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Control of Adenovirus Late Promoter Expression in Two Human Cell Lines

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We investigated the nucleotide sequence requirements of the adenovirus 2 late promoter when activated by either a *trans*-acting regulatory protein or a *cis*-acting enhancer element. Using deletion mutants in transient expression assays, we determined that the 5′ limit of the region required for activation by a *trans*-acting regulatory protein, the adenovirus early region 1a gene product, and the simian virus 40 enhancer is the same in both 293 and HeLa cells. Surprisingly, the 3′ limit of required sequences varied, depending on the mechanism of activation. Activation mediated by the early region 1a protein endogenous in 293 cells or produced after cotransfection of HeLa cells requires the region around the transcriptional start site, whereas activation brought about by an enhancer element in HeLa cells has no requirement for these sequences. Under no conditions tested did the simian virus 40 enhancer activate the late promoter in 293 cells, even when sequences sufficient for enhancer-mediated activation in HeLa cells, but not for early region 1a activation, were present. These results suggest the existence of at least two different mechanisms for positive regulation of promoter activity.

A major factor in gene regulation is differential promoter activation. Gene expression can be controlled at the level of transcription initiation by a number of mechanisms, all of which require either *cis*- or *trans*-acting factors, or both. Transcriptional enhancers are an example of *cis*-acting regulatory squences. (see references 18 and 35, for reviews.) The prototype for this class of controlling element, the simian virus 40 (SV40) 72-base-pair (bp) repeated sequence, was first identified as a far upstream component of the early promoter of this virus (4, 23). However, the so-called 72-bp repeats were soon shown to have unprecedented properties. When DNA fragments containing these sequences were excised from viral DNA and joined to recombinant molecules containing various heterologous genes, it was found that expression of the linked gene was dramatically enhanced, in a manner essentially independent of the position and orientation of the viral DNA fragment (3, 49).

Enhancers have since been identified in a number of viral genomes, including polyomavirus (13), retroviral long terminal repeats (20, 37), adenovirus (26, 31) and bovine papillomavirus (41). More recently, controlling elements that behave like enhancers have been found in or near certain cellular genes, including immunoglobulins (2, 17, 51) and insulin and chymotrypsin (62). The enhancers described to date show various degrees of specificity, ranging from a slight species specificity in the case of the papovavirus and retroviral enhancers (37) to an almost absolute cell-type specificity in the case of the immunoglobulin enhancers, which are functional only in lymphoid cells.

Enhancers in turn interact with factors in the cell nucleus (55). Such *trans*-acting factors constitute a second category of transcriptional regulators. One of the best studied is the SV40 T antigen, which stimulates DNA replication of the virus (11, 57), negatively regulates its own transcription (53, 58), and apparently positively regulates viral late transcription (8, 34). Other examples of *trans*-acting regulators include the glucocorticoid receptor protein, which interacts with the mouse mammary tumor virus long terminal repeat (10, 54), the heat shock activator protein (64), and the pseudorabies immediate-early gene product, which activates its own promoters (30) as well as the human β-globin promoter (22).

The adenoviral genome encodes a *trans*-acting factor in early region 1a (E1a). A protein product (hereafter referred to as the E1a protein) of this transcription unit has been shown to activate a variety of different promoters, including all other early adenoviral promoters (6, 32). Green et al. (22) have shown that the E1a protein produced constitutively in 293 cells or transiently in HeLa cells can activate the human β-globin promoter. In addition to its ability to activate genes, the E1a protein appears to be able to repress the expression of a class I major histocompatibility antigen-encoding gene (56).

To gain insight into the requirements for positive regulation of promoter activity, we have chosen to study the adenovirus type 2 (Ad2) late promoter and to analyze what nucleotide sequences are required for its activation by *cis*- and *trans*-acting regulators. It has been shown previously that the late promoter can be activated by the SV40 enhancer in HeLa cells. Elements previously shown to be a necessary part of this promoter in vitro and in vivo include the TATA box (12, 29) and further upstream sequences, centered around position −60 (29, 33, 65). In vitro experiments have failed to show a requirement for sequences defining the transcription start site (29).

The results presented here demonstrate that the Ad2 late promoter can be activated by either of two mechanisms, mediated by the SV40 enhancer in one case and the Ad2 E1a protein in the other. Furthermore, it appears that these two mechanisms proceed by distinct pathways.

MATERIALS AND METHODS

Plasmids. Plasmid pφ4-SVA was made from the plasmid pφ4, which contains adenoviral sequences from −405 to +33 relative to the transcriptional start site (+1) (29). An SV40 DNA segment extending from the *Hin*dIII site at nucleotide (nt) 5171 to the *Bam*HI site at nt 2533 (9), which includes the entire SV40 early region coding sequences and polyadenyla-

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tion signal, but no promoter or T-antigen binding sites, was attached between the *Hin*dIII and *Bam*HI sites of pφ4 to produce pφ4-SVA. Even though SV40 T antigen has been suggested to bring about positive as well as negative regulation of some genes, there is direct evidence from Ad-SV40 hybrid viruses, which argues against this protein having any effect on transcription from the late promoter of adenovirus (59; R. Tjian, personal communication). The same SV40 sequences were attached in a similar manner to several of the 5′ and 3′ deletion mutants previously described (29) to create the 5′ deletion mutants p601-SVA and p806-SVA and the 3′ deletion mutants p306-SVA, p208-SVA, and p307-SVA. The plasmid from which p210-SVA was derived has a *Pvu*II site at +33 instead of a *Hin*dIII site (29). Therefore, the *Hin*dIII site at the 5′ end of the SV40 early region fragment was filled in by DNA polymerase I large fragment before ligation to the *Pvu*II site of the parental plasmid. All plasmids were grown in *Escherichia coli* HB101.

The SV40 enhancer element was added to some of the plasmids and these constructs have an "e" added to their name; "ee" signifies that two enhancer elements were added. The DNA fragment containing the enhancer was obtained from a Bal 31 nuclease-generated deletion mutant (constructed by David Grass in this laboratory) that contains a *Bam*HI linker approximately 5 bp from the early region boundary of the SV40 enhancer. The enhancer-containing fragment was released from this plasmid by *Pvu*II and *Bam*HI digestion, made blunt ended with DNA polymerase large fragment and ligated into the appropriate plasmid at the filled-in *Eco*RI site, which is 405 bp upstream of the transcription start site.

The plasmid pE1aA was constructed by X.-Y. Fu in this laboratory from pHEB4 (obtained from S.-L. Hu), which contains all of the E1a protein coding sequences and intact enhancer (27) and promoter elements but lacks a polyadenylation signal. The SV40 early region polyadenylation signal, contained on a 120-bp *Bgl*II-*Bam*HI fragment from a Bal 31 deletion mutant (43), was inserted into pHEB4 to produce pE1aA. Preliminary experiments suggested that higher levels of expression of the gene expressing E1a could be obtained when this polyadenylation signal, which was used primarily for convenience, is present.

Cell culture and transfection. HeLa and 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO). For transfection, cells were split 1:10 for HeLa and 1:8 for 293 24 h before the addition of the DNA precipitate. Calcium phosphate precipitates were prepared by the method of Wigler et al. (63), except that 25 μg of plasmid DNA but no carrier DNA was used to transfect cells on a 150-mm plate. At 4 h after the addition of DNA to 293 cells, the DNA precipitates and medium were removed, and the cells were shocked with 10% glycerol for 1.5 min (15). HeLa cells were similarly glycerol shocked 18 h after the addition of the DNA precipitate. At 48 h after DNA was added, the cells were analyzed for the presence of SV40 early region products by indirect immunofluorescence, S1 nuclease mapping, or primer extension analysis. These experiments have all been repeated a minimum of seven times with several different preparations of plasmid DNA. Indirect immunofluorescence assays were performed as described in Lewis and Manley (E. D. Lewis and J. L. Manley, Nature, in press), using a monoclonal antibody to large T-antigen, Pab 416 (24).

RNA extraction and analysis. Total cytoplasmic RNA was extracted from cells after transfection with plasmid DNA as described previously (E. D. Lewis and J. L. Manley, in press). Probes for S1 nuclease analysis (6) were double stranded and labeled with ^{32}P at the 5′ ends with T4 polynucleotide kinase (Boehringer Mannheim). The following fragments were used as probes: pφ4-SVA *Bst*NI fragment (−126 to +115), p601-SVA *Taq*I-*Bst*NI fragment (the *Taq*I site in pBR322 to +115), p307-SVA *Bst*NI fragment (−126 to +80). Reaction mixtures containing 10 to 20 μg of total cytoplasmic RNA and 10 ng of probe were hybridized at 43°C for 4 h. S1 nuclease digestion was then carried out at 43°C for 30 min with 8×10^3 U of enzyme per ml (Boehringer Mannheim). The digestion products were electrophoresed through an 8% polyacrylamide–8 M urea sequencing-type gel (44). The DNA size marker was pBR322 digested with *Hpa*II and 5′ end labeled with polynucleotide kinase. Autoradiographic exposures were obtained without intensifying screens.

Primer extension analyses were carried out as described by McKnight and Kingsbury (45). The primer used was made on an Applied Biosystems DNA synthesizer and is complementary to the SV40 early region from nt 5131 to nt 5094 (numbering system of Buchman et al. [9]). The primer was 5′ end labeled with [^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim) and extended with avian myeloblastosis virus reverse transcription (Life Sciences). In all cases, autoradiograms were scanned with a Gilford model 250 densitometer.

RESULTS

E1a gene product activates the Ad2 late promoter. Previous studies have shown that a product of E1a is required for efficient expression of all other adenoviral early genes (5, 32). Although it is known that the late promoter is active at early times after infection, studies of the requirements for activation of the late promoter have yielded somewhat contradictory results. The work of Lewis and Mathews (40) suggested that the late promoter is active in the absence of any other viral factors, whereas studies by Nevins (50) suggested that an E1a product is required.

To address this problem, we first inserted into a plasmid containing the Ad2 late promoter an easily assayable eucaryotic gene, encoding the SV40 large T-antigen (Fig. 1). This plasmid (pφ4-SVA), which contains 405 bp of DNA upstream of the late transcriptional start site (at +1) and 33 bp downstream of it, was then used to transfect both HeLa and 293 cells. HeLa cells are human cells that are derived from a cervical carcinoma and contain no adenoviral sequences, and 293 cells (21) are human embryonic kidney cells that constitutively express Ad5 E1a gene products (1). The production of SV40 T-antigen, which is driven by the adenoviral late promoter, was assayed first by indirect immunofluorescence (Lewis and Manley, in press), using monoclonal antibodies directed against T-antigen (24). The results are expressed as the percentage of cells that contained detectable levels of T-antigen. In all experiments, the T-antigen immunofluorescence was confined to the nuclei of expressing cells, as it is in lytically infected or transformed cells. This measurement, although an indirect one, has been shown by others to be an accurate reflection of promoter activity (3, 49). Although no T-antigen-positive nuclei were found when pφ4-SVA was introduced into HeLa cells, readily detectable levels of expression were observed when this plasmid was used to transfect 293 cells (Table 1). These results suggest that the adenovirus late promoter can be activated by a *trans*-acting factor present in 293 cells, perhaps an E1a gene product.

Although both of these cell lines are human, they differ by

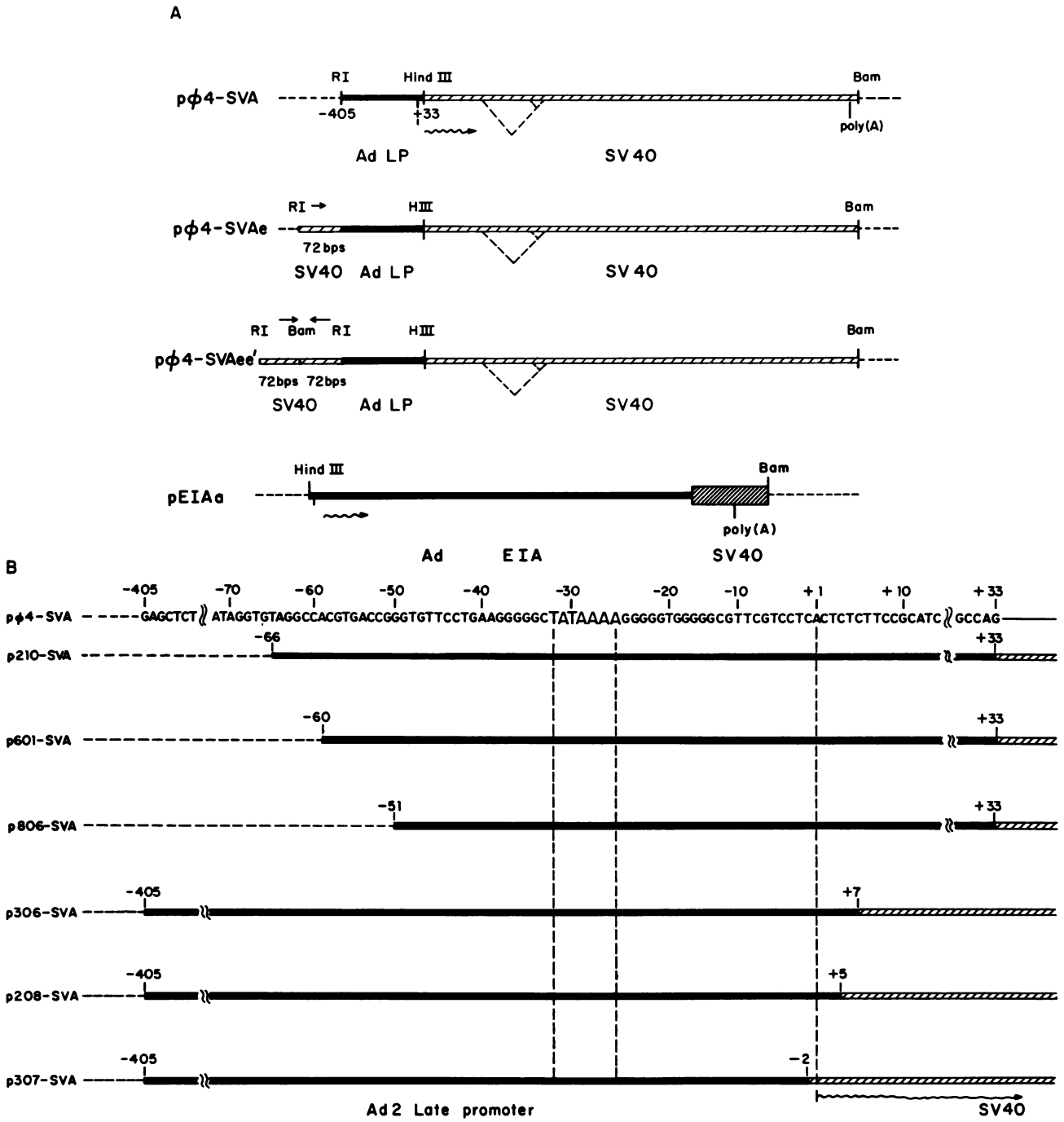

FIG. 1. Structures of the recombinant plasmids. (A) Plasmid pφ4-SVA contains 438 bp of Ad2 DNA from −405 to +33 relative to the transcription start site at +1. All adenoviral sequences are denoted by a thick line. The SV40 T-antigen-encoding sequences extend from the SV40 *Hin*dIII site at nt 5171 to the *Bam*HI site at nt 2533 (by the numbering system of Buchman et al. [9]) and are represented by a hatched line. The enhancer element used consists of the two SV40 72-bp repeats. The plasmid pE1aA contains approximately 230 bp 5′ to the Ad2 E1a cap site, the entire protein coding region, and the SV40 early region polyadenylation signals (see text for details of the constructions). (B) Details of the nucleotide sequences around the promoter and cap sites of the mutant plasmids used in this study. Ad2 late promoter deletion mutants were all attached to the SV40 T-antigen-coding region at the *Hin*dIII site at the SV40 nt 5171.

more than the presence or absence of E1a gene products. Therefore, to determine whether the late promoter can in fact be activated by an E1a-encoded function, an E1a containing plasmid, pE1aA, (Fig. 1) was cotransfected with pφ4-SVA into HeLa cells. Under these conditions, T-antigen was produced in a significant percentage of the cells, whereas no T-antigen was detected when pφ4-SVA was cotransfected with the plasmid vector, pBR322 (Table 1). These results strongly suggest that the E1a protein can activate the late promoter in *trans*.

TABLE 1. Transient expression of the late promoter plasmids in the presence or absence of E1a protein

Plasmid	% T-positive cells[a]	
	HeLa	293
p04-SVA + pBR322[b]	<0.05	8
p04-SVA + pE1aA	3.0	ND[c]
p306-SVA + pE1aA	<0.05	ND
p208-SVA + pE1aA	<0.05	ND
p307-SVA + pE1aA	<0.05	ND

[a] Indirect immunofluorescence analysis of the percentage of cells expressing T-antigen.
[b] The ratio of p04-SVA DNA to either pBR322 or pE1aA is 1:4.5.
[c] ND, Not done.

Identification of the 5′ boundary of nucleotide sequences required for *trans*-activation. We next asked what nucleotide sequences are required for the *trans*-activation observed in 293 cells. A series of deletion mutants generated by Bal 31 digestion of Ad2 late promoter upstream sequences (29) were attached to the SV40 T-antigen-coding region to create plasmids identical to pφ4-SVA, except for the absence in successive deletion mutants of sequences upstream of the transcription start site (Fig. 1B). All plasmids contain 33 bp of Ad2 sequence 3′ to the cap site. Indirect immunofluorescence analysis of cells transfected with these mutants revealed that deletion of all but 66 bp upstream of the start site did not affect the number of T-antigen-positive cells produced, compared to pφ4-SVA (Table 2). However, deletion of a further 6 bp, in mutant p601-SVA, reduced the number of cells producing T-antigen to an undetectable level. Transfection with a mutant that contains only 51 bp in the Ad2 late promoter region (p806-SVA) also failed to give rise to any T-antigen-positive cells. These results indicate that sequences upstream of the TATA box are necessary for expression of the late promoter in 293 cells.

Quantitative nuclease S1 analysis of the 5′ ends of total cytoplasmic RNA extracted from 293 cells after transfection with plasmid DNA was also performed to confirm the results obtained from immunofluorescence (Fig. 2). pφ4-SVA-transfected cells produced a high level of SV40-specific RNA with one transcriptional start site at the expected position (+1) in the adenovirus late promoter (Fig. 2, lane b). RNA from p210-SVA, which leaves 66 bp 5′ to the start site, gave rise to a band of similar intensity and with the same start site (Fig. 2, compare lanes b and c). The mutant p601-SVA produced very little Ad-SV40 specific RNA (Fig. 2, lanes a and d). The RNA produced from the p601-SVA template also begins at the predicted start site. RNA extracted from p806-SVA-transfected cells did not give rise to a detectable S1-resistant species (data not shown), suggesting that this mutant is transcriptionally inactive in 293 cells. These results demonstrate that nucleotide sequences up to the region between 66 and 60 bp upstream of the transcription start site are required for efficient promoter activity in 293 cells.

Identification of the 3′ boundary of nucleotide sequences required for promoter activation in 293 cells. We next examined the 3′ limit of the sequences required to obtain promoter activity in 293 cells. An additional series of Bal 31-generated mutations that deleted sequences from the 3′ direction around the late transcription start site (29) were attached to the SV40 T-antigen-coding region (Fig. 1B) and used in transient expression assays in 293 cells. All of these 3′ deletion mutants contain 405 bp upstream of the Ad2 transcription start site but have variable amounts of adenoviral

TABLE 2. Transient expression of 5′ deletion mutants in 293 cells.

Deletion end points[a]		Plasmid	% T-positive cells
5′	3′		
−405	+33	p04-SVA	8.0
−66	+33	p210-SVA	9.0
−60	+33	p601-SVA	<0.05
−51	+33	p806-SVA	<0.05[b]

[a] The deletion end point numbers are relative to the late promoter cap site at +1.
[b] No cells expressing T-antigen were seen, and <0.05% represents the limits of the resolution of this assay.

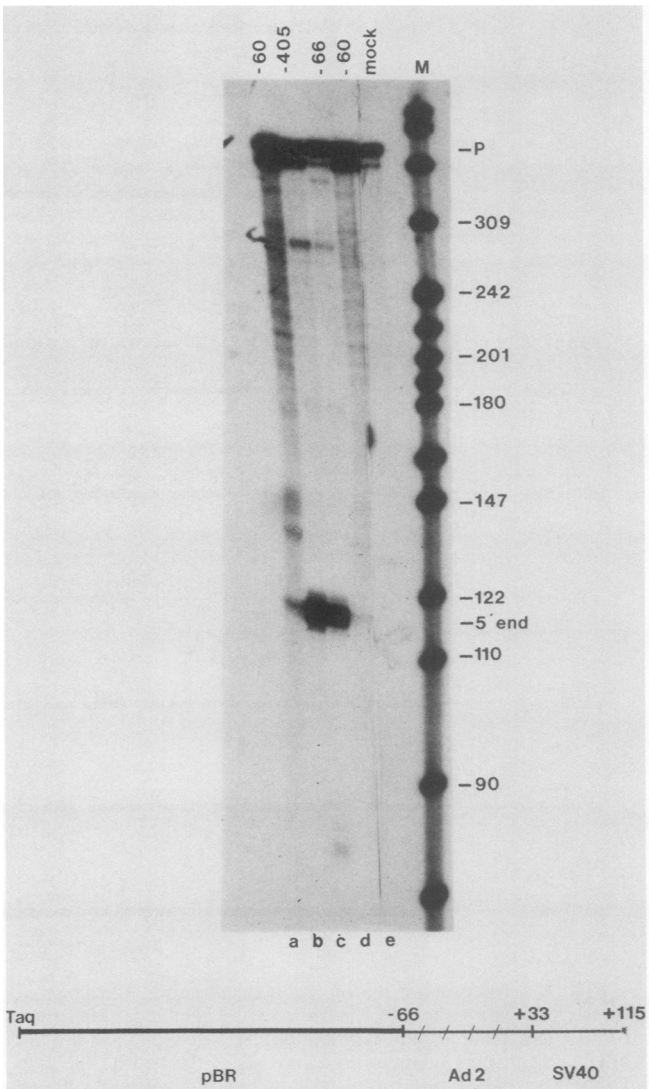

FIG. 2. Quantitative S-1 nuclease analysis of mRNA synthesized from 5′ deletion mutants. (Top) Total cytoplasmic RNA (10 μg) from 293 cells transfected with pφ4-SVA (lane b), p210-SVA (lane c), p601-SVA (lane d), or pBR322 (lane e) was analyzed. RNA (30 μg) from cells transfected with p601-SVA was used in lane a. The probe used is drawn at the bottom of the figure. The gel (top) was overexposed for lanes b and c so that the −60 mutant RNA samples were visible. Lane M contains pBR322 DNA digested with *Hpa*II and 5′ end-labeled for markers. The probe band is labeled P.

DNA downstream of the TATA box, depending on the extent of the deletion. The initial analyses were again carried out by using indirect immunofluorescence, and pφ4-SVA was again considered to be wild type. Upon transfection with this plasmid, about 8% of the cells expressed T-antigen (Table 3). Somewhat surprisingly, because of in vitro studies that showed no requirement for sequences downstream of the cap site for this promoter (29), transfection of 293 cells with the plasmid p306-SVA, which contains 7 bp past the start site, resulted in 1/10 the number of T-antigen-positive cells (Table 3). A deletion mutant lacking two additional nucleotides of adenovirus DNA, p208-SVA, gave almost no T-positive cells after transfection, and a deletion mutant that lacks all adenovirus sequences to 2 bp 5′ to the cap site, p307-SVA, produced no detectable T-positive nuclei (Table 3). These results, which have been obtained on numerous occasions, with several preparations of plasmid DNAs, suggest that nucleotides 3′ to the cap site are required to obtain transcriptional activity from the late promoter in 293 cells.

Primer extension analysis was used to assess the amount of SV40-specific cytoplasmic RNA produced in 293 cells after transfection with each of the 3′ deletion mutant plasmids. pφ4-SVA gave the highest level of SV40-specific RNA (Fig. 3A, lane a). RNA isolated from 293 cells transfected with p306-SVA yielded an extended product of significantly lower intensity than that from pφ4-SVA, approximately 30% of wild type (Fig. 3A, compare lanes a and b), consistent with the indirect immunofluorescence data. The amount of Ad2-SV40-specific RNA produced in cells transfected with this mutant has been found to be somewhat variable, ranging from 10% to the approximately 30% shown in Fig. 3A. Cells transfected with plasmids p208-SVA or p307-SVA produced barely detectable levels of specific RNA (Fig. 3, lanes c and d). Densitometer scanning of the autoradiogram revealed the reduction to be by a factor of approximately 20. These results demonstrate that at least 5 to 7 bp of nucleotide sequence downstream of the transcription start site are required to obtain late promoter activity in 293 cells. Results essentially identical to those shown in Fig. 3 have also been obtained by S1 nuclease analysis using a 5′ end-labeled DNA probe (see Fig. 6).

We also examined the nucleotide sequence requirements for activation of the Ad2 late promoter by the E1a protein in HeLa cells. In this set of experiments, the 3′ deletion mutants were cotransfected with an E1a-containing plasmid, pE1aA (see Fig. 1), and the levels of expression were measured by indirect immunofluorescence. As stated above, the introduction of pE1aA plus the wild-type plasmid, pφ4-SVA, into HeLa cells resulted in approximately 3% of the cells expressing T-antigen (Table 1). No cells expressing T-antigen were observed with any of the mutants containing less than 33 bp 3′ to the start site. These results are consistent with our observations in 293 cells, and suggest that specific nucleotide sequences around the transcription start site are required for activation of the late promoter by an E1a gene product.

TABLE 3. Transient expression of 3′ deletion mutants

Deletion end points		Plasmid	% T-positive cells	
5′	3′		293	HeLa
−405	+33	p04-SVA	8.0	<0.05
−405	+33	p04-SVAe[a]	10.5	26.6
−405	+33	p04-SVAee′	13.0	46.0
−405	+7	p306-SVA	0.4	<0.05
−405	+7	p306-SVAe	0.4	24.0
−405	+5	p208-SVA	0.1	<0.05
−405	+5	p208-SVAe	0.1	22.6
−405	−2	p307-SVA	<0.05	<0.05
−405	−2	p307-SVAe	<0.05	21.4

[a] The enhancer is located 405 bp upstream of the cap site in all cases when present.

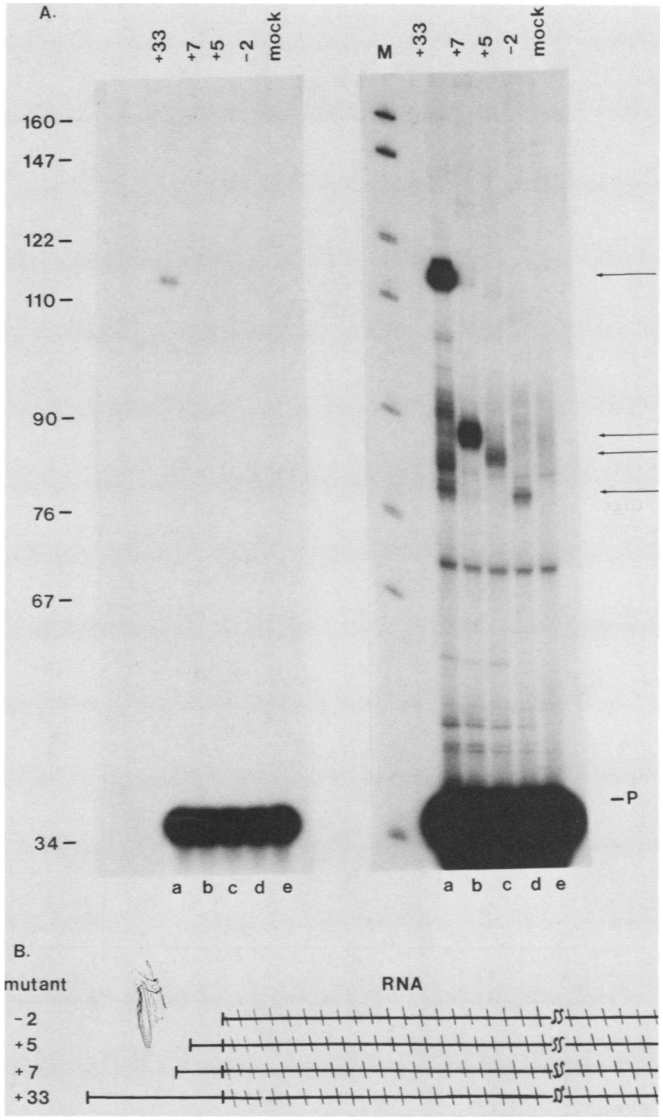

FIG. 3. Effect of 3′ deletions on Ad2 late promoter activity in 293 cells. (A) Samples (10 μg) of total cytoplasmic RNA from 293 cells transfected with 3′ deletion mutant DNAs were analyzed by primer extension. Extended products of 115 nt with pφ4-SVA RNA (lane a), 91 nt with p306-SVA (lane b), 89 nt with p208-SVA (lane c), and 82 and 81 nt with p307-SVA RNA (lane d), are indicated by arrows. Mock lane contains RNA from 293 cells transfected with pBR322 DNA (lane e). Lane M is pBR322 DNA digested with *Hpa*II and 5′ end labeled. P denotes primer. The autoradiogram on the left was exposed for 2 h; the one on the right was exposed for 3 days. (B) Schematic drawing of primer extension procedure. Hatched line represents SV40 sequences; solid line depicts adenovirus sequences. The primer was a 5′ end-labeled oligomer complementary to nt 5134 to nt 5095 of SV40 (numbering system of Buchman et al. [9]).

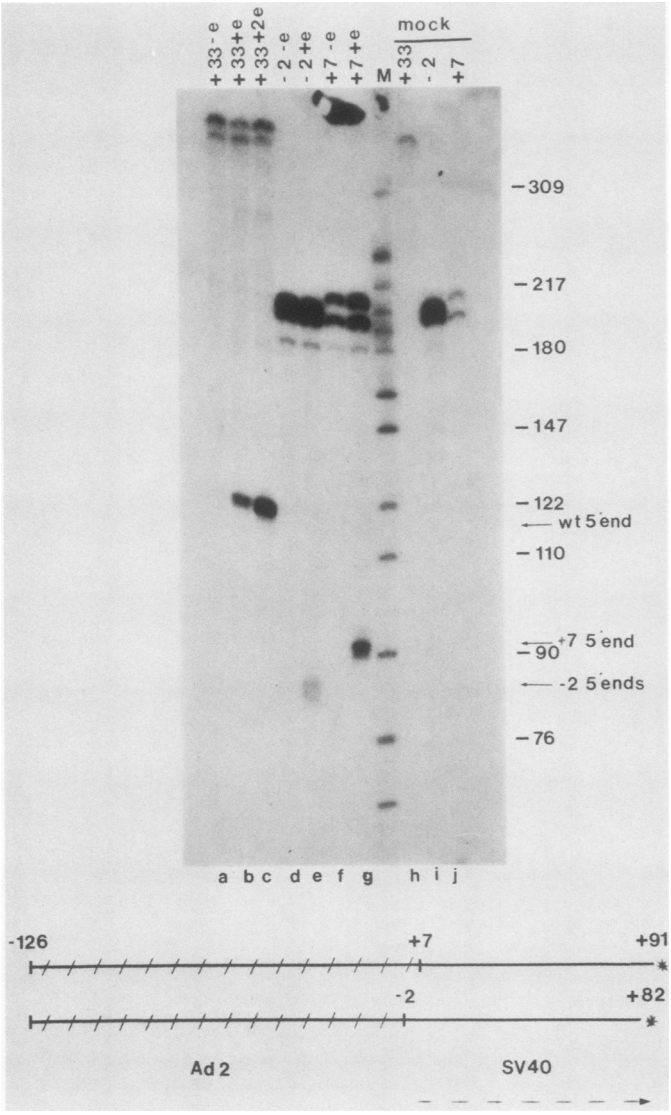

FIG. 4. Effect of 3′ deletions on activation by an enhancer element in HeLa cells. Quantitative S1 nuclease analysis on 10 μg of total cytoplasmic RNA extracted from HeLa cells after transfection with 3′ deletion mutants which either do or do not contain an enhancer element. The RNA used was isolated from HeLa cells after transfection with pφ4-SVA (lane a), pφ4-SVAe (lane b), pφ4-SVAee (lane c), p307-SVA (lane d), p307-SVAe (lane f), p306-SVA (lane g), p306-SVAe (lane h), and pBR322 (lanes i through k). The probe for lanes a through c is a 500-bp fragment from p601-SVA, extending from a *Taq*I site in pBR322 to the *Bst*NI site in SV40 at nt 5092. This probe produces a 118 nt protected DNA fragment. The probe for lanes d and e is a 212-bp *Bst*NI fragment from the p307-SVA mutant, which protects fragments of 81 and 82 nt and the probe for lanes f and g is the corresponding *Bst*NI fragment from the p306-SVA mutant, which protect a fragment of 91 nt. The +7 and −2 probes are shown at the bottom of the figure; SV40 DNA is drawn as a solid line, RNA is drawn as a dashed line, and adenoviral DNA is drawn as a hatched line. The 200-bp band common to lanes d through g is a contaminating fragment that copurifies with the probe. The probes were all 5′ end labeled with polynucleotide kinase. Lane M is pBR322 DNA digested with *Hpa*II and end labeled.

Identification of the 3′ boundary of nucleotide sequences required for *cis*-activation. To compare the sequences required for *trans*-activation with those required for *cis*-activation, a fragment of DNA containing the SV40 enhancer was inserted 405 bp upstream of the transcription start site in the series of plasmids containing 3′ deletions. Initial analyses were carried out by using indirect immunofluorescence. Transfection of HeLa cells with the wild-type plasmid, which now contains the SV40 enhancer (designated with an e, i.e., pφ4-SVAe), gave rise to about 25% of the cells expressing detectable levels of T-antigen (Table 3). Transfection of HeLa cells with a similar plasmid containing only 7 bp of adenoviral DNA downstream of the cap site, p306-SVAe, resulted in approximately the same percentage of cells expressing T-antigen, about 25%. Similar levels of expression were also seen in HeLa cells from the enhancer-containing plasmids that contained only 5 bp (p208-SVAe) or lacked the adenoviral start site (p307-SVAe). These results, which are similar to those obtained in vitro in HeLa cell extracts (29), suggest that sequences around the start site are not required to obtain wild-type levels of transcription when the late promoter is activated by an enhancer element.

These results were confirmed when the actual levels of SV40-specific RNA were examined. S1 nuclease analysis of the cytoplasmic RNA from HeLa cells transfected with pφ4-SVAe showed efficient expression from the late promoter, with the expected 5′ end (Fig. 4, lane b). A similar level of RNA, also with the authentic start site, was generated when p306-SVAe was used as the template (Fig. 4, lane g). The −2 deletion mutant, p307-SVAe, produced RNA from two adjacent start sites, (Fig. 4, lane e) as it did in 293 cells (see Fig. 6, lane f). However, densitometer scanning revealed that the total amount of RNA produced was not significantly affected in HeLa cells.

As expected from the result of others (28), no transcription from plasmids lacking the SV40 enhancer was observed in HeLa cells (Fig. 5, lane a). In sharp contrast, no enhancement (or repression) of transcription above the already high enhancer-independent level was seen in 293 cells when pφ4-SVAe was used as the template (Fig. 5, compare lanes e and f) or even when two SV40 enhancer elements were inserted into pφ4-SVA (Fig. 5, lane g, pφ4-SVAee). (See Fig. 1 for details of pφ4-SVAee.) We also observed that the addition of the SV40 enhancer element to the 3′ deletion mutants did not increase (or decrease) the low level of T-antigen-positive cells (Table 3) or RNA produced from the mutant templates (Fig. 6, compare lane b with c, and lane e with f). Thus, the SV40 enhancer is totally unable to affect expression from the Ad2 late promoter in 293 cells.

DISCUSSION

We have analyzed the nucleotide sequences required to obtain expression from the Ad2 late promoter when it is activated by either of two different mechanisms. Activation in *trans*, mediated by the Ad2 E1a protein, in either 293 or HeLa cells, requires between 60 and 66 bp of DNA upstream of the transcription start site (at +1) and approximately 7 bp downstream. Activation of the same promoter mediated by a *cis*-linked enhancer element in HeLa cells apparently requires the same nucleotide sequences 5′ to the start site (see reference 28) but has no requirement for sequences downstream of position −2.

It is highly unlikely that the significantly reduced levels of expression detected from the 3′ deletion mutants when activated by an E1a gene product were due to instability of the RNAs. The RNAs produced in HeLa cells from these mutants as a result of enhancer-mediated activation not only accumulate to the same level as does wild-type RNA (Fig. 4), but they are also apparently translated with roughly the same efficiencies (Table 3) and contain the same 5′ ends as

do the RNAs produced as a result of *trans*-activation (compare Fig. 3 and 4).

Some information about the mechanisms of promoter activation can be deduced from these results. The 5′ boundary of the late promoter has been found to be the same regardless of whether activation is brought about by a *trans*-acting regulatory protein, by a *cis*-acting enhancer sequence (28), or in vitro (28, 33). We suggest from this coincidence that a general transcription factor binds approximately 60 bp upstream of the transcription start site. Analysis of point mutants has demonstrated that the region around −55 and −57 is important for obtaining full expression from the late promoter in vitro (65) and in vivo (unpublished results). Studies with truncated templates in HeLa cell extracts also suggest that a distinct transcription factor binds to upstream sequences in this region (33, 47). It is tempting to speculate that this factor may be the general transcription factor, SP2, shown to be required for transcriptional activation of the Ad2 late promoter in vitro (14). Green et al. (22) have found that the upstream nucleotide sequence requirements for transcriptional activation mediated by the E1a protein of two other promoters, the SV40 early and the

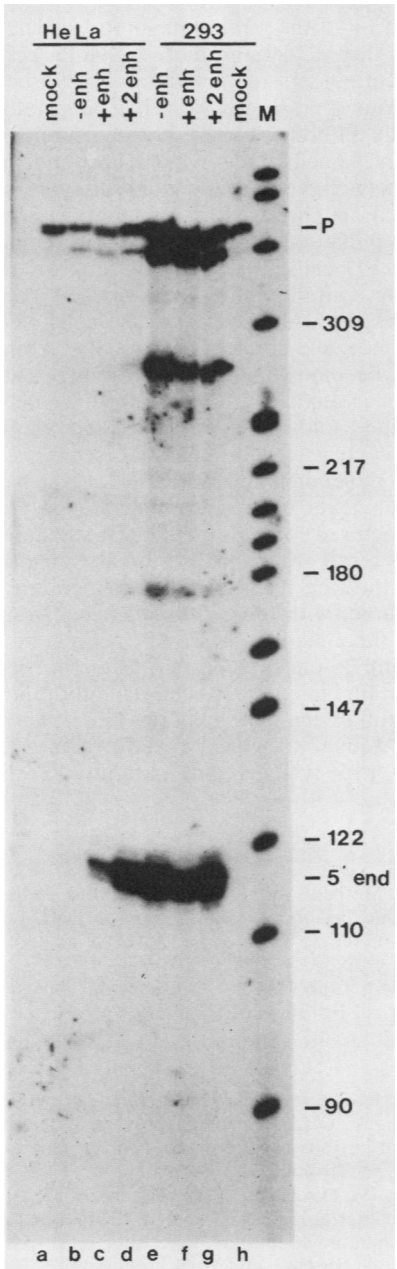

FIG. 5. Effect of the SV40 enhancer on pφ4SVA expression in 293 and HeLa cells. Quantitative S-1 nuclease analysis of 10 μg of RNA after transfection of 293 cells with pφ4-SVA containing 0, 1, or 2 enhancer elements from SV40, as noted above each lane. Each enhancer element is composed of two 72-bp repeats. Probe used is the 500-bp *Taq*I-*Bst*NI fragment from p601-SVA and is labeled P. Marker lane, M, is *Hpa*II-digested pBR322.

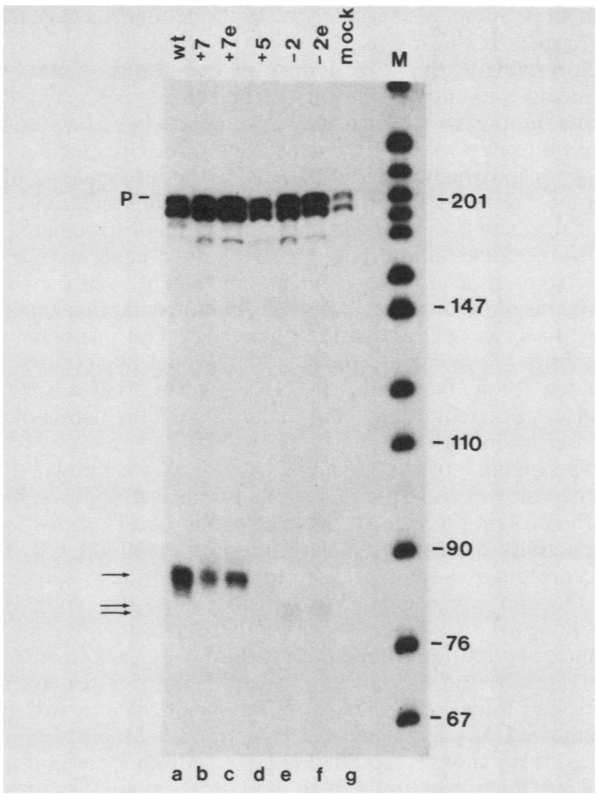

FIG. 6. Effect of the SV40 enhancer on 3′ deletion mutants in 293 cells. Total cytoplasmic RNA (10 μg) from 293 cells transfected with pφ4-SVA (lane a), p306-SVA (lane b), p306-SVAe (lane c), p208-SVA (lane d), p307-SVA (lane e), p307-SVAe (lane f), pBR322 (lane g) was used for S1 nuclease analysis. The protected fragments are indicated by arrows to the left of the figure. The probe, which is drawn schematically in the lower portion of this figure, is the *Bst*NI fragment from p307-SVA. The protected bands in lanes a through d are 2 nt longer than those in lanes e and f because the deletion mutants with endpoints at +7 and +5 contain 2 more nt of SV40 sequences in the RNA they encode than does the −2 mutant (see Fig. 1B). The probe used in this experiment contains only SV40 sequences, denoted by a hatched line, found at or 3′ to the transcription start sites for these RNAs. The same probe was used for all of the RNA samples to avoid any variations in band intensities that could arise from variations in the specific activities of different probes. The probe is labeled P, and the marker lane is labeled M.

human β-globin, also coincide with the sequences necessary for transcription in vitro.

Surprisingly, the 3′ boundary of the late promoter was found to vary depending on the mechanism of activation. Some, though so far only a few, promoters have been found to require nucleotide sequences around their respective cap sites for maximal activity (26, 38, 46; see reference 42 for a review). Here we show that a sequence around the transcription start site is essential in one case, activation mediated by a *trans*-acting factor, but not in another case, activation mediated by a *cis*-acting enhancer element. We conclude from this difference that two different molecular mechanisms for transcriptional activation of the Ad2 late promoter are possible. We speculate, because additional nucleotides are needed for activation by the E1a protein, that a specific transcription factor may bind to the DNA in the region of the transcription start site.

The identity of the postulated factor is not known. It is known, however, that an E1a gene product, probably a form of the protein predicted to have 289 amino acids (48), affects the activity of adenovirus early promoters (39, 48, 52). The E1a protein may interact directly with the primary sequence of the cap site region. We consider this idea somewhat unlikely for two reasons. First, there are no obvious sequence similarities around the cap sites of genes known to be activated by the E1a protein. Second, the E1a protein has not been found to be able to bind specifically to Ad2 late promoter DNA sequences (L. Ryner and J. Manley, unpublished results) or, in fact, to any DNA. Another possibility is that a cellular protein binds to the late promoter cap site. In this case, the role of the E1a protein might be to activate the cellular gene coding for the hypothetical factor.

Alternatively, the postulated factor may not recognize a specific nucleotide sequence, but instead recognize a structure assumed by the DNA in the region of the cap site. In this regard, it is interesting to note that Ziff and Evans (66) observed that a stem-loop structure could theoretically form between −26 and +8, relative to the cap site at +1, on the nontemplate strand. This stem is stabilized by several G–T bonds. The opposite or template strand would then place A's across from C's, which is a much less stable configuration. Ziff and Evans (66) therefore postulated that the template strand would remain in an open loop conformation. Such an open loop should create an S-1 nuclease-sensitive site. Goding and Russell (19) have in fact found such a site in this area of the late promoter. It is conceivable that the open loop could act as an entry site for RNA polymerase II or a transcription factor. We note that this is also a postulated role for enhancers (49). Nucleotide sequences to +8 are required for this theoretical stem-loop structure to form. The 3′ deletion mutants studied here successively cut into and destroy the potential stem-forming structure, suggesting an explanation for why sequences just past the cap site are important for transcription initiation. Experiments with point mutants are under way to test whether the proposed stem-loop structure exists and whether it is involved in the control of transcription.

An additional possibility is that the E1a protein modifies the transcriptional machinery in such a way that additional nucleotide sequences around the late promoter cap site are required for transcription initiation. Such a modification could either be enzymatic (e.g., phosphorylation) or structural (i.e., a sigma-like factor). However, again, no experimental evidence exists to support these ideas.

Green et al. (22) have examined the sequences upstream of the TATA box in the human β-globin promoter required for E1a-mediated activation. Their results show that for this promoter virtually no sequences upstream of the TATA box are necessary to obtain promoter activity. A comparison of these results with ours thus suggests that different mechanisms for E1a-mediated activation may exist, depending on the specific promoter. This notion is strengthened by studies that have either failed to detect any specific nucleotide sequences different from those needed for basal transcription required for E1a activation (36) or else, in one case (the Ad2 E4 promoter), identified sequences far upstream of the transcription start site that appear to be required for activation (16).

A comparison of the requirements for enhancer-mediated activation of the Ad2 late promoter in HeLa cells compared with the requirements for activation in 293 cells provides further evidence that the two mechanisms studied here are fundamentally different. The SV40 enhancer does not appear to function in 293 cells, although it is absolutely required for transcriptional activation of the late promoter in HeLa cells. Treisman et al. (60) have also shown that the SV40 enhancer does not stimulate transcription of the human β-globin promoter in 293 cells. Two explanations for these results were possible. One hypothesis is that the enhancer cannot elevate transcription above the already high level that occurs in 293 cells because some transcription factor, perhaps a general one, becomes limiting. The other possibility is that this enhancer is unable to function in these cells. We have shown that the SV40 enhancer cannot activate transcription in 293 cells even when all nucleotide sequences necessary for enhancer-dependent activation in HeLa cells are present, but when the level of transcription is very low because sequences required for E1a-mediated activation have been deleted. This result argues against the first hypothesis above, and implies that the transcriptional machinery in 293 cells does not interact with the SV40 enhancer. Alternatively, it may be that the enhancer is actively repressed in 293 cells, perhaps by an E1a-encoded protein. Experiments supporting this latter notion have in fact been recently reported (7, 61). Obtaining a full understanding of the molecular mechanisms of promoter activation will ultimately require the development of in vitro systems that faithfully reproduce these phenomena.

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