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# Analysis of an Activatable Promoter: Sequences in the Simian Virus 40 Late Promoter Required for T-Antigen-Mediated *trans* Activation

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**The late promoter of simian virus 40 (SV40) is activated in *trans* by the viral early gene product, T antigen. We inserted the wild-type late-promoter region, and deletion mutants of it, into chloramphenicol acetyltransferase transient expression vectors to identify promoter sequences which are active in the presence of T antigen. We defined two promoter activities. One activity was mediated by a promoter element within simian virus 40 nucleotides 200 to 270. The activity of this element was detectable only in the presence of an intact, functioning origin of replication and accounted for 25 to 35% of the wild-type late-promoter activity in the presence of T antigen. The other activity was mediated by an element located within a 33-base-pair sequence (simian virus nucleotides 168 to 200) which spans the junction of the 72-base-pair repeats. This element functioned in the absence of both the origin of replication and the T-antigen-binding sites and appeared to be responsible for *trans*-activated gene expression. When inserted into an essentially promoterless plasmid, the 33-base-pair element functioned in an orientation-dependent manner. Under wild-type conditions in the presence of T antigen, the activity of this element accounted for 65 to 75% of the late-promoter activity. The roles of the 33-base-pair element and T antigen in *trans*-activation are discussed.**

Transcription appears to be a key point for control of eucaryotic gene expression. Both the utilization of a transcriptional unit and the rate of its transcription can be controlled in response to environmental, metabolic, or developmental signals. Our present understanding of eucaryotic transcriptional control stems, in large part, from studies of the DNA viruses. One specific system, simian virus 40 (SV40), has repeatedly proven to be a useful model of both viral and cellular gene expression mechanisms. The circular, 5,243-base-pair (bp) SV40 genome is relatively simple, containing only two transcription units which are temporally expressed, early and late, during the lytic cycle (66). The early region is transcribed soon after final uncoating in the cell nucleus. The level of transcription from the early promoter is negatively autoregulated by the early gene product, T antigen (4, 34, 41, 51, 53, 61–63, 65). Conversely, late-region transcription is activated by T antigen (11, 36, 40), and late mRNA accumulates in large quantities in the later phases of the infection, after viral DNA replication (also activated by T antigen) has been initiated.

The promoter for early transcription has been studied in detail. In common with many other RNA polymerase II promoters, it contains a Goldberg-Hogness TATA sequence which directs the site of transcription initiation (5, 6, 24, 57). In addition, efficient transcription from the early promoter requires (i) specific binding of cellular factor(s) to guanine · cytosine-rich sequences within the 21-bp repeated region (see Fig. 1; 19, 20) and (ii) *cis*-activation by the 72-bp enhancer elements (22, 23, 31, 43, 71). Any cellular factor(s) necessary for the expression of this promoter appears to be relatively ubiquitous since the promoter is utilized in a wide variety of cells and species (66).

The SV40 late promoter, in contrast, is not efficiently utilized unless it is activated in *trans* by T antigen (11, 36, 40). Although the late-promoter region overlaps the 72-bp repeated enhancers of the early promoter, these enhancers do not appear to activate the late promoter. In addition, the late promoter has no recognizable TATA sequence, which may explain in part why late transcription initiates heterogeneously at sites between SV40 nucleotides 120 and 325 (see Fig. 1; 13, 15, 25). These dissimilarities between the SV40 early and late promoters suggest that the late promoter is prototypical of an alternate class of RNA polymerase II promoters.

In the present study we have sought to define the sequences within the late-promoter region which are necessary for SV40 late gene expression. Other researchers have addressed this question and have come to various conclusions. Promoter elements (see Fig. 1) have been proposed in the 21-bp repeated region (12, 22, 33, 35, 52), at the *BglI* site within the origin of replication (17), at a site 25 bp upstream of the major late transcriptional start site (12, 13), and within the 72-bp repeat region (21, 22, 23, 36). The different experimental protocols used (i.e., in vivo or in vitro, in the presence or absence of T antigen) may account for these seemingly disparate results. However, such differences point out the necessity of considering that the late promoter may contain multiple promoter elements. We defined two promoter elements which were active in the presence of T antigen. One element contributed ca. 25 to 35% of the late-promoter activity under wild-type conditions and was dependent on the presence of an intact origin of replication for function. The other element, which was necessary for *trans*-activated gene expression, was orientation dependent and remained active in the absence of the origin of replication and the T-antigen-binding sites. This element contributed 65 to 75% of the late-promoter activity under wild-type conditions and may be of general interest in the study of other genes activated in *trans*.

## MATERIALS AND METHODS

**Cells, enzymes, and reagents.** COS cells (26), a gift from Y. Gluzman, were grown in Dulbecco minimal essential medium supplemented with 5 to 10% fetal calf serum (FCS) and

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containing 4.5 g of glucose per liter. CV-1P cells, an established line of African green monkey kidney cells, were grown in Dulbecco minimal essential medium (1 g of glucose per liter) supplemented with 5 to 10% fetal calf serum.

Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were purchased from Bethesda Research Laboratories, New England Biolabs, Inc., or Amersham Corp. and were used as directed by the manufacturer. Purified chloramphenicol acetyltransferase (CAT) was purchased from P-L Biochemicals, Inc. [$^{14}$C]chloramphenicol was purchased from New England Nuclear Corp., and *Sal*I, *Xho*I, and *Hin*dIII linkers were purchased from P-L Biochemicals or New England Biolabs.

**Plasmids.** Plasmids pSV0-cat and pSV2-cat (32) were gifts from C. Gorman. Plasmid p6-1dL was constructed from p6-1 (31) by M. Sadofsky in this laboratory and was used as a source of T antigen in cotransfection experiments. It has a deletion of the SV40 late coding region, SV nucleotides 294 to 2533 (14), but retains the SV40 early region and the early promoter and contains a nonfunctional origin of replication (27). All other plasmids were constructed and isolated by standard recombinant DNA techniques (42) by using *Escherichia coli* HB101 (9, 10). Complete descriptions of the construction and characterization of each plasmid have been described elsewhere (Janis M. Keller, Ph.D. thesis, University of Pennsylvania, Philadelphia, Pa., 1984). Specific sequences in each plasmid are given in the figure legends.

Plasmids which contained the origin region were prepared in both a replicative and a nonreplicative context. The nonreplicative context was achieved by the introduction of a 6-bp deletion at the *Bgl*I site in the origin of replication (27; see Fig. 1).

**Transfection procedure, cell harvest, and CAT assay.** The calcium phosphate precipitation procedure, described previously (40), was used to introduce vector DNA into eucaryotic cells. The cells were generally harvested at 48 h after transfection and were split in two equal portions: one for CAT assay and the second for DNA analysis (described below). When RNA studies were done, a larger plate was used for each transfection (100-mm plates were used, plated with $8 \times 10^5$ cells); half the cells on a plate were extracted for RNA analysis (described below), and the second half were split evenly for CAT and DNA analyses.

The CAT assay method of Gorman et al. (28, 29), as modified and described previously (40), was used. The experimental results are reported as microunits of CAT enzyme activity, where 1 unit catalyzes the acetylation of 1 nmol of chloramphenicol per min at 30°C (pH 7.8). A standard curve of CAT enzyme activity versus percent acetylation of chloramphenicol was generated by using purified bacterial enzyme (P-L Biochemicals) and was found to be linear from 0.5 to 20% acetylation. Extracts were diluted whenever necessary to give assay results within the linear range of the curve. Therefore, a 1,000-fold range in CAT activity could be detected. Low levels of CAT activity are significant because there is no background level CAT activity in eucaryotic cells.

Transfection efficiencies occasionally varied from plate to plate within an experiment. To control for such variation each experiment was repeated several times, each time in duplicate. General trends were obvious in the overall results, and these are reported. In most experiments CAT activities were standardized for the amount of plasmid DNA in cells at the time of harvest (1). Cell counts were also determined from mock-transfectd plates to standardize activities for cell number.

**DNA quantitation and DNA synthesis inhibition studies.** Plasmid DNA was isolated from transfected cells by the procedure of Hirt (37). Plasmid DNA was analyzed and quantitated either by dot blot analysis as described previously (40) or by Southern blot-hybridization analysis (59) after cleavage with appropriate restriction enzymes and separation through agarose gels. Dot blots were quantitated by cutting the dots from the nitrocellulose and counting them in a scintillation counter. Southern blots were quantitated by densitometry.

Plasmid DNA replication was determined by restriction analysis by using the methylation-sensitive enzyme *Mbo*I. In studies requiring the inhibition of plasmid DNA synthesis by cytosine arabinoside (Ara C), plates of CV-1P cells were transfected in duplicate as described with p6-1dL to supply T antigen. After the glycerol shock the cells were fed with Dulbecco minimal essential medium containing 10% fetal calf serum, either with or without 20 μg of Ara C per ml. This Ara C concentration had been previously determined to be inhibitory to plasmid DNA replication but not lethal to cells during the course of the transfection (24 h). The normal period of 48 h was not used because some cell death could be detected by this time.

**RNA analysis.** Total RNA was extracted from cells 48 h after transfection by the method of Villarreal (69). The RNA preparations were freed of DNA by treatment for 45 min at 37°C with RNase-free DNase prepared by described methods (68). RNA blotting was performed as previously described (2, 64). Blots were pretreated and hybridized with a probe specific for CAT RNA, labeled in vitro with T4 DNA polymerase. Band intensities were quantitated by densitometry.

Primer extension was carried out by the method of Ghosh et al. (25). The primer (see Fig. 7) was labeled with $^{32}$P in vitro by using T4 DNA polymerase. Hybridization for primer extension analysis was carried out in 80% formamide conditions at 44.5°C for 13 h. After ethanol precipitation, the samples were incubated with 40 U of reverse transcriptase (Bethesda Research Laboratories) for 45 min at 44°C. Samples were electrophoresed on a 5% acrylamide-bisacrylamide (40:1) gel containing 7 M urea. The primer extension analysis is presented only as a qualitative analysis of 5′-end utilization by the various promoter deletion mutants. Quantitative comparisons of the primer extension data are not valid.

## RESULTS

**Description of the system.** The plasmids used for the transient expression studies contained the CAT gene from the pSV0-CAT vector (28), under the control of the SV40 late promoter. The primary late-promoter plasmid for all the studies with pL16 (in this paper, plasmids will be referred to without the cat suffix; e.g., pL16 is pL16-CAT) (Fig. 1). In pL16, the CAT gene is controlled by a 405-bp fragment of SV40 DNA (nucleotides 5171 to 333, through the origin of replication; Fig. 1) which contains an intact origin of replication, the 72- and 21-base-pair repeats, the T-antigen-binding sites, and the major late transcriptional start site (nucleotide 325). The SV40 fragment is oriented and positioned in pL16 such that the CAT gene translational initiation codon is 54 bp downstream of the SV40 major late transcriptional start site. In the presence of SV40 T antigen, pL16 can replicate in monkey cells; this is referred to as the replicative context. The plasmid pL16n contains the same SV40 fragment as does pL16, but it has a 6-bp deletion at the origin of

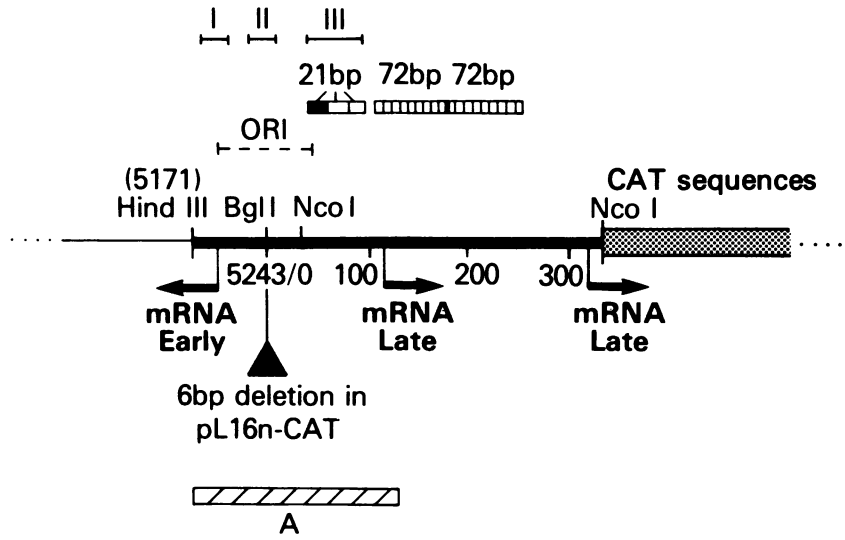


FIG. 1. Linear map of the SV40 promoter region in the plasmids pL16 and pL16n. The major features of the 400-bp SV40 control region (from nucleotides 5171 to 333) are shown. The numbers below the map refer to SV40 nucleotides, using the SV numbering system (14). The positions of important restriction sites, the 21- and 72-bp-repeat regions, the origin of replication (*ori*), and the three T-antigen-binding sites (indicated by I, II, and III) are shown above the map. Early and late mRNA initiation sites are indicated by arrows under the map. The location of the 6-bp deletion in pL16n is indicated by the solid triangle. The box marked "A" indicates the region of pL16 shown in Fig. 2.

replication (Fig. 1) which renders this plasmid nonreplicative (27, 40); all plasmids containing this defective origin are denoted by the addition of an "n" to their names or are referred to as the nonreplicative context. Deletions were constructed in pL16 and pL16n to test for late-promoter elements active in the presence of T antigen.

The comparison of deletions within the promoter region in both the replicative and nonreplicative contexts allowed us to differentiate true promoter effects from effects which might be caused by differences in replication efficiencies caused by the deletions. However, it should be pointed out that all of the mutants shown below (see Fig. 4 and 5), (except pL7, pT1h, and pT11), amplify with equal efficiency (data not shown). This agrees with previous results (7, 22), which demonstrate that the SV40 enhancers and the 21-bp repeats are not essential for replication from the SV40 origin.

Plasmids to be tested were introduced as supercoiled DNA into COS cells, which produce T antigen endogenously, or CV-1P cells. In CV-1P cells T antigen was provided by cotransfection with p6-1dl, a plasmid which encodes the SV40 early region. Similar results were generated by either procedure for supplying T antigen, indicating that the utilization of COS cells did not introduce unknown variables.

**Deletion of T-antigen-binding sites.** Starting with the parent plasmid, pL16, deletions were made in the T-antigen-binding sites (Fig. 1 and 2). It is predicted that if T antigen must bind to a given site on the DNA to activate transcription, then a plasmid lacking that site would give only basal levels of CAT activity regardless of the presence or absence of T antigen. This basal level would be comparable to the activity generated by pL16 or pL16n in the absence of T antigen (i.e., 50 to 60 μU of CAT activity per $10^5$ cells; Fig. 2, inset). The results shown in Fig. 2 indicate that all three binding sites could be removed without causing CAT activity to drop to the basal level. Binding site I was deleted in plasmid pT1c, and binding site III was deleted in plasmid pL533. In the presence of T antigen, both plasmids gave levels of CAT activity (5,000 and 6.070 μU of CAT activity per $10^5$ cells, respectively) which were comparable to the activity obtained with pL16 (8,000 μU of CAT activity per $10^5$ cells; Fig. 2, inset). All deletions which remove sequences between SV40 nucleotides 5225 and 25 (including all of binding site II) adversely affect the function of the viral origin of replication (7; personal observation). Plasmids pL551 through pL554 and pT1d through pT11 had deletions in this region, and all gave CAT activities (250 to 800 μU of CAT activity per $10^5$ cells) which were comparable to the level given by the parent nonreplicative plasmid, pL16n (740 μU of CAT activity per $10^5$ cells). CAT expression from all of these plasmids, including plasmid pT11 (419 μU of CAT activity per $10^5$ cells), which had all three T-antigen-binding sites removed, was activated in the presence of T antigen to levels well above the basal activity. Thus, plasmids lacking the origin-binding sites for T antigen maintained the ability to be *trans*-activated by T antigen.

**CAT activity when DNA replication is inhibited.** The above data indicate that there was little difference in T-antigen-mediated late gene expression whether 6 bp at the origin of replication were deleted, as in pL16n, or all three T-antigen-binding sites were removed, as in pT11. However, the T-antigen-activated level of CAT activity for pL16n was 10-fold less than for its replicative counterpart, pL16. There are two possible explanations for this difference: (i) the gene dosage in transfections with each plasmid were at least 10-fold different at the time of harvest, since pL16 can replicate in the presence of T antigen and pL16n cannot (40), or (ii) the deletion of only 6 bp at the origin, as in pL16n, was sufficient to cause the same effect as deleting all of the binding sites. The former explanation argues that interaction of T antigen with the origin-binding sites contributes to late gene expression by increasing the gene copy number, but is not the source of T-antigen-mediated *trans*-activation. The latter explanation argues that the origin region contains one of the elements involved in T-antigen-mediated *trans*-activation. These two possibilities can be distinguished by determining the level of T-antigen *trans*-activated gene expression with pL16 and pL16n under conditions where DNA synthesis is inhibited by Ara C. If the activities of pL16 and pL16n

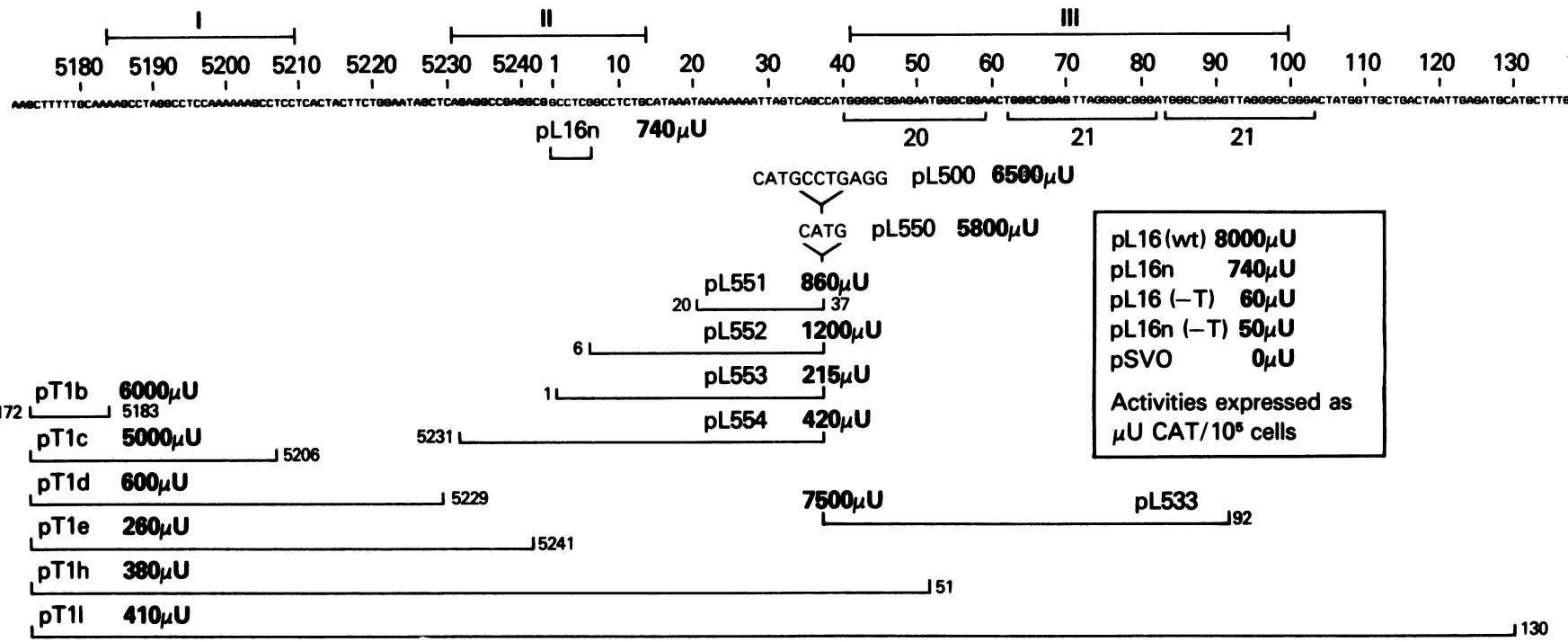


FIG. 2. Analysis of the affect of T-antigen-binding site deletions on late-promoter activation. An expansion of the region from pL16 marked "A" in Fig. 1 (SV40 nucleotides 5171 to 140) is shown. The three T-antigen-binding sites and the 21-bp repeats are indicated. The names of plasmids, their deleted regions (solid lines), and activities are given. The activities were assayed in COS cells and are given in microunits of CAT activity per $10^5$ cells. For comparison, the induced activities of pL16 and pL16n in COS cells (T antigen present) and the uninduced activities in CV-1P cells (−T, T antigen absent) are given in the inset. The activity of the promoterless plasmid, pSV0-cat (28), in COS cells is also given.

| Plasmid | T ag | μU CAT/10⁵ Cells | |
|---|---|---|---|
| | | -Ara C | +Ara C |
| pL16 | – | <10 | <10 |
| pL16 | + | 5869 (750) | 500 |
| pL16n | – | <10 | <10 |
| pL16n | + | 571 | 574 |

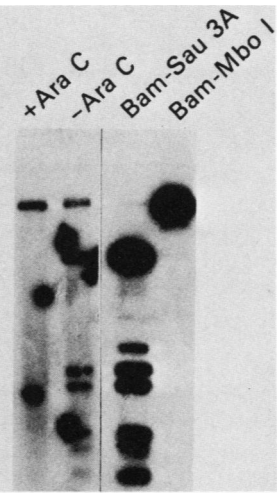


FIG. 3. CAT activity when DNA replication is inhibited. Plasmids pL16 and pL16n were transfected into CV-1P cells along with a T-antigen-supplying plasmid, p6-1dL (+) or an inactive filler plasmid, pSV0 (–). After transfection, half the plates were fed with medium containing 20 μg of Ara C per ml. All the plates were harvested and assayed for CAT at 24 h posttransfection. The gel shows Southern-blotted plasmid DNA. The two lanes on the left show plasmid DNA which was extracted from cells cotransfected with pL16 and p6-1dL in the presence or absence of Ara C and then digested with *Bam*HI and the methylation-sensitive endonuclease *Mbo*I. The two lanes on the right show pL16 DNA which was purified from *E. coli* and cleaved with *Bam*HI and either *Mbo*I or its methylation-insensitive isoschizomer, *Sau*3A.

were equivalent under these conditions, the former possibility would be supported. If the activity of pL16 remained greater than pL16n, the latter possibility would be supported. The results of this study are shown in Fig. 3. Replication was determined by cleavage with the methylation-sensitive restriction endonuclease *Mbo*I. In addition, the plasmids were cleaved with *Bam*HI to linearize nonreplicated plasmids. The Southern blot analysis of the DNA clearly showed that the replicative plasmid grown in the absence of Ara C was cleaved by *Mbo*I, the bands being comparable to those generated by purified pL16 cleaved with *Bam*HI and the methylation-insensitive endonuclease *Sau*3A. In contrast, the plasmid DNA isolated from the Ara C-treated cells was cleaved by *Bam*HI to form a linear plasmid but was not cleaved by *Mbo*I, indicating that the methylated plasmid DNA did not replicate in the treated cells. Comparison of the CAT activities indicated that T-antigens *trans*-activated expression with pL16, in the presence of Ara C, was equivalent to the *trans*-activated expression of pL16n either in the presence or absence of Ara C. However, when normalized to equivalent gene copy number (Fig. 3 [750 μU]), expression of pL16 under replicative conditions was 28 to 33% higher than the activities detected due to *trans*-activation without replication (i.e., 750 μU compared with 500 or 571 μU).

Overall the results shown in Fig. 2 and 3 indicate two sources of T-antigen-mediated late-promoter activity: (i) an activity which can function in the absence of both replication and an intact origin region and which accounts for ca. 65 to 75% of the total activity on a per genome basis and (ii) an activity which is dependent on replication and accounts for 25 to 35% of the total activity on a per genome basis. These promoter elements are defined below.

**Promoter deletional analysis.** To define the promoter elements indicated by the above experiment, we made deletions within pL16 (replicative context) and pL16n (nonreplicative context) at locations in the SV40 control region outside the origin region. The activities of these deletions and other pertinent deletions were determined in the presence of T antigen and are shown in Fig. 4. In the replicative context, the presence of T antigen produces maximal levels of CAT gene expression since replication, and thus genome amplification, can occur. Each plasmid in the replicative context (except pT1h, pT11, and pL7, which delete the origin region) can amplify with equal efficiency (7, 22; data not shown). In the nonreplicative context measured activities reflect promoter activity which is independent of amplification and replication. For all plasmids shown, the relative levels of CAT RNA, determined by Northern blotting (data not shown), corresponded with the relative levels of CAT enzyme activity; thus, CAT enzyme levels are an accurate indication of promoter activity.

The activity of each deletion was compared to the corresponding activity of pL16 in the replicative or nonreplicative contexts. The most interesting result was noted in the comparative activities of the series pL500 through pL563. In this set relatively high activities were maintained in both the replicative and nonreplicative contexts when deletions were made of the 21-bp-repeat region (pL533) as well as of sequences to a point 11 nucleotides before the junction of the 72-bp repeats (pL540). However, continuing the deletions 33 bp further (pL561), through the junction of the 72-bp repeats, caused a 62 to 68% drop in activity in the replicative context and a complete loss of activity in the nonreplicative context. Continuing the deletion to a point beyond the end of the 72-bp repeat (pL563) abolished activity in the replicative context. As in the previous experiment, these data indicate two promoter elements. One element, within SV40 nucleotides 200 to 270 (the difference between pL561 and pL563), was only detected when the origin was intact, i.e., the replicative context. The other element was located within the 33 nucleotides spanning the junction of the 72-bp repeats, SV40 nucleotides 168 to 200 (the difference between pL540 and pL561), which imparted activity in the nonreplicative context and also accounted for at least 65% of the activity in the replicative context.

The data shown in Fig. 4 suggest other interesting points. Plasmids pL163 and pL169 contained the region of the 21-bp

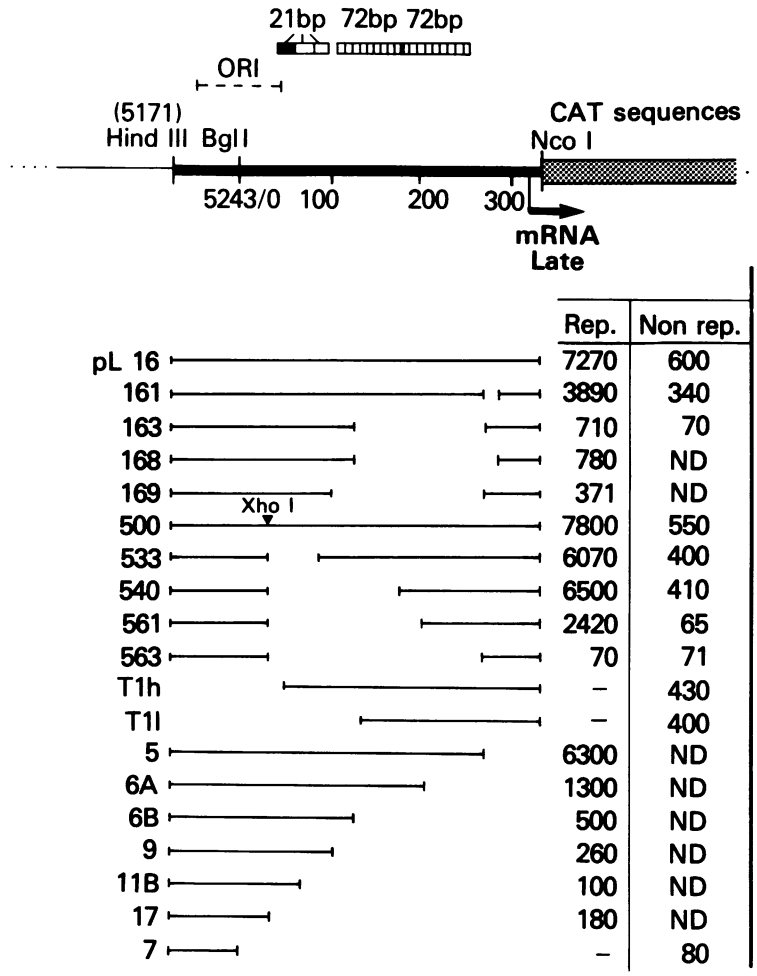


FIG. 4. Deletional analysis of the SV40 late-promoter region. The plasmids used in the deletional analysis of the SV40 late-promoter region are listed to the left of the figure, followed by maps of the deleted regions (indicated by open spaces) in each plasmid. CAT activity is given in microunits of CAT activity per $10^5$ cells. The replicative (Rep) and nonreplicative (Non rep.) contexts are explained in the text. The regions included in each construct are given below in SV nucleotide numbers. Deletions are indicated by an asterisk, and sites of inserted *Xho*I linkers are indicated by an X. Shown are: pL16, nucleotides 5171 to 333; pL161, nucleotides 5171 to 272*298 to 333; pL163, nucleotides 5171 to 132*272 to 333; pL168, nucleotides 5171 to 132*298 to 333; pL169, nucleotides 5171 to 108*272 to 333; pL500, nucleotides 5171 to 41X37 to 333; pL533, nucleotides 5171 to 41X*92 to 333; pL540, nucleotides 5171 to 41X*167 to 333; pL561, nucleotides 5171 to 41X*204 to 333; pL563, nucleotides 5171 to 41X*272 to 333; pLT1h and pLT1d are described in Fig. 2; pL5, nucleotides 5171 to 272; pL6A, nucleotides 5171 to 204; pL6B, nucleotides 5171 to 132; pL9, nucleotides 5171 to 108; pL11B, nucleotides 5171 to 72; pL17, nucleotides 5171 to 37; pL7, nucleotides 5171 to 1.

repeats but had deleted sequences within the 72-bp repeats. Whereas pL169 contained no 72-bp-repeat sequences, pL163 contained the first 22 bp of one 72-bp repeat. These sequences are the same as the last 22 bp of the 33-bp element defined above by PL540 and pL561. However, it was noted that pL163 and 169 had relatively low activity in the replicative context, and pL163 had basal activity in the nonreplicative context. These data indicate either a repressive effect of the 21-bp-repeat region or that the last 22 bp of the 33-bp element were not sufficient to mediate the activity indicated by the comparison of pL540 and pL561. At this point we cannot differentiate between these possibilities.

Plasmid pL161 was notable because it deleted a small region beyond the 72-bp repeats which resulted in a 50% reduction in activity. We did not consider this region further in this study; however, Brady et al. (12) have suggested that a sequence with functions analogously to a TATA box may be located in this position.

Plasmids pL5, pL6A, pL6B, pL9, pL11B, pL17, and pL7 do not contain the major late transcriptional initiation site, but have the CAT gene placed within the late-promoter region. The levels of CAT activity obtained from these plasmids generally reflected levels obtained when similar deletions were made within the promoter region in plasmids which retain the major mRNA start site and the sequences immediately upstream; for example, compare pL6B and pL163, pL5 and pL16, pL6B, and pL163. These results indicate that in many cases the deletion of the major late mRNA start site at nucleotide 325 had little effect on T-antigen-activated gene expression.

**Unidirectional promoter element within the 72-bp-repeat region.** The results shown in Fig. 4 indicate that a 33-bp sequence between SV40 nucleotides 168 and 200 was necessary for late promoter *trans*-activation by T antigen. The activity of this sequence was tested by inserting it into promoterless plasmids (Fig. 5). Some data shown in Fig. 4 is repeated in Fig. 5 for comparison. Plasmids pL563 and pL568 contain the origin of replication (nucleotides 5171 to

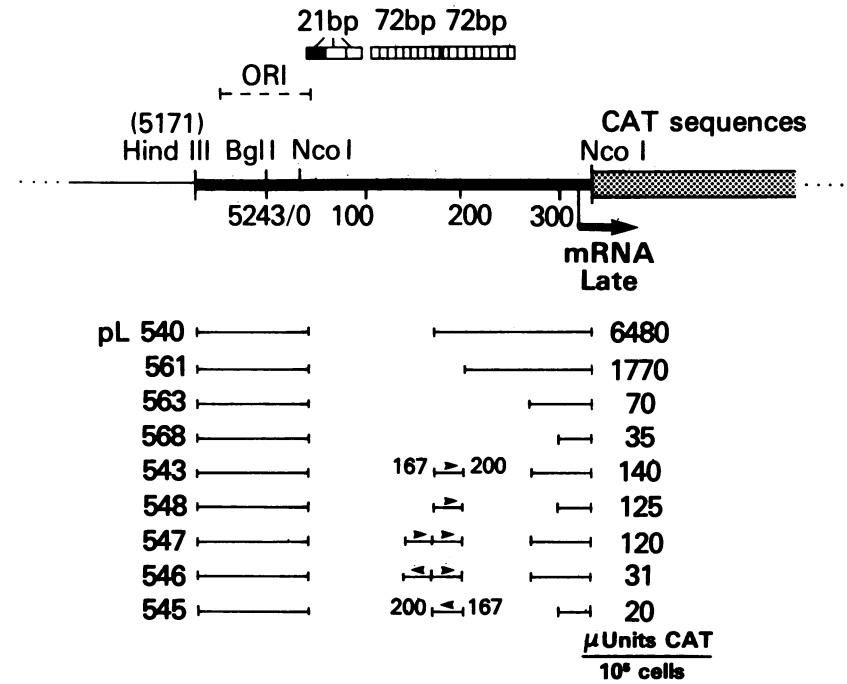


FIG. 5. Analysis of the CAT activity generated by a 33-bp late-promoter element. Solid lines indicate regions included in the plasmids. CAT activity was determined by using COS cells and is presented in microunits of CAT activity per $10^5$ cells. The arrowheads above the 33-bp fragment indicate the 5'-3' orientation of the inserted DNA. The regions included in each construct are denoted as described in the legend to Fig. 4.: pL540, nucleotides 5171 to 41X*167 to 333; pL561, nucleotides 5171 to 41X*204 to 333; pL563, nucleotides 5171 to 41X*272 to 333; pL568, nucleotides 5171 to 41X*298 to 333. The end points of the inserted fragments are given in the figure.

37) and the sequences surrounding the major late transcriptional initiation site (nucleotides 272 to 333 in pL563 or nucleotides 298 to 333 in pL568). These plasmids lack all of the major elements of the late promoter; thus, their activities were basal. Plasmids pL543 and pL548 have the 33-bp fragment inserted into pL563 and pL568, respectively, in the late orientation (orientation is indicated by the arrowheads in Fig. 5). This resulted in a two- to fourfold increase in CAT expression. Though small, this was a repeatable effect mediated by the 33-bp fragment in the absence of its normal surrounding sequences. The relative change in promoter activity elicited by the presence of this fragment (two to fourfold) was the same whether the remainder of the 72-bp-repeat region was retained in the plasmid or was deleted (e.g., there was a threefold difference between the activities of pL540 and pL561; likewise, there was a fourfold difference between the activities of pL548 and pL568). Tandem duplication of this fragment, as in pL547, caused no additional increase in CAT activity.

In plasmid pL545 the 33-bp fragment was inserted in the reverse orientation. It was clear from the low level of activity that the promoter element operated in an orientation-specific manner. To confirm and expand this result, we constructed plasmid pL546, which had a tandem tail-to-tail duplication of the 33-bp fragment. The activity of pL546 was equivalent to or less than the level produced by pL563 despite the presence of one copy of the 33-bp fragment in the correct orientation for late-promoter activity. These results again indicate that the 33-bp sequence contained a promoter element which operated in a unidirectional manner and which may be inhibitory to upstream initiation if present in the opposite orientation.

**RNA studies.** We stated above that among the plasmids tested the relative CAT activities and relative CAT mRNA levels, as determined by Northern analysis, varied in a proportional manner. The experiments presented in this section used primer extension analysis to determine the fidelity of 5'-end utilization in a number of representative plasmids. These results were compared to the known 5'-end utilization of the virus. As noted above, the primer extension analysis is presented as a qualitative analysis only. Transcription from the SV40 late region initiates at a number of locations (15, 25, 32). The major initiation site is at nucleotide 325, with minor sites at nucleotides 295, 264, 192, and 120. These viral initiation sites are indicated by solid triangles on the top map in Fig. 6. In addition, very minor species of RNA were found with 5' ends throughout the region from nucleotides 270 to 320; a few of the more significant species in this class are shown by open triangles on the top map in Fig. 6.

The primer which was used for primer extension analysis of plasmid-derived RNA was a 158-bp fragment extending from the CAT-SV40 junction to a *Pvu*II site within the CAT coding sequence (Fig. 6). The results of a typical primer extension analysis are shown in Fig. 7. Bands indicating discrete extension products were seen for each plasmid and demonstrate that transcription was initiated within the SV40 control sequence 5' to the CAT-SV40 junction. Nuclease S1 analysis of these same RNA samples confirmed that 5' ends within the CAT sequence were rare and that the ends mapped by primer extension were not due to strong stops for reverse transcriptase (data not shown). The SV40 nucleotides to which the major bands mapped for pL16 are indicated to the right of the gel in Fig. 7. The positions of the 5' ends which are represented by bands in Fig. 7 are indicated by solid triangles in the lower part of Fig. 6, taking into account deletion size. We found that the major and minor mRNA initiation sites used for authentic viral late

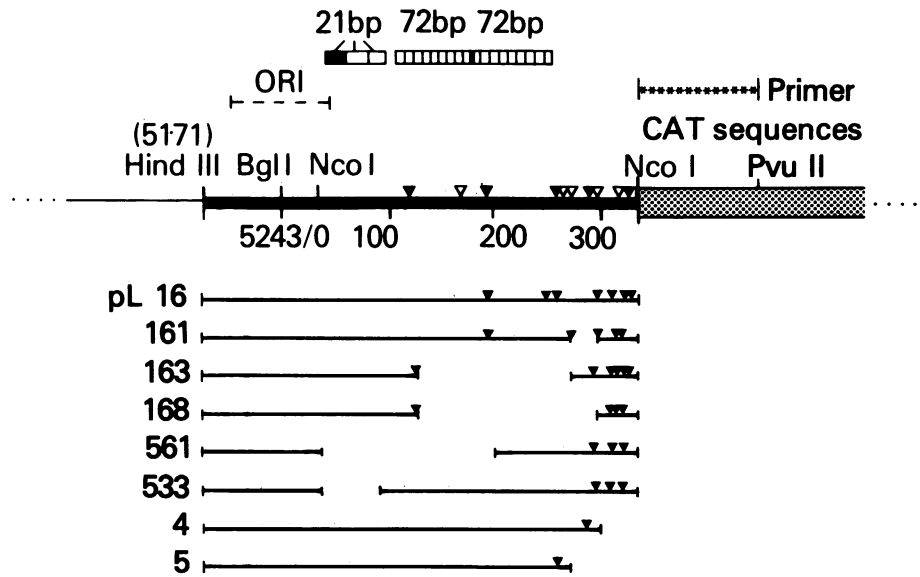


FIG. 6. Map positions of the 5′ ends of RNA transcribed from CAT plasmids. The map positions of the major 5′ end in SV40 late mRNA (nucleotide 325) and the minor sites at nucleotides 120, 192, 264, and 294 as determined for viral RNA are indicated by solid triangles on the linear map of the SV40 control region in pL16 (at the top of the figure). The open triangles indicate the positions of very minor transcriptional initiation sites which are also found in wild-type viral RNA (14, 15, 25). The primer which was used to study 5′-end utilization is shown. In the lower portion of the figure the positions of the 5′ ends which were detected by primer extension analysis (shown in Fig. 7) are indicated by solid triangles on the maps of the plasmids from which RNAs were derived.

transcription were used in our plasmids. However, shifts in the extent of utilization of these sites were seen. With plasmids pL16 and pL163 we found that the wild-type initiation site at nucleotide 325 was utilized (Fig. 7, band 1). The minor wild-type site at nucleotide 295 was also used in both plasmids, and sites at nucleotides 264 and 192 were used in pL16. In all the plasmids tested there was a major start site at ca. 13 bases upstream of the SV40-CAT sequence junction (Fig. 7, band 2). In plasmids which have the CAT gene starting at SV40 nucleotide 333 (pL16, pL161, pL163, pL168, pL533, and pL561), the start site indicated by band 2 mapped to a very minor SV40 initiation site at nucleotide 320. For pL4 and pL5 the start site indicated by band 2 mapped to minor SV40 initiation sites at nucleotides 284 and 260, respectively.

The accentuated use of minor start sites in our plasmids was predictable. Piatak et al. (47, 48) have found that mutations within the SV40 late leader sequence cause upstream shifts in the localization of the most abundantly utilized 5′ ends. Placement of the CAT gene at SV40 nucleotide 333 essentially eliminated all of the leader sequence and evidently caused similar shifts in initiation site utilization to occur in the plasmids. Upstream mutations (i.e., deletions of nucleotides 25 to 30 bases upstream of the start site at nucleotide 325) have also been shown to cause shifts in utilization of the major mRNA initiation sites in a 5′ direction (13). Our results are consistent with these findings. Plasmids pL161 and pL168, which had deletions starting at 27 bases upstream from the major mRNA initiation site, did not have bands corresponding to a 5′ end at the major start site (Fig. 7, band 1), but utilized minor sites instead.

Overall, our primer extension results together with S1 nuclease results (data not shown) indicate that transcription from our plasmids initiated from major and minor transcriptional initiation sites known to be utilized in authentic viral late transcription. Shifts in the emphasis of utilization of 5′ ends were found due to the nature of our constructions, but these shifts were predicted from studies of viable SV40 deletion mutants (13, 32, 47, 48).

## DISCUSSION

The mechanisms which control the early and late promoters of SV40 emphasize contrasting means to accomplish gene expression. The early promoter is immediately active through the utilization of *cis*-acting enhancer elements, whereas late gene expression is relatively silent until the late promoter is activated, in *trans*, by the early gene product, T antigen (6, 11, 40). For the virus, this sequence of events insures that an efficient temporal expression of gene products is maintained. T antigen and other viral *trans*-activating proteins, e.g., adenovirus E1A and herpesvirus immediate-early proteins, are quite efficient at activating their homologous viral promoters (8, 18, 39, 40, 44, 49, 70) and have been shown to activate cellular gene expression as well (1, 30, 38, 45, 55, 56, 58, 67). Such results suggest that (i) under normal conditions, cellular gene expression may involve *trans*-activation mechanisms, and (ii) expression of the viral *trans*-activator proteins, or the aberrant expression of putative cellular *trans*-activator proteins, may result in deviant *trans*-activation of cellular gene expression. Thus, it is of general interest to define the elements of promoters which respond to *trans*-activation.

Using the CAT transient expression system and deletion analysis, we identified elements of the SV40 late-promoter region (SV40 nucleotides 5171 to 5243 and 1 to 333; Fig. 1 and 8) needed for T-antigen-mediated *trans*-activation. As mentioned above, many previous works (12, 13, 17, 21–23, 33, 35, 36, 54) have examined the structure of the late promoter. These experiments utilized varied experimental approaches and indicated late-promoter elements within the origin of replication, the 21-bp repeats, the 72-bp repeats, and other regions. Examination with reference to the present data makes clear that these previous studies characterized various aspects of the promoter elements described here,

each aspect being emphasized by the specific experimental approach used. We acknowledge these studies; however, we will not attempt to discuss each result in comparison to the present data.

Our deletion mutants were tested in both a replicative and a nonreplicative context in the presence of T antigen. Thus, effects of replication could be differentiated from *trans*-activation or the action of promoter elements which might coincide with the origin of replication. The overall results suggest that the late promoter is a complex structure composed of overlapping elements which define at least two promoter activities. Figure 8 diagrams the elements we have located. One of the promoter activities was mediated by sequences between SV nucleotides 200 and 270 (element III). This activity required an intact origin region (element I) and replication to be detected. Our quantitative data suggest that the activity mediated by the element I-III combination contributed ca. 25 to 35% of the late-promoter activity under wild-type conditions in the presence of T antigen. A second T-antigen-dependent promoter activity was mediated by sequences within SV nucleotides 168 to 200 (element A). This promoter element could function independently of the origin and the T-antigen-binding sites; additionally, our data indicate that element A was highly orientation dependent. Under wild-type conditions in the presence of T antigen, the presence of element A contributed 65 to 75% of the late-promoter activity. In Fig. 8 the region of the 21-bp repeats is noted as element II. As indicated by the data of Fig. 4, this region had an effect on the activity of the late promoter in the replicative context when assayed in the absence of elements III and A (e.g., pL163 and pL169). However, the nature and significance of this effect remains to be determined.

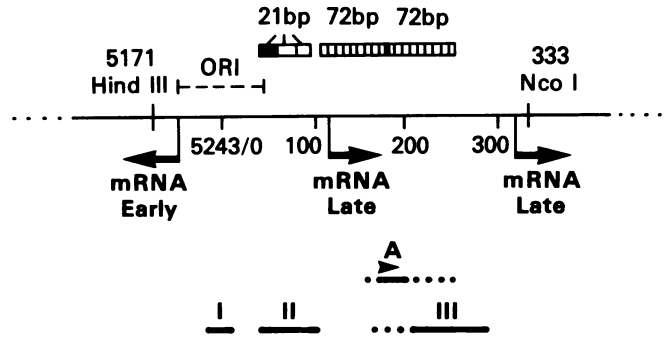


FIG. 8. Elements of the SV40 late promoter. The diagram indicates the elements of the late promoter as indicated by the data presented. Element A (SV40 nucleotides 168 to 200) is the element necessary for *trans*-activation and is origin independent. The arrowhead indicates the orientation dependence of element A. Element I is the origin region centering on the *BglI* site. Element II covers the 21-bp repeat region. Element III denotes the region which is an active promoter element in the presence of T antigen when the origin (element I) is intact. See the text for details.

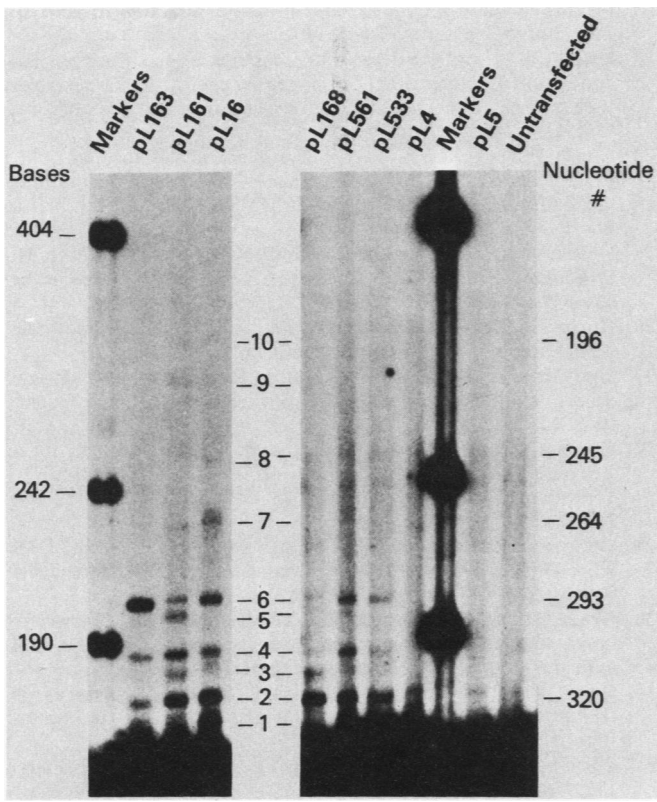


FIG. 7. Qualitative primer extension analysis of 5′ ends of plasmid-derived RNA. The primer for primer extension analysis was a 158-bp fragment of CAT DNA (shown in Fig. 6) from the *SalI* restriction site at the 5′ end of the CAT sequence (marked as *NcoI*, the original SV40 site at this location) to the *PvuII* restriction site within the gene. Ten micrograms of total cell RNA from transfected cells was assayed. The markers are 5′-end-labeled *HpaII* fragments of pSV2-cat DNA (28). Nucleotide # refers to SV40 nucleotide numbers for the SV40 fragment within pL16 (see Fig. 1).

The observation of two SV40 late-promoter activities is not unique to this promoter. Both the herpes simplex virus immediate-early genes (49, 50, 65) and the mouse mammary tumor virus long terminal repeats (16) have relatively efficient minimal promoters, as well as distinct promoter elements which are activated in *trans* by herpes virion proteins and glucocordicoid hormones, respectively. At this point we believe that the activity mediated by the element I-III combination is specific for the late promoter, since it appeared to require the unique interaction of T antigen with the origin-binding sites. In contrast, the activity mediated by element A did not depend on the specific origin sites; therefore, this element may be indicative of a more general type of promoter element activated in *trans*. It has been suggested previously that SV40 late gene expression requires a cellular factor either induced by or working in conjunction with T antigen (3, 46). More recently two observations suggest that *trans*-activation may be mediated directly by a cellular factor rather than T antigen. First, in vitro analysis of SV40 late mRNA transcription complexes show that these complexes do not contain T antigen (60). Second, T antigen appears to be a promiscuous activator of nonviral genes (1). These observations can best be explained by the assumption that T antigen induces a cellular factor (or a small number of factors) which directly mediate the activation of many gene promoters, including the viral late

```
CTTTCCACACC TGGTTGCTGACTAATTGAGATG   SV40 Element A
            TAAT-GAGATa              HSV IE
```

FIG. 9. *Trans*-activable element A of the SV40 late promoter. The underlined sequences denote the region of the enhancer core as defined for early-promoter activity (71). The space denotes the junction of the 72-bp-repeat enhancer regions. The herpes simplex virus (HSV) sequences, defined for activity of the immediate-early (IE) gene (50), are described in the text.

promoter, by utilization of element A. Thus, element A may be indicative of the structure of *trans*-activable elements within promoters. In this regard, Fig. 9 shows an interesting homology region comparing element A sequences with sequences which have been shown to be necessary for the virion protein-mediated activation of a herpesvirus immediate-early gene (50). Examination of several promoters which are activated by T antigen in transient expression analysis (1) reveal limited homologies with this TAAT(T)GAGAT sequence (J. C. Alwine, personal observation); however, it is premature to consider this a generalized *trans*-activable element.

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