Constraints on spacing between transcription factor binding sites in a simple adenovirus promoter

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The adenovirus 2 E1B transcription unit has an extremely simple promoter consisting of a TATA box and a closely situated GC box. The interaction between the TATA box and the GC box was analyzed using insertion mutations which expand the distance between the two elements. We observed that the E1B promoter has an unusually rigid architecture. When the GC box, which is a binding site for transcription factor Sp1, was separated further from the TATA box than in the wild-type promoter, in vivo transcription quickly diminished to a level comparable to the elimination of the Sp1 site. Yet all the insertion mutants bound Sp1 factor in vitro with an affinity approximately equal to that of the wild-type promoter. From these results, we argue that Sp1 binding alone is not sufficient to stimulate transcription. The increased distance might disrupt direct contacts between Sp1 and transcription factors bound at the TATA box, contacts required for transcription stimulation by Sp1. The insertion mutations do not interfere with the transcription activation process mediated by the adenovirus large E1A protein or the pseudorabies virus immediate early protein.

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Transcription control signals of eukaryotic genes transcribed by RNA polymerase II are composed of distinct sequence elements usually located upstream of the transcription start site (for reviews, see McKnight and Tjian 1986; Maniatis et al. 1987). The first prominent promoter element is the commonly found TATA box, which is located approximately 30 bp upstream of the transcription start site. Ample evidence indicates that the TATA box plays a fundamental role in transcription initiation (Breathnach and Chambon 1981). In many promoters, mutations in the TATA box cause a greater decrease in transcription than mutations in other promoter elements do. This is the case for the herpes simplex virus type-1 thymidine kinase (HSV tk) (Coen et al. 1986), mouse β -globin (Charnay et al. 1985), adenovirus 2 (Ad2) E1B (Wu et al. 1987) and major late promoters (Corden et al. 1980; Hu and Manley 1981; Concino et al. 1983). An RNA polymerase II transcription factor has been shown to bind to the TATA box region (Parker and Topol 1984; Sawadogo and Roeder 1985). The second class of promoter signals are the so-called upstream elements that appear to be binding sites for transcription factors. One particularly well-studied example is a GC-rich element found in many cellular and viral promoters (Kadonaga et al. 1986). The cellular transcription factor, Sp1, which binds to this element has been purified to homogeneity (Briggs et al. 1986; Kadonaga and Tjian 1986). Addition of Sp1 to in vitro transcription reactions stimulates transcription from promoters containing Sp1

binding sites (Dyner and Tjian 1983a,b; Jones et al. 1985). However, the mechanism by which Sp1 and other upstream transcription factors act to modulate transcription initiation remains unclear.

Two modes of upstream transcription factor interaction seem very likely to be crucial to transcription stimulation. The first, a protein-DNA interaction, requires that the transcription factor recognizes and binds to its cognate DNA sequence. In two well-studied systems, the simian virus 40 (SV40) early and HSV tk promoters, DNA sequence mutations that depress RNA synthesis also inhibit transcription factors from binding (Jones et al. 1985; Wildeman et al. 1986; Lee et al. 1987a,b; Mitchell et al. 1987). Second, both biochemical and in vivo genetic analyses suggest that protein-protein interactions between transcription factors are important to the transcription process. Biochemical studies indicate that binding of the upstream factor MLTF (Chodosh et al. 1986) to the adenovirus major late promoter is greatly stabilized by binding of the adjacent TATA-box transcription factor (TFIID) (Sawadogo and Roeder 1985). This observation supports the hypothesis that TFIID and MLTF make protein-protein contacts with each other when they are bound concurrently (Sawadogo and Roeder 1985).

Expansion of the distance between upstream elements and the TATA box can reduce transcription (McKnight 1982; Takahashi et al. 1986). In addition, in the SV40 promoter/enhancer early region, inserting half-turns of a

DNA helix between transcription factor binding sites is more detrimental to transcription than inserting full turns (Takahashi et al. 1986). These results all point toward the involvement of direct functional interactions between transcription factors. More specifically, the latter result suggests that proper transcription initiation requires stereospecific alignment between upstream factor(s) and the protein(s) bound to the TATA box.

In light of the indications that transcription factors need to interact to augment transcription, it might seem surprising that considerable changes in the distance between the TATA box and the upstream elements can be tolerated (McKnight 1982). Thus, to accommodate the necessary interactions, the plasticity in the allowed positions of promoter elements must stem from either flexibility in the intervening DNA (Ptashne 1986) or in the transcription factor proteins (Takahashi et al. 1986), or both.

In an effort to study the interactions between transcription factors, we have analyzed the influence of alterations in the spacing between transcription factor binding sites in the unusually simple Ad2 E1B promoter. The principle E1B promoter elements are a binding site for the Sp1 transcription factor (Kadonaga et al. 1987) and a consensus TATA box (Fig. 1; Wu et al. 1987; also see data below). Disruption of the Sp1 site and TATA box reduced the rate of E1B transcription *in vivo* by 6- and 20-fold, respectively. Deletion of both elements eliminated detectable E1B transcription. Transcription from the E1B promoter is greatly stimulated by the Ad2 large E1A protein (reviewed in Berk 1986) and the pseudorabies virus immediate early protein (IEP), a process termed *trans*-activation. Here, we also analyzed the influence of altered spacing between the E1B promoter elements on E1A and IEP *trans*-activation.

We found that the distance between the Sp1 and TATA-box transcription factor binding sites required for maximal promoter activity is unusually constrained in the E1B promoter. This result suggests that direct interactions between these two factors are required for transcription stimulation by Sp1. All insertion mutants were activated well by E1A and IEP. This result indicates that disruption of the interaction between Sp1 and TATA-box transcription factors does not interfere with the transactivation process.

Figure 1. Sequence of the adenovirus 2 E1B promoter region and mutants. The GC box and TATA box are underlined in the wild-type and mutant promoters.

Results

No significant E1B promoter elements lie between the Sp1 site and TATA box

An earlier study (Wu et al. 1987) suggested that the Ad2 E1B promoter was comprised principally of single, closely situated binding sites for host cell transcription factors Sp1 and a TATA-box transcription factor. The high-affinity Sp1-binding site GGGGCGGGC (Kadonaga et al. 1986) occurs between 48 and 39 bp upstream of the Ad2 E1B RNA cap site (Fig. 1) and is bound by purified Sp1 *in vitro* (Wu et al. 1987). Deletion of Ad2 sequence from within the neighboring E1A transcription unit to just upstream of this sequence (from -127 to -55 with respect to the E1B cap site) does not depress E1B transcription significantly, whereas deletions and linker-scanning mutations that impinge on the GGGGCGGGC sequence depress E1B transcription to 15–20% the level of the wild-type promoter (Wu et al. 1987). However, none of the mutants analyzed previously tested the function of the 8-bp sequence between the TATA box and the consensus Sp1-binding site (Fig. 1). To determine whether this 8-bp sequence con-

Figure 2. Transcription from E1B promoter linker-scanning mutants. An autoradiogram shows an S1 analysis of RNA isolated 16 hr post-infection of HeLa cells. Cells were coinfecting with the mutant indicated at the top of each lane plus the control S13B mutant containing a wild-type E1B promoter and a small deletion at +74 in the E1B transcription unit. Correctly initiated E1B transcripts from the E1B promoter mutant viruses and wild-type protect a 219-base fragment from S1 digestion. E1B transcripts from the internal control S13B mutant produce a 145-base protected fragment. The S1-protected fragment generated from LS -5/+5 is slightly shorter than for the other mutants because of the region of nonhomology between the first 5 bases of the mRNA and the probe generated from wild-type Ad2 DNA. S1 analyses performed with the homologous probe prepared from LS -5/+5 show that the 5' end of LS -5/+5 E1B RNA maps at the +1 position (data not shown).

tained a significant promoter element, we constructed the mutant LS - 38/ - 31 in which this region is substituted with an unrelated DNA sequence (Fig. 1). We also constructed mutant LS - 30/ - 23, which has a precise substitution of the TATA box, and mutant LS - 48/ - 39, which has a precise substitution of the consensus Sp1-binding site.

Figure 2 shows an S1 analysis of E1B RNA isolated 16 hr postinfection of HeLa cells with LS - 48/ - 39, LS - 38/ - 31, and LS - 30/ - 23, as well as the E1B promoter LS mutants constructed earlier (Wu et al. 1987). To provide an internal control for transcription from the wild-type E1B promoter, the cells were coinfecting with the mutant S13B, which has a small deletion at +74 in the E1B transcription unit. Correctly initiated E1B transcripts from the E1B-promoter mutant viruses protect a 219-base fragment from S1 digestion, whereas E1B transcripts from the S13B virus produce a 145-base protected fragment. All mutations disrupting the GGGGCGGGGC Sp1-binding site consensus sequence (LS - 55/ - 46, LS - 48/ - 38, and LS - 45/ - 36) or the TATA box (LS - 35/ - 26, LS - 30/ - 23, and LS - 25/ - 16) depressed E1B transcription significantly. However, mutations that did not disrupt the Sp1 consensus sequence or the TATA box, including LS - 38/ - 31, had only minor effects on E1B transcription (Fig. 2). The level of transcription from each of these mutants relative to the wild-type E1B promoter was similar when RNA was isolated during the early phase of infection (6 hr postinfection; data not shown).

These results demonstrate that sequences in the interval between the Sp1 consensus sequence and the

TATA box have only a minor influence on E1B transcription. However, a slight depression in E1B transcription from mutant LS - 38/ - 31, compared to the wild-type promoter, was reproducibly observed. This effect was probably due to a slight decrease in the affinity of Sp1 for the LS - 38/ - 31 template, compared to the wild-type promoter. Figure 3 shows a titration of the DNA-binding activity of purified Sp1 on the wild-type E1B promoter and LS - 38/ - 31, as assayed by DNase I footprinting. In this and other experiments, concentrations of Sp1 four to eight times higher were required to completely protect an approximately 20 bp region centered over the GGGGCGGGGC sequence in LS - 38/ - 31, compared to the wild-type promoter. As expected, no protection by Sp1 was observed for the mutant LS - 48/ - 39. A similar decrease of four- to eight-fold in the affinity of Sp1 for the LS - 38/ - 31 mutant was observed in competition assays where unlabeled DNA fragments were used to compete for Sp1 binding to a labeled wild-type fragment (as in Fig. 4 below; data not shown). These data indicate that nucleotides just 3' to the 10-bp consensus sequence have a small influence on Sp1 binding. These results, together with those presented previously (Wu et al. 1987), demonstrate that the principle E1B promoter elements are the single Sp1-binding site and the closely situated TATA box.

Mutants with expanded distances between the Sp1 site and TATA box

Insertion mutations in the E1B promoter region were generated by linker insertion (Barany 1985), as described

Figure 3. Sp1 DNase I footprinting on wild-type and E1B promoter mutants. The DNA probes used in the footprinting analyses are indicated at the top. Probes of equal specific activity were incubated in binding reactions with microliters of purified Sp1 indicated at the top of each lane and then digested with DNase I, as described in Materials and methods. [M] A lane of markers. Maxam-Gilbert sequencing of the LS - 38/ - 31 probe is shown in the four lanes at the right.

Figure 4. DNase I footprinting competition by the E1B promoter insertion mutants. DNase I footprinting shown in each panel was performed using the wild-type promoter region as probe and partially purified Sp1, as described in Materials and methods. (Lanes no) Reactions without added Sp1. All other lanes contained the same amount of Sp1 and the indicated molar excess over the labeled probe of the equivalent restriction fragments from the wild-type or mutant promoter regions, as indicated. The unlabeled, competing fragments extend from -362 to $+74$.

in Materials and methods. The precise sequence alterations in these mutants were determined by DNA sequencing (Fig. 1). The insertion mutations were designed to merely expand the distance between, while maintaining the integrity of, the Sp1 consensus (Kadonaga et al. 1986) and TATA-box sequences. Furthermore, the insertions are multiples of approximately half-turns of the DNA helix, allowing us to investigate the stereospecificity (Takahashi et al. 1986) of Sp1 function. The Sp1 affinity and the transcriptional activities of these insertion mutants were compared with the wild-type promoter and the LS $-48/-39$ mutant whose Sp1 site was substituted precisely. To facilitate the accurate analyses of E1B transcription *in vivo*, we chose to rebuild all mutants into the adenoviral genome. In this way, the E1B promoter mutations can be introduced into HeLa cells by viral infection. These mutant viruses were also designed to contain a deletion in their E1A gene (dl1505; Osborne et al. 1984), which rendered them deficient in E1A transactivation function. E1A activation of the mutants was examined by coinfection with adenovirus mutant S13B, which provides wild-type E1A function *in trans* but expresses a distinguishable E1B message. Likewise, transactivation by IEP of pseudorabies virus was examined by coinfection with wild-type pseudorabies virus.

Binding of Sp1 to the promoter mutants

The first point to establish regarding the effects of the insertions was whether the Sp1 transcription factor recognizes and binds to the mutated promoters with an affinity equal to that of the wild-type promoter. The Sp1-binding affinity to different mutants was compared by a DNase I footprinting competition assay, as shown in Figure 4. Using a wild-type E1B promoter fragment

as probe, partially purified Sp1 (Briggs et al. 1986) clearly protected a DNA segment containing the GGGCGGGC element from DNase I cleavage. The experiments also show the titration of excess unlabeled wild-type or mutant competitor promoter fragment. When 10- to 30-fold molar excess of wild-type fragment was added, the Sp1-binding was entirely competed away. Notably, the insertion mutants IN5, IN10, IN16, and IN22 competed as efficiently as the wild-type E1B promoter because the same titration endpoint was reached with 10- to 30-fold molar excess of the mutant promoter fragments. We also observed in direct DNase I footprinting analyses of each of the labeled mutant promoters that the partially purified Sp1 bound to the wild-type and insertion mutant promoters at equal Sp1 concentrations (data not shown). These results indicate that Sp1 has similar affinity (within a factor of 2) for each of the insertion mutant promoters as for the wild-type sequence. On the other hand, the GC-box substitution mutant LS $-48/-39$ did not compete (Fig. 4) and did not bind Sp1 in direct footprinting experiments (Fig. 3), as expected.

E1B transcription of the insertion mutants

The DNase I footprinting results clearly show that Sp1 can recognize and interact with the insertion mutants. To determine what effect the increased distance between the Sp1 site and TATA box has on E1B transcription, HeLa cells were infected with mutant viruses and cytoplasmic RNA was harvested after 6 hr, during the early phase of infection. E1B RNA concentrations were examined either without expression of transactivator protein or in the presence of E1A or IEP. E1B RNA was quantitated by S1 analysis. Much less E1B RNA was expressed from IN16 and IN22 than from the wild-type E1B promoter (Fig. 5; Table 1). This was true both for the

unstimulated promoter and under conditions of transactivation by E1A or IEP (Fig. 5; Table 1). In fact, E1B RNA from these mutants was reduced to a similar level as observed for the Sp1-site substitution mutant LS - 48/

Figure 5. Transcription from the wild-type and E1B promoter insertion mutants. S1 analyses of cytoplasmic RNA isolated 6 hr postinfection were performed as described in Materials and methods. In the +E1A panel, cells were coinfecting with Ad5 mutant S13B, which provided wild-type E1A proteins *in trans*. In the +IEP panel, cells were coinfecting with pseudorabies virus to provide IEP *in trans*. The infections were performed at the same time, the S1 analyses were performed with the same probe, and the autoradiograms were exposed for the same period. Consequently, the intensities of the bands represent the relative concentrations of E1B mRNAs. Quantitation of this data is shown in Table 1, experiment 1. The reproduction of the upper panel shown here appears to show the intensity of the IN5 S1-protected fragment equal to that of the IN16 mutant, but this is an artifact of the reproduction process. The correct quantitations obtained from longer exposures of the original autoradiogram are listed in Table 1.

Table 1. Transcriptional activity of the E1B mutants relative to wild type (WT)

		Experiment				Mean $\pm$ S.D.
		1	2	3	4	
No activator	WT	1.00	1.00	1.00	1.00	1.00
	IN5	0.71	1.06	0.67	0.97	0.85 $\pm$ 0.17
	IN10	1.55	1.00	0.64	1.09	1.07 $\pm$ 0.32
	IN16	0.18	0.22	0.17	0.31	0.22 $\pm$ 0.055
	IN22	0.15	0.08	0.13	0.11	0.12 $\pm$ 0.025
	LS - 48	0.18	0.14	0.13	0.27	0.18 $\pm$ 0.055
+ E1A	WT	1.00	1.00	1.00	1.00	1.00
	IN5	0.38	1.00	0.32	0.38	0.52 $\pm$ 0.28
	IN10	0.47	1.22	0.48	0.34	0.63 $\pm$ 0.35
	IN16	0.11	0.19	0.07	0.08	0.11 $\pm$ 0.047
	IN22	0.11	0.17	0.06	0.05	0.10 $\pm$ 0.048
	LS - 48	0.26	0.17	0.06	0.23	0.18 $\pm$ 0.082
+ IEP	WT	1.00	1.00			1.00
	IN5	0.40	0.98			0.69
	IN10	1.00	1.19			1.09
	IN16	0.27	0.31			0.29
	IN22	0.34	0.18			0.26
	LS - 48	0.20	0.10			0.15

–39 (~ 0.18 of wild-type, Table 1). Insertion of 5 or 10 bp was better tolerated. E1B RNA produced by IN10 was not reduced significantly compared to wild-type (Table 1). In the experiment shown in Figure 5 (Experiment 1 of Table 1), IN5 produced less E1B RNA than IN10 or wild-type did. However, this phasic variation was not observed in all experiments (Table 1). Finally, the insertions did not alter the start site of E1B transcription (Fig. 5).

Transcription activation by E1A and IEP

To determine whether the insertions interfere with transactivation, the influence on E1B transcription of E1A and IEP expression was compared between the wild-type and mutant promoters (Fig. 5). E2A RNA expression was examined in each experiment as a control for recovery of mRNA and for the function of E1A and IEP transactivators. Equivalent levels of E2A RNA were detected in each of the coinfections with S13B (to provide E1A) and pseudorabies virus (to provide IEP) (data not shown). In the absence of a viral transactivator, insufficient E2A RNA was produced to be detected under the conditions used ($\leq 2\%$ the level of E2A RNA observed in the S13B coinfection). The E1B RNA produced from the wild-type promoter was activated about 10-fold by E1A. All mutants were activated similarly by E1A, ranging from 7- to 14-fold. Similarly, the pseudorabies virus IEP stimulated the mutant promoters, as well as the wild-type E1B promoter (Fig. 5). Therefore, the insertions do not interrupt transactivation mediated by either E1A or IEP.

Discussion

To understand the functional relationship between the

Sp1 site and TATA box of the E1B promoter, we investigated the effects of expanding the distance between the two sites. We observed that when the Sp1 site was positioned farther than 18 bp from the TATA box, its effect on transcription diminished significantly. However, we showed that the affinity of Sp1 for the displaced binding sites in each of the insertion mutants was unaltered. In contrast, the LS - 48/ - 39 mutant, whose Sp1 site was substituted precisely, could not bind Sp1. In vivo transcription from this E1B promoter mutant fell to $18 \pm 5.5\%$ of wild-type during the early phase of infection. As observed for other promoter regions containing Sp1 sites, such as the SV40 early (Dyan and Tjian 1983b) and HSV tk (Jones et al. 1985) promoters, we conclude that for the Sp1 transcription factor to participate in the transcription initiation process, it must first recognize and bind to the promoter region. However, Sp1 binding to the promoter region alone is not sufficient for its function. In the simple E1B promoter, the influence of Sp1 fell off rapidly, comparing a promoter with 18 bp between the Sp1 site and TATA box and promoters with 24 and 30 bp separating these transcription factor binding sites.

A simple interpretation could be that besides binding to the DNA, the Sp1 factor must also contact the TATA box factor directly to mediate its influence on transcription. The added distance in IN16 and IN22 might prevent such contact between the two transcription factors. Consequently, the transcription phenotype of IN16 and IN22 is depressed comparably to the Sp1 site-deficient mutant, LS - 48/ - 39. Interestingly, the IN5 and IN10 mutations cause a much smaller depression in transcription. Perhaps these smaller insertions are better tolerated because of flexibility in the transcription factor proteins. For example, a flexible protein hinge might separate a DNA-binding domain from a domain that contacts a neighboring transcription factor, as suggested by Takahashi et al. (1986).

Inserting a half-turn of DNA helix between the Sp1 site and TATA box (IN5) was more detrimental to E1B transcription than insertion of a full turn (IN10) in some experiments (Fig. 5), as might be expected even for 'flexible' transcription factors. However, the effect was somewhat smaller and was observed less consistently (Table 1) than for similar insertions between transcription factor binding sites in the SV40 early promoter (Takahashi et al. 1986). Perhaps this is because multiple Sp1 factors bind along one side of the helix in the SV40 21-bp repeat region (Gidoni et al. 1984). The additive effects of these multiple, assymmetrically arranged transcription factors might increase the stereospecificity of their interactions with the TATA-box transcription factor or enhancer-binding factors (Wildeman et al. 1986; Lee et al. 1987b; Mitchell et al. 1987).

In the early SV40 promoter, Takahashi et al. (1986) observed that insertions between the Sp1 sites and the TATA box result in new transcription start sites. These are displaced upstream by a distance approximately equal to the size of the insertion, so that it appears that the Sp1 binding sites are more important than the TATA

box in positioning the transcription start sites in the early SV40 promoter. In the E1B promoter insertion mutants studied here, no displacement in the transcription start site was observed. Nor is there a change in the transcription initiation site in HSV I tk promoter insertion mutants (McKnight 1982). Both the E1B and tk promoter TATA boxes are a good match to the consensus TATA-box sequence (Breathnach and Chambon 1981) and probably function as binding sites for a host cell transcription factor (Parker and Topol 1984; Sawadogo and Roeder 1985). On the other hand, the SV40 early promoter TATA box is a poor match to the consensus sequence. In both the E1B and tk promoters, mutations in the TATA box depress transcription significantly (McKnight and Kingsbury 1982; Wu et al. 1987), whereas mutations in the SV40 TATA box have only a small effect on the level of transcription (Benoist and Chambon 1981; Fromm and Berg 1983; Wasylyk et al. 1983). Apparently, the SV40 TATA box functions quite differently from the E1B and tk promoter TATA boxes that fix the initiation site.

One distinctive feature of the E1B promoter is its rigid architecture, in that relatively small increases in distance between its promoter elements are not tolerated. In contrast, the spatial relationship between the upstream promoter elements and the TATA box is much more flexible in the extensively analyzed HSV tk promoter. The proximal Sp1 site in the HSV tk promoter (McKnight and Kingsbury 1982; Jones et al. 1985) is separated from the TATA box by 19 bp. Introducing an extra 30 bp between the Sp1 site and TATA box does not compromise tk transcription significantly [McKnight 1982]. Therefore, separation of 49 bp in total between the proximal Sp1 site and the TATA box in the tk promoter is well tolerated. Yet, in the E1B promoter, separations of just 24 or 30 bp reduce transcription equivalently to a complete loss of the Sp1 site. What characteristics distinguish these two promoters, permitting the extensive spatial flexibility of the tk promoter in contrast to the much more constrained spacing requirements in the E1B promoter?

The upstream elements of the tk promoter are more complex than for the unusually simple E1B promoter. Two binding sites for transcription factor Sp1 and one binding site for transcription factor CTF have been identified (Jones et al. 1985) in the upstream control elements defined and studied by McKnight and co-workers (McKnight and Kingsbury 1982; McKnight 1982). Transcription factor CTF binds to a CCAAT box at -81 to -85, which is flanked by two Sp1 sites (Jones et al. 1985). Perhaps multiple interactions between the upstream transcription factors and the TATA box factor bound to the tk promoter provide sufficient stability to permit deformation and bending of the intervening DNA helix, a mechanism suggested for the long-distance influence of enhancer regions (Pisahn 1986). Interactions between the single Sp1 factor and the TATA-box factor bound on the E1B promoter might not be sufficient to permit bending of the intervening DNA. Consistent with this idea, insertion of multiples of a single tran-

scription factor binding site often stimulate transcription *in vivo* to a much greater extent than insertion of a single site (Angel et al. 1987; Lee et al. 1987b). Alternatively, the CTF factor bound to the tk promoter region might mediate the influence of the neighboring Sp1 factors, allowing Sp1 interactions over a longer distance.

Transcription from the E1B promoter is stimulated about 10-fold by the large adenovirus E1A protein (Berk et al. 1979; Jones and Shenk 1979a; Nevins 1981) and by the pseudorabies virus IEP (Fig. 5, Feldman et al. 1982). The mechanism of this transactivation is not yet well understood. If these transactivating viral proteins functioned to increase the stimulating effects of upstream factors or to influence interactions between upstream factors and the TATA-box factor, we might have expected that the IN16 and IN22 insertions that nearly eliminate the influence of the E1B Sp1 site would show diminished transactivation compared to the wild-type promoter. This was not observed. On the other hand, the results are in agreement with the suggestion, based on studies of E1B promoter linker-scan mutants (Wu et al. 1987) and analyses of transcription factor activities in extracts of adenovirus-infected HeLa cells (Leong et al. 1988), that E1A expression increases transcription from the E1B promoter by stimulating the activity of the TATA-box transcription factor. Many of the hypotheses raised by these studies can be tested directly through further manipulations of the E1B promoter sequence and analyses of transcription factor interactions *in vitro*.

Materials and methods

Cells and viruses

Suspension cultures of HeLa and 293 cells (Graham et al. 1977) were grown in minimal essential medium (SMEM, GIBCO) supplemented with 5% newborn calf serum (NCS). The 293 cells were also grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% NCS.

Adenoviruses containing the E1B promoter mutations were propagated in suspension cultures of 293 cells grown in SMEM containing 2% NCS. Viral stocks harvested from 293 cells were assayed by plaque titration on 293 monolayers in DMEM with 0.7% agarose, 2 mM L-glutamine, MEM nonessential amino acids (GIBCO), 25 mM MgCl₂, and 2% NCS. Wild-type pseudorabies virus was obtained from L. Feldman, propagated on rabbit skin cells, and assayed by plaque titration on rabbit kidney cells.

Mutant plasmid construction

The pBR322 derived plasmids used in this study were propagated in *Escherichia coli* HB101, and plasmid DNA was purified by the alkaline-SDS method, as described (Birnboim and Doly 1979). Enzymes for cloning were used according to the manufacturers' recommendations.

The E1B promoter insertion mutant IN10 (insertion of 10 bp) was created by combining two previously constructed linker scan mutants, LS - 45/ - 36, LS - 35/ - 26 (Wu et al. 1987). The LS mutants have the specified sequence (e.g., from -45 to -36 upstream of the E1B transcription start site) replaced by an *Xho*I linker sequence (CCTCGAGGAA). Fusing the small *Xba*I-*Xho*I fragment of pBE5 dl1500 LS - 35/ - 26 to the large

*Xba*I-*Xho*I fragment of pBE5 dl1500 LS - 45/ - 36 resulted in the 10-bp insertion CCTCGAGGAA at -35. Insertion at -35 does not disrupt either the GC (-49 to -39) or TATA boxes (-30 to -23).

IN5, IN16, and IN22 mutants were all generated from the IN10 mutant. IN5 was generated by cutting IN10 at the *Xho*I site and trimming the protruding single-stranded ends with nuclease S1 (BRL). One clone resulting from the blunt end ligation had five residues removed and was designated IN5. IN16 and IN22 were derived by ligating one or two copies, respectively, of a 6-nucleotide single-stranded linker (Pharmacia) into the *Xho*I site of IN10. The pTCGAAT linker converts the *Xho*I protruding ends to an *Eco*RI site (Barany 1985). The precise sequence of each mutation was determined by the dideoxy-chain termination methods of sequencing (Sanger et al. 1977; Smith 1980). M13 GG1 LS - 48/ - 39 was created by oligonucleotide-directed mutagenesis, as described previously (Zoller and Smith 1983; Wu et al. 1987). A 40-mer oligonucleotide that precisely substituted the 10 residue GC box with an *Xho*I site (TTCTCGAGAA) was utilized to prime the mutagenesis. M13 GG1 LS - 38/ - 31 and M13 GG1 LS - 30/ - 23 were constructed similarly using 40-mer mutagenic oligonucleotides with the multiple-base substitutions near the center.

To facilitate the construction of these E1B promoter mutations into the adenoviral genome, the *Xba*I-*Sac*I fragment of each E1B promoter mutant was cloned between the equivalent sites of pBE1505. This plasmid contains Ad2 sequence (1-3329 nucleotide) from the E1A mutant virus dl1505 (Osborne et al. 1984) inserted into the *Pst*I site of pBR322. Mutant dl1505 has an out-of-frame deletion following amino acid 22 in the E1A proteins. The plasmids thus derived for the mutants are designated pBE1505/IN5, pBE1505/IN10, pBE1505/IN16, pBE1505/IN22, pBE1505/LS - 48/ - 39, pBE1505/LS - 38/ - 31, and pBE1505/LS - 30/ - 23.

Construction of E1B promoter mutant virus

All E1B promoter mutant adenoviruses were generated by *in vivo* recombination after transfection of DNA into 293 cells (Stow 1981). Three micrograms of *Xba*I-digested dl309 (Jones and Shenk 1979b) viral DNA and 5 µg of *Bam*HI-digested pBE1505-derived mutant plasmid DNA were cotransfected. Virus plaques isolated from the transfections were propagated, and viral DNA isolated and digested with *Xho*I and *Eco*RI to identify the desired mutants. Two further rounds of plaque formation on 293 cells were performed for each mutant virus to ensure purity.

Quantitative S1 analysis of E1B RNA

HeLa suspension cells were infected with mutant viruses at 20 plaque forming units (pfu)/cell. To analyze the transcriptional activation effect of E1A or IEP, the E1B promoter mutant viruses were coinfecting with helper adenovirus S13B (20 pfu/cell) or pseudorabies virus (10 pfu/cell), respectively. S13B virus (obtained from J. Sambrook) provided functional E1A protein *in trans*, but its E1B gene contains a small deletion that distinguishes the S13B E1B RNA from the E1B RNA expressed from the E1B promoter mutants (Wu et al. 1987). To commence infection, absorption of adenovirus and pseudorabies virus was performed by adding virus stocks to suspension cultures of exponentially growing HeLa cells concentrated to 4 × 10⁶ cells/ml for 1 hr and 0.5 hr, respectively. Infected HeLa cells were harvested 6 hr postinfection, and cytoplasmic RNA was extracted as described (Berk et al. 1979).

The S1 probe was the Ad2 *Bst*EII (nucleotide 1918) (Gingras

et al. 1982)–*Xba*I (nucleotide 1336) fragment 5' labeled with 32 P at the *Bst*II site (+220 with respect to the E1B cap site). Fifty micrograms of cytoplasmic RNA was hybridized with 75 ng probe fragment at 46°C for 16–20 hr in 50 μ l, as described (Berk and Sharp 1977). S1 digestion was performed by adding 500 μ l of solution containing 0.25 M NaCl, 0.03 M Na acetate (pH 4.5), 1 mM ZnCl₂, 5% glycerol, and 400–500 units of nuclease S1 (BRL) at 22°C for 60 min. Protected probe fragments were separated by electrophoresis on a 5% denaturing polyacrylamide gel. Direct or preflashed–intensified autoradiograms were analyzed with a Hoefer densitometer, and peak integration was performed by computer (Data Acquisition Program for Acorn BBC).

DNase I footprinting competition

The DNase I footprint probes were prepared from clones of Ad2 wild-type or mutant sequences from 1336 (*Xba*I) to 1769 (*Sac*I) into the corresponding sites of pUC18. The plasmids were digested at the *Eco*RI site of the pUC18 polylinker next to the *Sac*I site (+70) of the wild-type E1B promoter fragment. Phosphatase and kinase treatments followed by secondary digestion with *Xba*I produced a probe labeled at the *Eco*RI site next to +70 on the transcribed strand extending to –363. The concentrations of the competing fragments were quantitated carefully by spectrophotometry and checked by gel electrophoresis and ethidium bromide staining of aliquots.

Partially purified Sp1 (the 0.3 M KCl eluate from heparin agarose) was prepared according to Briggs et al. (1986) and used in the analysis of Figure 4. Extensively purified Sp1 prepared by three passages over a specific-sequence DNA affinity column (Kadonaga and Tjian 1986) was used in the experiments of Figure 3. Binding reactions were carried out with 10 fmole probe, 3.5 μ g of Sp1 fraction, and 1 μ g of [poly(dI-dC)]·[poly(dI-dC)] (BRL) in a 50- μ l volume, as described (Jones et al. 1985). Titration of the Sp1 fraction showed that 1.4 μ g was sufficient to protect the probe completely. Reactions were incubated on ice for 15–20 min with or without the cold competitor fragments, followed by 2 min at room temperature. DNase I digestion was carried out for 1 min at room temperature by adding 2.5–4 ng DNase I (Worthington, freshly diluted) in 50 μ l of 5 mM CaCl₂, 10 mM MgCl₂. The reactions were processed further, as described (Jones et al. 1985), and resolved on an 8% polyacrylamide sequencing gel.

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Constraints on spacing between transcription factor binding sites in a simple adenovirus promoter.

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