viruses contains the most extensive deletion of DNA sequences 3′ to the late promoter that we have tested. We compared the relative efficiencies of transcription and translation of the SV40 sequences carried by these hybrid viruses. To our surprise, each hybrid SV40 mRNA was translated with the same efficiency, regardless of its 5′ leader sequences. Unexpectedly, DNA sequences that lie downstream from the major late promoter were found to contain an element that significantly increases the frequency of transcription initiation from the late promoter. Comparison of the late promoter sequences present in each virus indicates that the downstream transcription element extends into the intron that follows the first leader segment. A transient expression assay was used to demonstrate that the effect of these downstream sequences is mediated by a virus-encoded or -induced *trans*-acting factor.

MATERIALS AND METHODS

Cells and viruses. Monkey CV-1 and COS7 cells and human HeLa and 293 cells were cultured as previously described (49, 57). The recombinant viruses Ad-SVI+ and Ad-SVIII, previously termed Ad-SVR26 and Ad-SVR284, as well as the method for the construction and amplification of Ad-SVI and Ad-SVII, have been described previously (56, 57). Several independently derived isolates of Ad-SVI and Ad-SVII gave results identical to those reported here. The hybrid virus stocks consist of both defective recombinants and an adenovirus type 2-adenovirus type 5 recombinant helper virus designated 1 × 51i (19, 47). To ensure comparable multiplicities of infection, the virus stocks were titrated by SV40 T antigen indirect immunofluorescence in infected CV-1 cells, as well as by plaque assay on HeLa cells. We chose conditions (approximately 20 PFU per cell of helper, 4 PFU per cell of hybrid) that were sufficient to cause all of the infected cells to become T antigen positive as assayed by indirect immunofluorescence. Cells infected in this way contained identical amounts of recombinant viral genomes, as determined by blot hybridization of extracted DNA with an SV40 probe (data not shown; 22, 51).

Plasmids. Plasmids pAd-SVI+ and pAd-SVIII (formerly called pALE and p9B, respectively) have been described previously (56, 57), Plasmids pAd-SVI and pAd-SVII were constructed by standard cloning procedures (32). The vector in each case was pBR322, and the viral DNA was inserted at the *Bam*HI site. All four plasmids contain the SV40 early coding region extending from the *Hin*dIII site at nucleotide (nt) 5171 to the *Bam*HI site at nt 2533 (59). pAd-SVI contains adenovirus type 2 DNA sequences (15) from the *Sma*I site at nt 4112 (converted to *Bam*HI with linkers) to the *Pvu*II site at nt 6071 in the first leader segment. pAd-SVI+ (56) contains adenovirus type 2 DNA sequences from the same converted *Sma*I site to the *Hin*dIII site at nt 6231 in the intron after the first leader segment. pAd-SVII contains adenovirus type 2 DNA sequences from the *Sac*I site at nt 5638 (converted to *Bam*HI with linkers) to nt 7167 in the second leader segment (reached by BAL 31 digestion from the *Bgl*I site at nt 7210). pAd-SVIII (57) contains adenovirus type 2 DNA sequences from the same converted *Sac*I site to the *Xho*I site at nt 9689 in the third leader segment (converted to *Hin*dIII with linkers).

To construct plasmids pAd-SVI-ori and pAd-SVIII-ori (see Fig. 6A), the viral DNA inserts from pAd-SVI and pAd-SVIII were inserted into the *Bgl*II site of pSVT2 (45) which contains the minimal SV40 origin of replication (nt 5171 to 41) in a small pBR322-derived vector.

Immunoprecipitation of SV40 T antigen. CV-1 cells were infected with about 20 PFU of each hybrid virus stock per cell and incubated at 37°C. Infected cells were pulse-labeled for 3 h with 100 μCi of L-[^{35}S]methionine at 28 h postinfection. The labeled cells were washed twice with phosphate-buffered saline and lysed with 1 ml of ice-cold buffer B (50 mM Tris [pH 7.9], 200 mM LiCl, 0.5% Nonidet P-40) containing 125 μg of phenylmethylsulfonyl fluoride per ml. Portions containing 10^7 cpm were subjected to immunoprecipitation with PAb905 (a gift from M. Tevethia), a monoclonal antibody directed against the carboxy terminus of SV40 T antigen, as described previously (55). The immune complexes were fractionated on a 7 to 15% gradient of polyacrylamide containing sodium dodecyl sulfate (52). Relative amounts of T antigen were determined by densitometry of the autoradiogram.

RNA extraction and analysis. Total cytoplasmic RNA was isolated at 24 to 26 h postinfection as described previously (3). A 36-nt primer complementary to early SV40 RNA was prepared from plasmid pSV07 as described previously (45); it extends from SV40 nt 5136 to 5171 (5′ to 3′). A 24-nt primer was synthesized with an Applied Biosystems machine; it extends from SV40 nt 5146 to 5169 (5′ to 3′). The primers were 5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (35). For primer extension analysis, samples containing 10 to 15 μg of RNA were mixed with 50 fmol of end-labeled primer. The RNA-primer mixture was annealed for 60 min at 60°C (36-mer) or 45°C (24-mer) and then extended with 10 U of avian myeloblastosis virus reverse transcriptase for 60 min as described previously (36). The extension products were fractionated on an 8% polyacrylamide–8 M urea gel and subjected to autoradiography without an intensifying screen.

Northern blot analysis of 10 μg of glyoxalated, cytoplasmic RNA was carried out as described previously (54). In preliminary experiments, the nitrocellulose filter was hybrid-

ized with nick-translated SV40 DNA (33, 44) and then was subjected to autoradiography. The first probe was removed by boiling the filter in 20 mM Tris (pH 7.9); the filter was then hybridized with a nick-translated pUC8 clone, pAdH/X, containing adenovirus DNA sequences extending from map units 51.0 (*Hin*dIII) to 53.8 (*Xho*I) and reexposed. In the particular experiment shown in Fig. 4, the filter was hybridized with both radiolabeled DNAs simultaneously and subjected to autoradiography without an intensifying screen. Relative amounts of RNA in both primer extension and Northern blot experiments were determined by densitometry of autoradiograms.

Pulse-labeling of RNA in infected cells. CV-1 cells were infected with approximately 20 PFU of each hybrid virus stock per cell. After 24 to 26 h, the cells were made permeable with digitonin and concentrated as described by Ucker and Yamamoto (60), except that 200 μg of digitonin per ml was required to render all the cells trypan blue permeable. Each transcription reaction (125 μl) contained 2.5×10^6 infected cells and 80 μCi of [α-^{32}P]UTP in a reaction mixture as described previously (60). Transcription was terminated by the addition of 0.75 ml of 7.5 M guanidinium hydrochloride–25 mM sodium acetate (pH 7.0), and the RNA was isolated by the method of Chirgwin et al. (9). Then, 80,000 cpm of labeled RNA was hybridized with gel-fractionated restriction fragments from pSV121 (56) and pAdH/X which were immobilized on nitrocellulose (51). pSV121 was restricted with *Bam*HI to yield pBR322 and the SV40 early region. pAdH/X was restricted with *Xho*I and *Hin*dIII to liberate adenovirus late region sequences (map units 51.0 to 53.8) from pUC8. After 2 days at 42°C, the nitrocellulose strips were washed as described previously (61) and exposed to X-ray film for 1 week at −80°C with an intensifying screen.

Transfection of COS7 cells. COS7 cells were first infected with 10 PFU of adenovirus per cell. Mock infections were done with phosphate-buffered saline. After 60 min, the virus was removed and replaced with medium, and the cells were incubated at 37°C for 1 h. The cells were then transfected with 1 μg of plasmid DNA per 2×10^6 cells by the DEAE-dextran method (50). Briefly, the DEAE-dextran-DNA mixture was left on the cells for 30 min at 33°C. It was then removed and replaced with medium containing 100 μM chloroquine (31) and incubated for 8 h at 37°C (49). The chloroquine-containing medium was then removed, and the cells were incubated in standard medium for another 14 to 16 h at 37°C. Cells (8×10^6) were used for cytoplasmic RNA isolation as described previously (3). Cells (8×10^5) were extracted by the method of Hirt (22) to obtain low-molecular-weight DNA.

RESULTS

Construction of adenovirus-SV40 recombinant viruses. The recombinant adenovirus-SV40 plasmids and the viruses derived from them are depicted in Fig. 1. Plasmids pAd-SV1+ and pAd-SVIII have been described and were previously named pALE and p9B, respectively (56, 57). Their adenovirus derivatives, Ad-SVI+ and Ad-SVIII, were formerly known as Ad-SVR26 and Ad-SVR284, respectively. Two new plasmids were constructed for insertion into the adenovirus genome. Plasmid pAd-SVI contains the SV40 early coding region fused to the first leader segment downstream of the adenovirus major late promoter. Similarly, pAd-SVII contains the SV40 A gene fused to the second late leader segment (Fig. 1A). The viral DNA from each plasmid was positioned within the adenovirus genome by in vitro and in vivo recombination as described previously (56). The genome structures of the resulting defective adenovirus-SV40 recombinants, Ad-SVI and Ad-SVII (Fig. 1B), were determined by restriction digestion and Southern blot hybridization of viral DNA (data not shown; 22, 51). The structure of each recombinant genome was the same as that predicted on the basis of the initial plasmid construction and constituted approximately 10 to 20% of the virus population. Together with Ad-SVIII, in which the T antigen coding region is fused to the third late leader segment, these new viruses form a matched set expected to encode hybrid SV40 mRNAs that differ only in the number of adenovirus leader segments attached to their 5′ ends.

Expression of SV40 T antigen in infected cells. CV-1 monkey cells infected with Ad-SVI, Ad-SVII, and Ad-SVIII were pulse-labeled with L-[^{35}S]methionine at 24 h postinfection. T antigen was immunoprecipitated from infected-cell extracts with a monoclonal antibody directed against the carboxy terminus of SV40 T antigen and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results show that Ad-SVII produced the same amount of SV40 T antigen as that produced by Ad-SVIII, whereas Ad-SVI produced 5- to 10-fold-less T antigen (Fig. 2, lanes 4 to 6). Identical results were obtained by using a monoclonal antibody directed against the amino terminus of T antigen or by staining the gel shown in Fig. 2 with Coomassie blue (data not shown). This difference in T antigen production was also observed when labeled proteins from infected cells were compared without immunoprecipitation (Fig. 2, lanes 1 to 3). The amounts of other late viral proteins, such as hexon (120 kilodaltons) and the 100-kilodalton protein, were similar in all three cases.

Analysis of recombinant virus-encoded hybrid mRNAs. It was important to determine whether the difference in the amount of T antigen produced by the hybrid viruses was caused by the different tripartite leader sequences carried by the SV40 mRNAs or by the relative abundance of the hybrid mRNA species. First, total cytoplasmic RNA was prepared from hybrid virus-infected cells at 24 h postinfection and analyzed by primer extension with a 36-base oligonucleotide complementary to early SV40 RNA. The reverse transcription products were fractionated on a urea-polyacrylamide sequencing gel and subjected to autoradiography (Fig. 3). Each virus produced RNA that gave an extension product of exactly the size predicted from the virus structure, thus confirming that the hybrid RNAs had the expected spliced late leader structure. In addition, the amount of the Ad-SVI extension product was 5 to 10 times lower than the amount of either the Ad-SVII or Ad-SVII extension products. Therefore, the relative amounts of the extension products correlate with the relative amounts of T antigen expressed by the three recombinant viruses. Identical results were obtained with poly(A)$^+$ mRNA or with nuclear RNA isolated from hybrid virus-infected cells (data not shown).

To quantitate more precisely the amounts of hybrid SV40 RNA in infected cells, we analyzed the RNAs by Northern blot hybridization. RNA extracted from Ad-SVI+-infected cells was also included in these experiments to help identify sequences responsible for the apparent reduction in SV40 RNA produced by Ad-SVI. Cytoplasmic RNA extracted from cells at 24 h postinfection was glyoxalated, fractionated on an agarose gel, and transferred to nitrocellulose. The filter was probed with nick-translated SV40 DNA to detect hybrid SV40 RNAs and with a control DNA fragment from the late region of adenovirus (map units 51.0 to 53.8) that detects

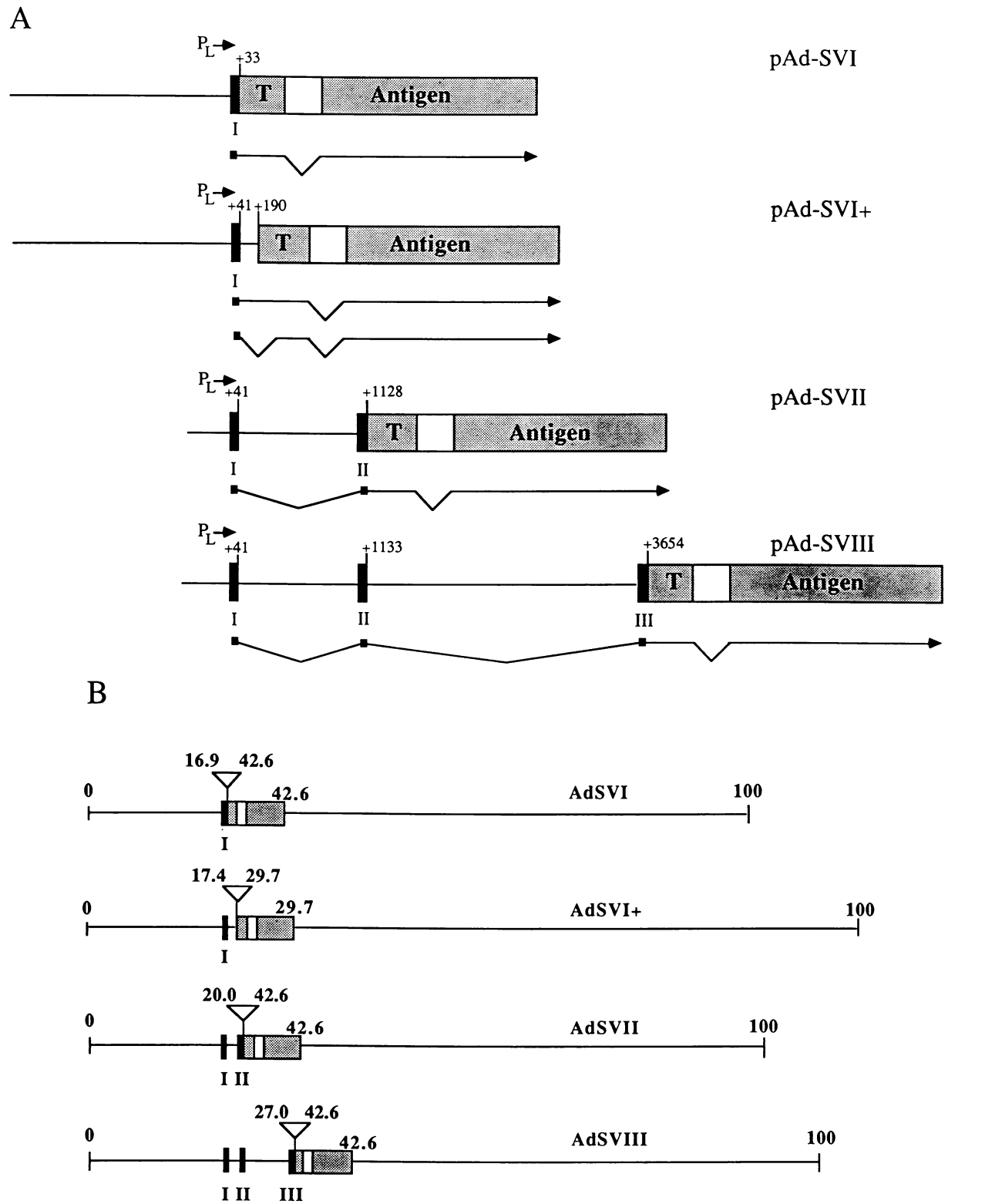

FIG. 1. (A) Structure of the recombinant plasmids used to construct hybrid viruses. Only the viral DNA insert is shown. The thin lines represent adenovirus DNA; the direction of transcription from the major late promoter (P_L) is indicated by an arrow. The tripartite leader segments (■) are shown with roman numerals beneath; shown also are SV40 T antigen coding sequences (▣) and the large T intron (□). The numbers above the boxes show the distance in base pairs from the late RNA start site to the junction between adenovirus and SV40 sequences. Beneath each plasmid insert are depicted the expected structures of large T antigen mRNAs produced from the corresponding viruses. The positions of the tripartite leader segments are indicated (■). (B) Structure of recombinant virus genomes. Relative positions of late leader segments (■), deleted adenovirus DNA (▽), and SV40 DNA (▣) are shown. The large T intron is also indicated (□). Numbers designate adenovirus map units (1 map unit = 360 base pairs).

some of the L3 family of late RNAs. Densitometric analysis of the data shown in Fig. 4 revealed that Ad-SVI expressed 10-fold-less SV40 RNA than did Ad-SVI+, Ad-SVII, and Ad-SVIII. In contrast, cells infected with each of the four virus stocks expressed the same amount of late RNA that hybridizes to the adenovirus L3 probe. Thus, the differences in T antigen production among Ad-SVI, Ad-SVII, and Ad-SVIII can be accounted for by the difference in the relative amounts of hybrid SV40 RNAs that they produce. We conclude that the number of late leader segments attached to the 5′ end of the SV40 T antigen coding region has little, if any, effect on the translation of this mRNA. In addition, adenovirus *cis* regulatory sequences that affect the amount of hybrid SV40 RNA detected in infected cells are located within the transcribed region and extend no further downstream from the late promoter than the SV40 insertion site in Ad-SVI+ (+190).

Pulse-labeling of hybrid virus-encoded RNAs. The reduction in the amount of hybrid SV40 RNA in Ad-SVI-infected cells could be caused by a reduction in the level of transcription initiation from the recombinant adenovirus major late promoter or by a posttranscriptional event. To distinguish between these two possibilities, hybrid virus-infected cells were made permeable with digitonin, and RNA was pulse-labeled with [α-^{32}P]UTP. Incorporation of label into RNA was linear for at least 8 min (data not shown). Total cellular RNA was isolated after labeling a period of 2 min and used to probe identical Southern blots of SV40, adenovirus, and pBR322 DNA. An autoradiograph of the blots (Fig. 5) showed that the amount of nascent SV40 RNA initiated from the late promoter of Ad-SVI was much less than that initiated from the late promoters of Ad-SVI+, Ad-SVII, and Ad-SVIII, in accordance with the measurements of relative steady-state RNA concentration. In contrast, equivalent amounts of labeled RNA hybridized to a DNA fragment from the adenovirus L3 region.

We were concerned that the reduction in transcription initiation from the late promoter of Ad-SVI might be caused by the inadvertant introduction of a mutation into the late promoter of pAd-SVI, the plasmid used to construct Ad-SVI. To test this possibility, the pAd-SV plasmids were transcribed in vitro, with an extract in which plasmids that carry mutations in the TATA box or upstream element of the adenovirus late promoter exhibit a mutant phenotype (21, 34). No difference between the pAd-SV plasmids in the ability to direct transcription in vitro was observed (data not shown), indicating that the previously defined late promoter sequences contained in these plasmids were intact and functional.

Taken together with the in vitro results, the pulse-labeling experiment suggests that DNA sequences downstream from the adenovirus major late promoter, between the SV40 insertion site in Ad-SVI (+33) and that in Ad-SVI+ (+190),

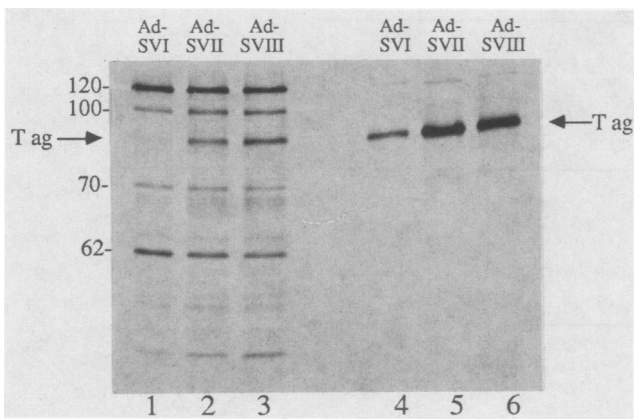

FIG. 2. Immunoprecipitation of hybrid virus-encoded SV40 large T antigen. CV-1 cells were infected with Ad-SVI (lanes 1 and 4), Ad-SVII (lanes 2 and 5), or Ad-SVIII (lanes 3 and 6), labeled with L-[^{35}S]methionine, and harvested. Portions containing 10^7 cpm were subjected to immunoprecipitation with monoclonal antibody PAb905, directed against SV40 T antigen, fractionated on a 7 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel, and subjected to autoradiography (lanes 4 to 6). Samples containing 10^5 cpm of labeled extract were fractionated on the same gel (lanes 1 to 3). SV40 large T antigen (T ag) is identified. Numbers to the left of the gel indicate the molecular mass, in kilodaltons, of some prominent adenovirus late proteins.

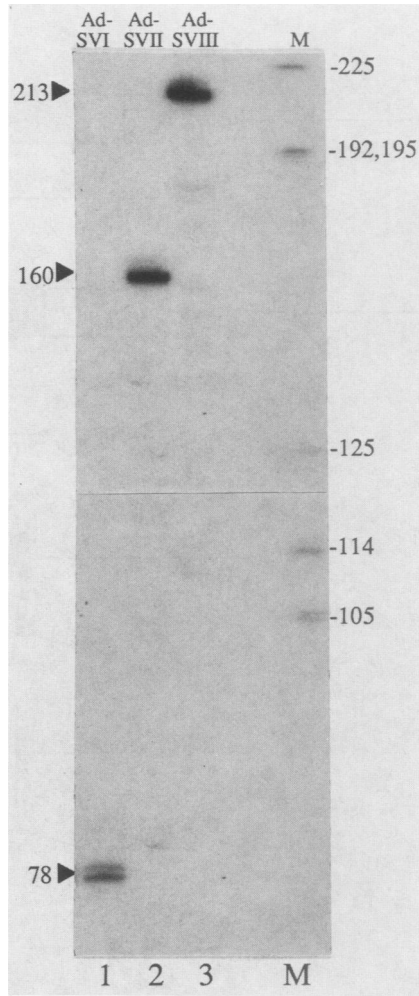

FIG. 3. Structural analysis of 5′ ends of hybrid SV40 RNAs encoded by recombinant viruses. Samples (10 μg) of cytoplasmic RNA extracted from cells infected with each recombinant virus were annealed to the ^{35}P-end-labeled 36-nt SV40 primer which was extended with reverse transcriptase and fractionated on an 8% polyacrylamide–8 M urea gel. Extended products of 78 nt with Ad-SVI RNA (lane 1), of 160 nt with Ad-SVII (lane 2), and of 213 nt with Ad-SVIII (lane 3) are indicated by arrowheads. The lengths (in base pairs) of ^{35}P-labeled DNA standards are indicated to the right of lane M.

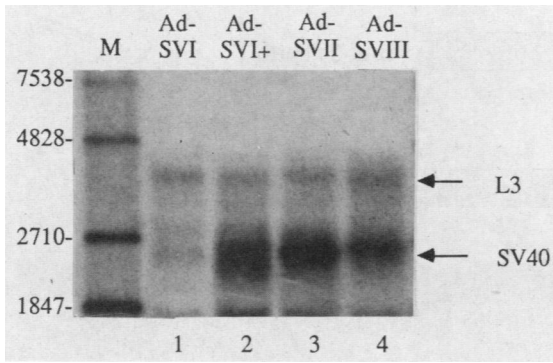

FIG. 4. Northern blot hybridization of viral RNAs from hybrid virus-infected cells. A 10-μg amount of cytoplasmic RNA extracted from cells infected with each of the hybrid viruses was denatured by treatment with glyoxal and fractionated on a 1% agarose gel. The RNA was transferred to nitrocellulose and probed with nick-translated SV40 DNA, as well as with DNA from adenovirus map unit 51.0 to 53.8 (L3). Lanes: 1, Ad-SVI RNA; 2, Ad-SVI+ RNA; 3, Ad-SVII RNA; 4, Ad-SVIII RNA. The lengths (in nucleotides) of DNA standards are indicated to the left of lane M.

influence the rate of RNA initiation from this promoter in adenovirus-infected cells.

Transient expression assay for 3′ activating sequences. The results of in vitro transcription also suggested that uninfected cells could be missing a factor that discriminates between templates that contain sequences in the intron after the first leader and those that do not. To determine whether the transcriptional control sequences downstream from the major late promoter are active in uninfected cells or whether their activity depends upon a virus-coded protein or virus-induced host factor, we examined transcription from plasmid-borne copies of the late promoter in uninfected and infected cells. Previous work suggested that transcription from the adenovirus late promoter carried on the pAd-SV plasmids would be undetectable in HeLa cells (21, 29). We confirmed this result and also found that transcription from the plasmid-borne late promoter is not detectable 24 h after adenovirus infection of transfected HeLa cells (data not shown).

The difficulty in detecting transcripts expressed from the pAd-SV late promoters could be caused by the low copy number of these nonreplicating plasmids in transfected cells. A more interesting possibility is suggested by the work of Thomas and Mathews (53), who showed that the late promoter requires a *cis*-acting alteration caused by viral DNA replication to become active during the late phase of infection. Therefore, to permit replication of the pAd-SV plasmids without introducing the complication of additional enhancer or promoter elements, we inserted the minimal SV40 origin of replication into these plasmids. The resulting pAd-SV-ori plasmids are depicted in Fig. 6A. We used COS7 cells as recipients in the transient expression studies because they constitutively express SV40 T antigen from an integrated copy of the SV40 genome and thus support replication of SV40 origin-containing plasmids (17). In addition, the COS7 cell line, unlike other monkey cell lines, is permissive for adenovirus infection because of the helper function activity of SV40 T antigen (data not shown).

COS7 cells were mock infected or infected with 10 PFU of adenovirus per cell. After 1 h, the cells were transfected with the pAd-SV-ori plasmids or mock transfected and, 24 h later, total cytoplasmic RNA, as well as low-molecular-weight DNA, were prepared. We used a primer extension assay to distinguish between the endogenous SV40 RNA produced by the COS7 cells and the plasmid-encoded hybrid SV40 RNAs. A synthetic 24-nucleotide SV40 primer was made that hybridizes to sequences common to the various SV40 RNAs. The products of reverse transcription differ in size because COS7 RNAs initiate from the SV40 early promoter, whereas pAd-SV-ori RNAs initiate from the adenovirus late promoter. The results of such an experiment are shown in Fig. 6B. Little or no difference was observed in the amount of SV40 RNA produced by pAd-SVI-ori or pAd-SVIII-ori in mock infected COS7 cells (compare lanes 1 and 4). How-

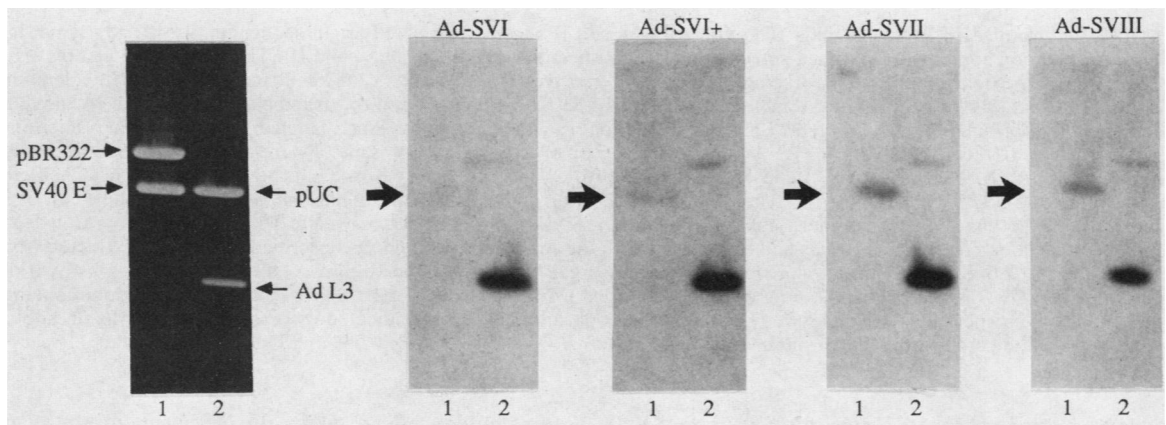

FIG. 5. Hybridization of pulse-labeled RNA to SV40 and adenovirus DNA. CV-1 cells infected with each hybrid virus were made permeable with digitonin and prepared for transcription. Each transcription reaction contained 2.5×10^6 infected cells and 80 μCi of [α-^{32}P]UTP. Transcription was terminated after 2 min, and the RNA was isolated. Plasmid pSV121 was cleaved with *Bam*HI, and plasmid pAdH/X was cleaved with *Hin*dIII and *Xho*I. The DNA fragments were divided into portions and fractionated in pairs on a 1% agarose gel (Lanes: 1, pSV121; 2, pAdH/X). The DNA was stained with ethidium bromide (left-most panel; the identity of each DNA fragment is indicated) and then blotted onto nitrocellulose which was cut into identical strips. The strips were hybridized separately with labeled RNA derived from cells infected with the hybrid virus indicated above each panel. After being washed, the strips were subjected to autoradiography. An arrow marks the location of the SV40 DNA on each strip. The faint hybridization to a high-molecular-weight band seen in each lane marked 2 is the result of partial digestion of pAdH/X.

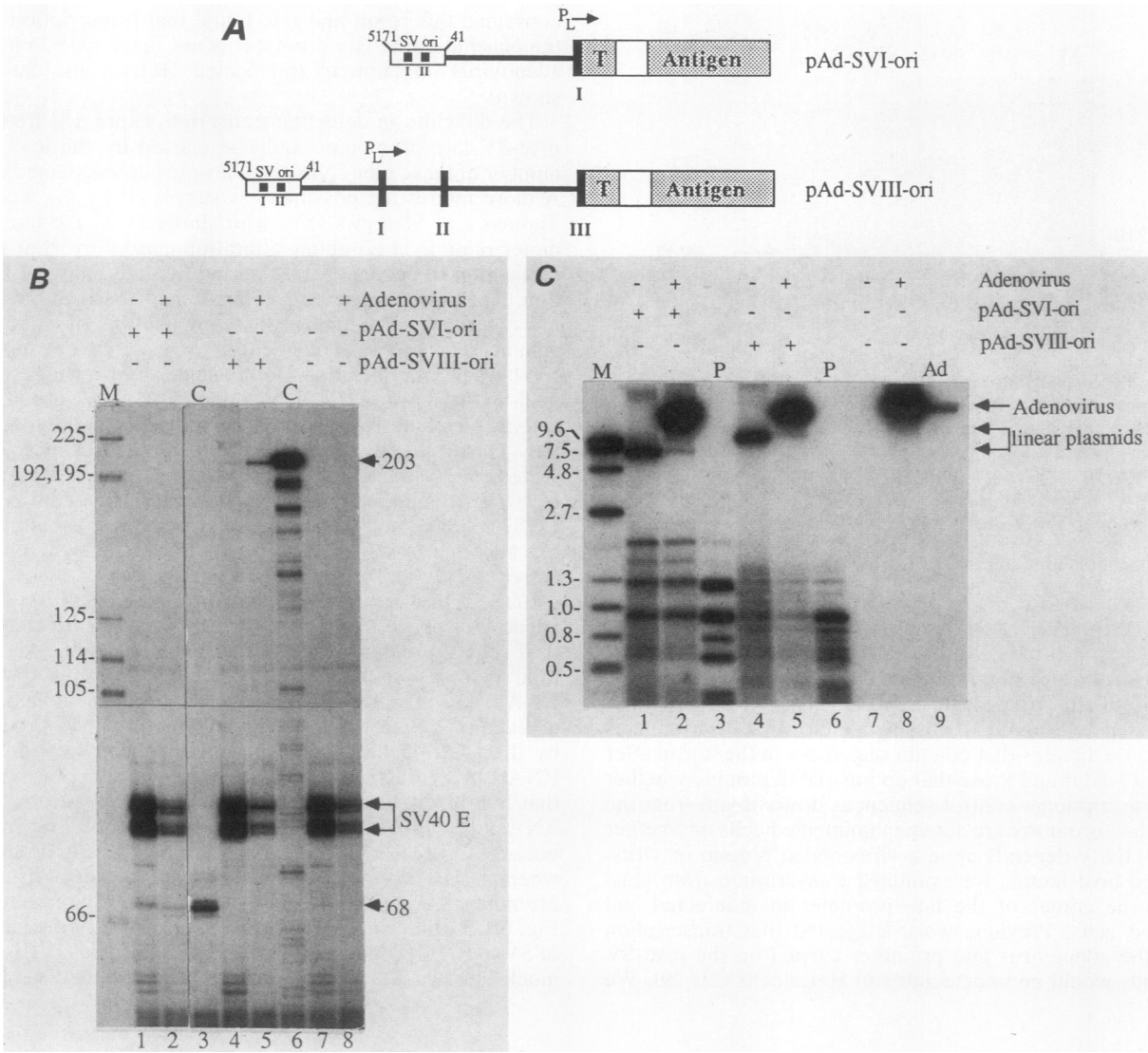

FIG. 6. (A) Structure of Ad-SV-ori plasmids. The viral DNA insert is shown. The SV40 origin sequence (nt 5171 to 41, not to scale) is shown as a small open box containing two black squares that indicate T antigen binding sites (I and II). The rest of the symbols are the same as in Fig. 1A. (B) Primer extension analysis of RNA extracted from transfected COS7 cells. COS7 cells were infected with adenovirus (lanes 2, 5, and 8) and transfected with pAd-SVI-ori (lanes 1 and 2) or pAd-SVIII-ori (lanes 4 and 5), as indicated (by + and −) above the lanes. A 15-μg amount of cytoplasmic RNA extracted 24 h posttransfection was annealed to a ^{32}P-end-labeled 24-nt synthetic primer and reverse transcribed. As controls (C), 0.5 μg of Ad-SVI RNA (lane 3) and 0.5 μg of Ad-SVIII RNA (lane 6) were also subjected to primer extension. cDNAs of 68 nt from pAd-SVI-ori and Ad-SVI and of 203 nt from pAd-SV-ori and Ad-SVIII are indicated by arrows. cDNAs derived from reverse transcription of COS7 SV40 RNA (SV40 E) are also indicated by arrows. The lengths (in base pairs) of ^{32}P-end-labeled DNA standards are indicated to the left of lane M. (C) Southern blot hybridization analysis of low-molecular-weight DNA isolated from transfected COS7 cells. COS7 cells were infected with adenovirus and transfected with the Ad-SV-ori plasmids as described in B. DNA extracted from 2×10^5 cells was cleaved with *Cla*I and *Dpn*I, fractionated on a 1% agarose gel, and transferred to nitrocellulose. The filter was hybridized with nick-translated pAd-SVIII DNA. As controls, 6 ng of each of purified pAd-SVI (lane 3), pAd-SVIII (lane 6), and adenovirus (lane 9) DNA were treated similarly. The positions of adenovirus DNA and linear plasmid DNAs are indicated by arrows. The lengths (in kilobase pairs) of DNA standards are indicated to the left of the marker lane.

ever, in adenovirus-infected COS7 cells, fivefold-more SV40 RNA was produced by pAd-SVIII-ori than by pAd-SVI-ori (compare lanes 2 and 5). In addition, adenovirus infection resulted in a fivefold decrease in SV40 RNA expressed from the SV40 early promoter in COS7 cells (compare lanes 1 and 2, lanes 4 and 5, and lanes 7 and 8). This effect is probably a result of the E1a-mediated repression of SV40 early transcription reported by others (5, 61).

We also isolated low-molecular-weight DNA from the transfected cells used in this experiment to measure the amount of replicated plasmid DNA. The extracted DNA was first linearized by digestion with *Cla*I and then restricted with *Dpn*I, which cleaves only the input, bacterially methylated DNA that has not replicated in mammalian cells. DNA that has replicated cannot be cleaved with *Dpn*I (40). A Southern blot of the gel-fractionated DNA was probed with nick-translated pAd-SVIII DNA and subjected to autoradiography (Fig. 6C). As expected, adenovirus DNA was only

detected in samples prepared from infected cells (lanes 2, 5, and 8). Each plasmid DNA replicated to the same extent in uninfected cells (lanes 1 and 4). However, in infected cells, each plasmid DNA replicated to a much lower extent (1/20 in each case) than in uninfected cells (compare lane 1 with lane 2 and lane 4 with lane 5). This last result could be a consequence of the reduction in the amount of endogenous SV40 RNA and, therefore, of T antigen in infected COS7 cells.

Taking the RNA and DNA analyses together, and assuming that the amount of plasmid DNA measured by Southern blotting is proportional to the amount of active template in both infected and uninfected cells, we found that, in infected cells, there was a 20-fold increase in transcription from pAd-SVI-ori and a 100-fold increase in transcription from pAd-SVIII-ori on a per-template basis. We do not know whether the downstream sequence-independent component of the transcriptional induction (20-fold) in infected cells is caused by adenovirus per se or by the recently described phenomenon of incompatibility between transcription and replication in SV40 origin-containing plasmids (25, 28). Nevertheless, there is a downstream sequence-dependent (fivefold) induction of transcription from pAd-SVIII-ori relative to pAd-SVI-ori in adenovirus-infected cells.

DISCUSSION

We constructed two new adenovirus-SV40 hybrid viruses to evaluate the effects of the tripartite leader sequences on adenovirus late gene expression. Ad-SVI contains SV40 T antigen coding sequences inserted into the first late leader segment, and Ad-SVII contains the T antigen gene inserted into the second late leader segment. Together with Ad-SVIII, in which the T antigen gene is inserted into the third late leader, these viruses produce a matched set of hybrid SV40 mRNAs that differ only in the number of adenovirus late leader segments attached to the full-length T antigen coding region. Cells infected with Ad-SVI produce 5- to 10-fold-less T antigen than those infected with Ad-SVII or Ad-SVIII. Ad-SVI-infected cells also contain 5- to 10-fold-less hybrid SV40 mRNA than cells infected with Ad-SVII or Ad-SVIII. Therefore, altering the 5′ late leader structure does not appear to affect the translation of hybrid SV40 mRNAs in adenovirus-infected cells but does influence the efficiency of transcription from the late promoter.

This work extends our previous analysis of the role of the tripartite leader in late mRNA translation (57). In those experiments, one, two, or all three segments of the tripartite leader were attached to T antigen coding sequences by way of a splice that removes 48 nucleotides of the SV40 sequence, including the normal T antigen initiation codon. Translation of these hybrid mRNAs in infected cells is also unaffected by changes in the 5′ leader structure. However, the amount of T antigen translated from these mRNAs is much lower than that transfected from equivalent amounts of Ad-SVIII hybrid SV40 mRNA which contains a nearly complete copy of the tripartite leader joined to the full-length T antigen coding region. These results, taken together with the results reported here, indicate that the 48-nucleotide sequence that includes the normal T antigen initiation codon, not the tripartite leader, is a critical element for optimal translation of T antigen in adenovirus-infected cells.

In contrast, Logan and Shenk (30) constructed hybrid translation units consisting of the adenovirus major late promoter and various portions of the tripartite leader that were derived from a cDNA clone and positioned upstream from the adenovirus E1a coding region. The hybrid transcription units were substituted for the normal E1a region of adenovirus. Equivalent amounts of E1a-encoded mRNA are found in infected cells regardless of the number of late leader segments present at the 5′ end of the hybrid E1a mRNAs. During the late phase of infection, however, a nearly complete tripartite leader was found to enhance the translation of E1a mRNA four-to fivefold relative to similar mRNAs carrying only one or two segments of the tripartite leader.

Berkner and Sharp (4) have obtained similar results by substituting for the adenovirus E1a region a hybrid transcription unit containing the adenovirus major late promoter and one or all three leader segments fused to a mouse dihydrofolate reductase (DHFR) cDNA. Hybrid DHFR mRNA that carries the complete tripartite leader is more efficiently translated than hybrid DHFR mRNA that carries only the first segment of the tripartite leader. However, cells infected with these viruses also produce hybrid adenovirus protein IX mRNAs, which arise by readthrough transcription from the inserted late promoter and splicing into the adjacent early 1b region that encodes protein IX. These hybrid protein IX mRNAs contain either one segment or all three segments of the tripartite leader, depending on the particular virus structure. Nevertheless, these readthrough mRNAs are translated with equal efficiency, much as we observed with hybrid T antigen mRNAs.

We have no data that would reconcile the observed differences in the effect of the tripartite leader on the translation of T antigen and protein IX on one hand and E1a and DHFR on the other. We can only suggest that if the tripartite leader is involved in selective translation of late mRNAs, it alone is not sufficient. Perhaps specific sequences surrounding the translation initiation site play an additional role in influencing translation and, in the case of T antigen and protein IX, are more important than the tripartite leader in determining translational efficiency. Another possibility is that T antigen mRNA carrying two segments of the tripartite leader is already translated so efficiently that it is not possible to increase its utilization in the infected cell by adding additional leader segments.

We were surprised to find that sequences downstream from the late promoter, in the intron between the first and second leader segments, influence the amount of hybrid SV40 mRNA synthesized in recombinant virus-infected cells. Ad-SVI, in which the SV40 sequences are located at +33 relative to the start site of transcription, gives rise to 5 to 10 times less SV40 RNA than Ad-SVI+, Ad-SVII, or Ad-SVIII in which the SV40 insertions are located at +190, +1128, and +3654, respectively. Therefore, the 3′ boundary of a downstream control region lies between +33 and +190. The 5′ boundary has not yet been determined. Measurements of pulse-labeled SV40 RNA in hybrid virus-infected cells showed that removal of these control sequences results in a reduction in transcription initiation from the adenovirus major late promoter.

These 3′ control sequences lie, at least in part, within the intron adjacent to the first late leader segment. Thus, they are missing from all of the hybrid E1a transcription units described by Logan and Shenk (30) that carry various portions of a tripartite leader cDNA fused to the E1a coding region. For this reason, and perhaps also because of the E1a enhancer located upstream from the hybrid transcription units, no differences are detected in the production of hybrid E1a mRNA between viruses that contain only the first segment of the tripartite leader downstream from the inserted late promoter and those that contain additional leader

segments (30). When the activities of the endogenous late promoter and of the late promoter inserted in the E1a region carried on the same virus are compared in a pulse-labeling experiment, however, the former is five times more active than the latter, consistent with our results (J. Logan and T. Shenk, personal communication).

We used a transient expression assay to compare the effects of alterations in 3′ DNA sequences on transcription initiation from the late promoter in infected and uninfected cells. In agreement with previous results (21, 29), no expression of SV40 T antigen under the control of the major late promoter was detected from any of the pAd-SV plasmids after their transfection into HeLa cells. However, by adding the minimal SV40 origin of replication to the pAd-SV plasmids and transfecting them into COS7 cells which allow plasmid replication (17), expression from the plasmid-borne promoters was readily detected. After transfection of adenovirus-infected COS7 cells, we observed five times more SV40 RNA derived from pAd-SVIII-ori than from pAd-SVI-ori. This difference was not observed in mock-infected COS7 cells transfected with the same plasmids. Therefore, the influence of downstream sequences on transcription initiation from the major late promoter appears to be dependent on some *trans*-acting factor encoded or induced by adenovirus.

These results lead us to propose that a factor or factors present in adenovirus-infected COS7 cells influences transcription initiation from the major late promoter and that the action of this factor(s) is dependent on DNA sequences located 3′ to the start site of transcription. The 3′ boundary of these sequences lies between +33 and +190. Further analysis of mutations in sequences 5′ to +33 will be required to establish the 5′ boundary.

The DNA sequences that affect adenovirus late promoter activity in uninfected cells have been extensively analyzed by using transient expression systems and in vitro transcription (10, 11, 21, 23, 38, 62). The conclusions from the analysis of 5′ DNA deletions and point mutations are that both an upstream element and the TATA box are important contributors to late promoter activity. Specific DNA-binding proteins that interact with sequences in the upstream element and the TATA box and that are required for late promoter activity have been identified (7, 48; W. Morgan, personal communication).

The downstream control sequences we describe here appear to function in infected but not in uninfected COS7 cells. Recently, Lewis and Manley (29) have demonstrated a requirement for DNA sequences downstream from the adenovirus late promoter from the cap site to at least position +33 for transcriptional activity in 293 cells or in HeLa cells cotransfected with an E1a-expressing plasmid. These transient expression systems cannot be compared directly with ours because of differences in transfection efficiency, plasmid structure, and plasmid replicaton. If the 3′ boundary of that control element actually extends beyond position +33, it is possible that the control sequences we have defined are part of a single-factor binding site that includes the sequences identified by Lewis and Manley (29).

The proposed factor is clearly either induced or activated in adenovirus-infected COS7 cells. Our results do not implicate any particular protein. One possible candidate is the adenovirus E1a 289-amino acid protein, which is known to *trans*-activate adenovirus early promoters (2, 6, 12, 24, 26, 39, 43). Although the mechanism of E1a *trans*-activation is not known, it is unlikely that this protein binds directly to DNA (13). The E1a protein may induce or activate other specific DNA-binding transcription factors. It is unlikely that the SV40 T antigen present in COS7 cells is the activating factor because its concentration is reduced by adenovirus infection. We cannot exclude the possibility that some other protein induced by T antigen expression in COS7 cells contributes to the activation of the late promoter in these experiments. A combination of in vitro and in vivo approaches can now be used to identify the factor that interacts with the sequences we identified and to determine whether or not the regulation of the adenovirus major late promoter described here is related to E1a or T antigen *trans*-activation.

Regulation of transcription elements located downstream from the RNA start site appears not to be a phenomenon restricted to the adenovirus major late promoter. The regulated pattern of expression of certain cellular genes, such as the immunoglobulin heavy and light chain genes (1, 41), the chicken thymidine kinase gene (37), and the β-globin gene (8), seems to be modulated in part by sequences 3′ to the transcription start site. We expect that upon further examination many other examples of this type of promoter regulation will be uncovered.

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LITERATURE CITED

1. **Banerji, J., L. Olsen, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
2. **Berk, A. J., F. Lee, T. Harrison, T. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus-5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
4. **Berkner, K. L., and P. A. Sharp.** 1985. Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. Nucleic Acids Res. **13**:841–857.
5. **Borrelli, E., R. Hen, and P. Chambon.** 1984. The adenovirus-2 E1a products repress stimulation of transcription by enhancers. Nature (London) **312**:608–612.
6. **Carlock, L. R., and N. C. Jones.** 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1a mRNA species. J. Virol. **40**:657–664.
7. **Carthew, R. W., L. A. Chodosh, and P. A. Sharp.** 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**:439–448.
8. **Charnay, P., R. Treisman, P. Mellon, M. Chao, R. Axel, and T. Maniatis.** 1984. Differences in human α- and β-globin gene expression in mouse erythroleukemia cells: the role of intragenic sequences. Cell **38**:251–263.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5299.
10. **Concino, M., R. A. Goldman, M. H. Caruthers, and R. Weinman.** 1983. Point mutations in the adenovirus major late promoter with different transcriptional efficiencies *in vitro*. J. Biol. Chem. **258**:8493–8496.
11. **Corden, J., B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, and P. Chambon.** 1980. Promoter sequences of eukaryotic protein-coding genes. Science **209**:1406–1413.

12. **Ferguson, B., N. Jones, J. Richter, and M. Rosenberg.** 1984. Adenovirus E1a gene product expressed at high levels in *Escherichia coli* is functional. Science **224**:1343–1346.
13. **Ferguson, B., B. Krippl, O. Andrisani, N. Jones, H. Westphal, and M. Rosenberg.** 1985. E1a 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind DNA. Mol. Cell. Biol. **5**:2653–2661.
14. **Fey, G., J. B. Lewis, T. Grodzicker, and A. Bothwell.** 1979. Characterization of a fused protein specified by the adenovirus type 2-simian virus 40 hybrid Ad2$^+$ND1 dp2. J. Virol. **30**:201–217.
15. **Gingeras, T., D. Sciaky, R. E. Gelinas, J. Bing-Dong, C. E. Yen, M. M. Kelly, P. A. Bullock, B. L. Parsons, K. E. O'Neill, and R. J. Roberts.** 1982. Nucleotide sequences from the adenovirus-2 genome. J. Biol. Chem. **257**:13475–13491.
16. **Ginsberg, H. S., L. J. Bellow, and A. J. Levine.** 1967. Control of biosynthesis of host macromolecules in cells infected with adenovirus, p. 547–572. *In* J. S. Colter and W. Paranchych (ed.), Molecular biology of viruses. Academic Press, Inc., New York.
17. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
18. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **35**:59–72.
19. **Grodzicker, T., C. Anderson, J. Sambrook, and M. B. Matthews.** 1977. The physical locations of structural genes in adenovirus DNA. Virology **80**:111–126.
20. **Grodzicker, T., J. B. Lewis, and C. W. Anderson.** 1976. Conditional lethal mutants of adenovirus type 2-simian virus 40 hybrids. II. Ad2$^+$ND1 host-range mutants that synthesize fragments of the Ad2$^+$ND1 30K protein. J. Virol. **19**:559–571.
21. **Hen, R., P. Sassone-Corsi, J. Corden, M. P. Gaub, and P. Chambon.** 1982. Sequences upstream from the TATA box are required *in vivo* and *in vitro* for efficient transcription from the adenovirus serotype 2 major late promoter. Proc. Natl. Acad. Sci. USA **79**:7132–7136.
22. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
23. **Hu, S.-L., and J. Manley.** 1981. DNA sequences required for initiation of transcription *in vitro* from the major late promoter of adenovirus 2. Proc. Natl. Acad. Sci. USA **78**:820–824.
24. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
25. **Lebkowski, J. S., S. Clancy, and M. P. Calos.** 1985. Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression. Nature (London) **317**:169–171.
26. **Leff, T., R. Elkaim, C. R. Goding, P. Jalinot, P. Sassone-Corsi, M. Perricaudet, C. Kedinger, and P. Chambon.** 1984. Individual products of the adenovirus 12S and 13S E1a mRNAs stimulate viral EIIA and EIII expression at the transcriptional level. Proc. Natl. Acad. Sci. USA **81**:4381–4385.
27. **Levine, A. S., M. J. Levin, M. N. Oxman, and A. M. Lewis, Jr.** 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VII. Characterization of the simian virus 40 RNA species induced by five nondefective hybrid viruses. J. Virol. **11**:672–681.
28. **Lewis, E. D., and J. L. Manley.** 1985. Repression of simian virus 40 early transcription by viral DNA replication in human 293 cells. Nature (London) **317**:172–175.
29. **Lewis, E. D., and J. L. Manley.** 1985. Control of adenovirus late promoter expression in two human cell lines. Mol. Cell. Biol. **5**:2433–2442.
30. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA **81**:3655–3659.
31. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
34. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole cell extract. Proc. Natl. Acad. Sci. USA **77**:3855–3859.
35. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
36. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science **217**:316–324.
37. **Merrill, G. F., S. D. Hauschka, and S. L. McKnight.** 1984. tk enzyme expression in differentiating muscle cells is regulated through an internal segment of the cellular tk gene. Mol. Cell. Biol. **4**:1777–1784.
38. **Miyamoto, N. G., V. Moncollin, M. Wintzerith, R. Hen, J. M. Egly, and P. Chambon.** 1984. Stimulation of *in vitro* transcription by the upstream element of adenovirus-2 major late promoter involves a specific factor. Nucleic Acids Res. **12**: 8779–8799.
39. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the function of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. Nature (London) **295**: 380–384.
40. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209**:1392–1396.
41. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
42. **Rabson, A. S., G. T. O'Conor, K. Berezesky, and F. J. Paul.** 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exp. Biol. Med. **116**:187–190.
43. **Ricciardi, R. P., R. L. Jones, C. L. Cepko, P. A. Sharp, and B. E. Roberts.** 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. USA **78**:6121–6125.
44. **Ribgy, P., M. Rhodes, M. Dieckmann, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
45. **Rio, D. C., and R. Tjian.** 1983. SV40 T antigen binding site mutations that affect autoregulation. Cell **32**:1227–1240.
46. **Russell, W. C., and J. J. Skehel.** 1972. The polypeptides of adenovirus-infected cells. J. Gen. Virol. **15**:45–57.
47. **Sambrook, J., J. Williams, P. A. Sharp, and T. Grodzicker.** 1975. Physical mapping of temperature-sensitive mutants of adenovirus. J. Mol. Biol. **97**:369–390.
48. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
49. **Smale, S. T., and R. Tjian.** 1985. Transcription of herpes simplex virus *tk* sequences under the control of wild-type and mutant human RNA polymerase I promoters. Mol. Cell. Biol. **5**:352–362.
50. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78**:7575–7578.
51. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
52. **Studier, R. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
53. **Thomas, G. P., and M. B. Mathews.** 1980. DNA replication and the early to late transition in adenovirus infection. Cell **22**:523–533.
54. **Thomas, P.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**: 255–266.
55. **Thummel, C. S., T. L. Burgess, and R. Tjian.** 1981. Properties of simian virus 40 small t antigen overproduced in bacteria. J.

Virol. **37**:683–697.
56. **Thummel, C., R. Tjian, and T. Grodzicker.** 1982. Construction of adenovirus expression vectors by site-directed *in vivo* recombination. J. Mol. Appl. Genet. **1**:435–446.
57. **Thummel, C., R. Tjian, S.-H. Hu, and T. Grodzicker.** 1983. Translational control of SV40 T antigen expressed from the adenovirus late promoter. Cell **33**:455–464.
58. **Tjian, R., G. Fey, and A. Graessman.** 1978. Biological activity of purified simian virus 40 T antigen proteins. Proc. Natl. Acad. Sci. USA **72**:1031–1037.
59. **Tooze, J. (ed).** 1981. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
60. **Ucker, D. S., and K. R. Yamamoto.** 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. J. Biol. Chem. **259**:7416–7420.
61. **Velcich, A., and E. Ziff.** 1985. Adenovirus E1A proteins repress transcription from the SV40 early promoter. Cell **40**:705–716.
62. **Yu, Y. T., and J. L. Manley.** 1984. Generation and functional analyses for base-substitution mutants of the adenovirus-2 major late promoter. Nucleic Acids Res. **12**:9309–9321.