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Sp1 Activates Transcription without Enhancing DNA-Binding Activity of the TATA Box Factor

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We have studied the interactions of the Sp1 and IID transcription factors with a simple RNA polymerase II promoter. The adenovirus E1B core promoter consists essentially of a GC box and a TATA box, binding sites for the Sp1 and IID transcription factors, respectively. The E1B promoter is accurately transcribed in vitro using a mammalian transcription system. Sp1 activates E1B transcription in vitro in reactions using IID factor isolated from either human or yeast cells. In DNase I footprinting studies, Sp1 bound rapidly to its recognition sequence even at 0°C ($t_{1/2}$ < 1 min). In contrast, yeast IID bound more slowly ($t_{1/2}$ ~ 6 min at 25°C) and required thermal energy for stable binding to the TATA box sequence. Dissociation rates were measured by the addition of specific oligonucleotide competitors to preformed DNA-protein complexes. Sp1 dissociates rapidly ($t_{1/2}$ < 1 min) at 25°C, while yeast IID dissociates with an estimated $t_{1/2}$ of 1 h at 25°C. Sp1 and yeast IID bound to the E1B promoter simultaneously but independently. The rates of binding and dissociation of these factors were not significantly affected by the presence of the other factor. Bound Sp1 factor did not alter or enhance the yeast IID footprint. Oligonucleotide challenge of in vitro transcription reactions indicated that Sp1 also did not enhance the binding of the human IID factor to the E1B promoter. Thus the Sp1 factor activates transcription of the E1B gene by a mechanism that does not enhance the DNA-binding activity of the IID factor. Sp1 factor activates E1B transcription by 5- to 10-fold in vitro. Under these in vitro transcription conditions, transcripts due to reinitiation from an individual promoter complex contribute only a small portion of the total yield of E1B transcripts. Thus Sp1 cannot activate transcription by increasing the rate of initiation events per complex. Instead it appears that Sp1 acts by increasing the number of productive transcription complexes formed in vitro.

Gene expression is most often controlled through the regulation of transcription initiation (22). However, a detailed understanding of the molecular mechanism of transcription initiation and its regulation is not yet complete. The initiation reaction at eucaryotic mRNA genes involves the assembly at the promoter site of a multicomponent complex containing a molecule of RNA polymerase II, a set of general transcription factors (IIA, IIB, IIE, IIF, and IID), and one or more of a set of gene-specific transcriptional activators (9, 11). The availability of in vitro transcription systems as well as the purification of some transcription factors has allowed extensive characterization of the initiation reaction.

Gene-specific transcriptional activators are thought to enhance transcription by binding to their DNA recognition sequences at the promoter and thus bringing an "activation domain" to the proximity (26). In some cases the DNA-binding domain and the activation domain can be physically separated and even mixed in protein fusion experiments (3, 14). Thus gene-specific transcription factors have at least two activities, a sequence-specific DNA-binding activity and a transcriptional activation activity. Still it is not at all clear how these activation domains stimulate transcription. Do activation domains interact with the polymerase molecule, with the TATA box factor (IID), or with other transcription factors? Do they affect the formation of the transcription complex, or do they affect the number of initiation events per complex? We have tried to answer some of these questions by studying a relatively simple eucaryotic polymerase II promoter.

The adenovirus type 2 E1B promoter contains essentially two *cis*-acting promoter elements, a TATA box and a GC box. These elements constitute binding sites for the IID and Sp1 transcription factors, respectively. Both of these elements are required for wild-type levels of transcription in vivo and in vitro (25, 33). Further upstream (−250 to −125 base pairs) there are additional binding sites for transcription factors; however, these distal elements make only a modest contribution to transcription in vivo (31) and have no effect on the wild-type core promoter in vitro (25). For that reason we have focused our attention on the 60 base pairs (bp) composing the E1B core promoter and, in particular, on the DNA-binding and transcriptional activities of the Sp1 and IID factors.

The Sp1 factor activates transcription from a number of cellular and viral polymerase II genes which contain its DNA recognition sequence, the GC box. Sp1 activity is contained in two metalloproteins (M_r 105,000 and 95,000) which are posttranslationally modified by the addition of O-linked *N*-acetylglucosamine (17). Sp1 can readily be purified from HeLa cells (4), and its cDNA has been cloned and sequenced (19). The Sp1 protein seems to fit well to the two-domain model of gene-specific transcription factors. The DNA-binding domain, including three sequences homologous with the zinc finger motif, is encoded by the C-terminal 168 amino acids and is by itself not sufficient to activate transcription. The transcriptional activation domain is contained in glutamine-rich sequences of the N-terminal 400 amino acids (8). While much has been learned about the structure of the Sp1 protein, the mechanism by which its activation domain stimulates transcription is still not defined.

The TATA box factor (IID) is usually considered a general transcription factor since its recognition sequence is present

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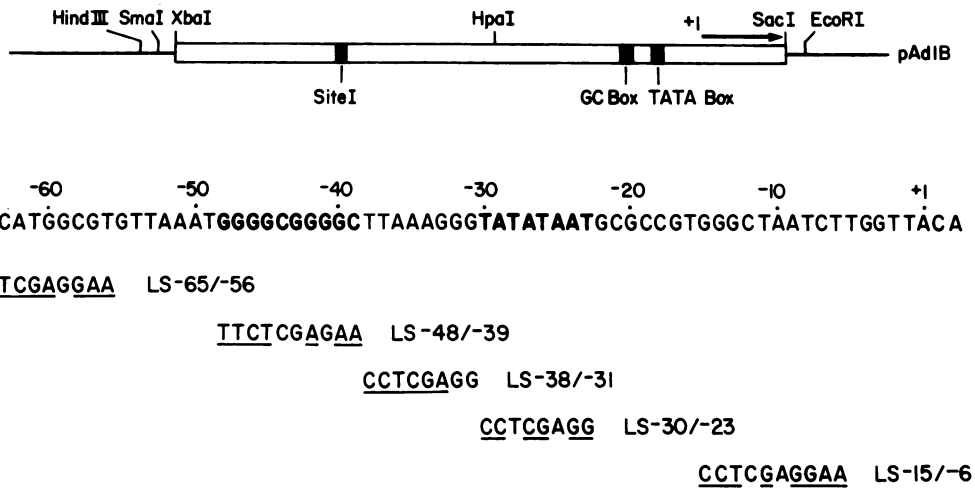

FIG. 1. Adenovirus E1B promoter and linker scan mutations. Plasmid pAd1B containing the E1B promoter of adenovirus type 2 inserted into pUC18 is diagrammed schematically. The TATA box sequence (−30 to −23), the proximal GC box (−48 to −39), and site I (−250 to −241 [25]) are shown as solid bars. The E1B cap site (+1) is located at nucleotide 1699 of the adenovirus type 2 genome (1, 16). All restriction sites shown cut only once in pAd1B. The sequence of the adenovirus E1B core promoter is shown with the GC box and TATA box sequences in boldface type. The sequences of the linker scan mutations used in this study are shown; the nucleotides that are different from wild type are underlined.

in most polymerase II promoters. IID isolated from HeLa cells (HIID) is required for in vitro transcription of polymerase II genes and binds to the TATA box sequence (28). While the IID factor has been partially purified from HeLa cells (23), the polypeptide has not yet been identified or cloned. Our own preparations of HIID are active when assayed for the reconstitution of in vitro transcription; however, we consistently fail to detect any TATA box binding activity by using DNase I footprinting experiments. For this reason, we have also isolated IID from the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is a convenient source of this factor since yeast IID (YIID) is easy to purify, complements mammalian in vitro transcription systems, and readily binds to the TATA box sequence in DNase I footprinting experiments (6).

We have used highly purified preparations of YIID and Sp1 as well as partially purified HIID to analyze the DNA-binding and transcriptional activities of these factors on the adenovirus E1B promoter. Our results indicate that Sp1 does not activate transcription by altering the DNA-binding activity of the IID factor. However, it does appear to increase the number of productive transcription complexes formed in vitro.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The plasmid pAd1B (Fig. 1) contains the adenovirus type 2 *Xba*I-to-*Sac*I fragment (nucleotides 1336 to 1767 [16]) inserted into the *Xba*I and *Sac*I sites of pUC18 (24). The same *Xba*I-*Sac*I fragment containing an E1B promoter linker scan mutation (32, 33) was likewise inserted into pUC18 and designated pAd1BLS−65/−56, for example. A smaller plasmid, designated pAd1BΔ, lacks the upstream distal binding sites (25) and was constructed by cleavage of pAd1B at the *Hpa*I site (adenovirus nucleotide 1561) and the *Sma*I site in the polylinker, followed by religation. To generate an E1B promoter with a distinguishable primer extension product, plasmid pAd1BΔ was cleaved with *Eco*RI and *Sac*I, treated with mung bean nuclease, and religated to yield a plasmid with a 10-bp deletion, designated pAd1BΔRS.

Oligonucleotide B_{1750} (oligo B_{1750}) and oligo UC_{475} were used for primer extension assays. Oligo B_{1750} was identical to the bottom strand of adenovirus type 2 nucleotides 1750 to 1731: 5′-GCACAGCAGAAAATCTTCC-3′. Oligo B_{1750} was complementary to RNA synthesized in vivo and in vitro and yielded a 52-base primer extension product. Oligo UC_{475} contained sequences 475 to 450 of pUC18: 5′-AGGAAA CAGCTATGACCATGATTACG-3′. Oligo UC_{475} was complementary to E1B RNA synthesized in vitro and yielded a 105-nucleotide primer extension product from pAd1B and a 95-base primer extension product from plasmids bearing the 10-bp *Eco*RI-to-*Sac*I deletion.

The double-stranded TATA box oligonucleotide was formed by the hybridization of two complementary 29-mers with the following sequences: TATA 1, 5′-TCGACTTAA AGGGTATATAATGCGCCGTG-3′, and TATA 2, 5′-TC GACACGGCGCATTATATACCCTTTAAG-3′. The double-stranded Sp1 oligonucleotide was formed by the hybridization of two complementary 14-mers with the following sequences: Sp1, 5′-TCGAGGGGCGGGGC-3′, and Sp2, 5′-TCGAGCCCCGCCCC-3′. The double-stranded oligonucleotide was then phosphorylated and ligated to yield multimers of the Sp1-binding site. An oligonucleotide used as a negative control contained sequences from the *Xenopus* somatic 5S gene and was formed by the hybridization, phosphorylation, and ligation of two complementary 25-mers with the following sequences: 5S1, 5′-TGCTCGCCTACGGCCATA CCACCCT-3′, and 5S2, 5′-CGAGCAAGGGTGGTATGGC CGTAGG-3′.

Extracts and factors. HeLa cell nuclear extracts were prepared and chromatographed on phosphocellulose (9). Sp1 was purified by chromatography on Sephacryl S-300 followed by three successive passes over an oligonucleotide affinity column (17). HIID was prepared as described by Nakajima et al. (23); the DEAE fraction was used in these studies. YIID was prepared by the method of Buratowski et al. (6); the Superose 12 fraction was used in these studies.

Transcription assays. Transcription reactions (50 μl) contained 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 60 mM KCl, 12% (vol/

vol) glycerol, 7.5 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.6 mM of each of the four ribonucleoside triphosphates, 2 μg of supercoiled plasmid, and 50 μg of nuclear extract protein. Reactions with reconstituted factors contained, in place of nuclear extract, 5 μg of phosphocellulose B fraction, 5 μg of phosphocellulose C fraction (9), and either 7 μg of HIID or 0.06 μg of YIID. Reactions were incubated at 30°C for 1 h, after which 0.1 ml of stop mix (18) was added. RNA was purified by extraction with an equal volume of phenol-chloroform (1:1), precipitated with ethanol, and dried. RNA pellets were suspended in 10 μl of a solution containing 10 mM Tris hydrochloride (pH 8), 1 mM EDTA, 0.25 M KCl, and 100 fmol of oligonucleotide primer. Primers were phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP and had a specific activity of $\sim 6 \times 10^3$ cpm/fmol. Reactions were incubated at 80°C for 5 min and then at 37°C for 30 min. A 25-μl volume of a solution containing 20 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.3 mM of each of the four deoxyribonucleoside triphosphates, and 10 μg of actinomycin D per ml was added, and the reaction was then warmed to 45°C. Twenty units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) was added, and the reaction was incubated for an additional 30 min at 45°C. Primer extension products were collected by ethanol precipitation, suspended in formamide, and resolved on 10% polyacrylamide–8 M urea gels.

E1B RNA synthesized in vivo was prepared from adenovirus-infected HeLa cells (33). Total cytoplasmic RNA (100 μg) was primer extended as described above except that the hybridization and primer extension reactions were in twice the volume as was used for in vitro transcription reactions. The RNA was digested with RNase before gel electrophoresis to prevent distortion of the mobility of the primer extension products.

DNase I footprinting assays. Footprinting assays were conducted essentially as described by Jones et al. (18). Reactions (50 μl) contained 12 mM HEPES-KOH (pH 7.9), 12% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.6 mM dithiothreitol, 1% (vol/vol) polyvinyl alcohol, and ~2 ng of end-labeled DNA probe. No carrier DNA was included in these reactions. Reactions with YIID contained 1 μl of the Superose 12 peak fraction (~0.03 mg of protein per ml). Reactions with Sp1 contained 5 μl of the third-pass affinity fraction (<0.01 mg of protein per ml). Proteins were added on ice and then incubated for 1 h at 25°C unless indicated otherwise. An equal volume of 10 mM $MgCl_2$–5 mM $CaCl_2$ was added immediately before the addition of 1 μl of freshly diluted 1-μg/ml DNase I (Worthington). After 1 min at 25°C, DNase I digestion was stopped with the addition of 0.1 ml of stop mix (18). DNA pellets were suspended in 3 μl of formamide–0.1 M NaOH (2:1) and subjected to electrophoresis on 6% polyacrylamide–8 M urea sequencing gels.

E1B probes were prepared by cleavage of pAd1B wild-type or linker scan promoter plasmids with *Eco*RI (Fig. 1). The top strand was labeled by filling in the *Eco*RI termini with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [α-^{32}P]dATP and [α-^{32}P]dTTP (see Fig. 3, 4A, 5, and 6B). The bottom strand was labeled by treatment of the *Eco*RI termini with calf intestine alkaline phosphatase and phosphorylated by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (see Fig. 6A). In both cases, DNAs were then cleaved with *Hin*dIII and resolved on a nondenaturing 5% polyacrylamide gel. The 450-bp fragment was excised and eluted. Detailed procedures for the purification and labeling of plasmid DNAs have been described (21).

RESULTS

In vitro transcription of the E1B promoter. To establish the relevance of these in vitro studies to in vivo transcription, we analyzed transcription from the wild-type E1B promoter and a set of linker scan mutations which have been previously studied in vivo (32, 33). Accurate in vitro transcription of the adenovirus E1B promoter by HeLa whole-cell extracts has been described using runoff (10) and S1 nuclease (25) assays. In these studies, we used HeLa cell nuclear extracts (9) followed by primer extension analysis. The adenovirus E1B promoter, the *Xba*I (−362)-to-*Sac*I (+67) fragment, was cloned into pUC18, and the supercoiled plasmid was used as a template. A ^{32}P-labeled oligonucleotide complementary to E1B mRNA at positions +52 to +33 was used to primer-extend RNA synthesized in vitro as well as cytoplasmic RNA isolated from adenovirus-infected HeLa cells. Both RNA samples yielded a predominant primer extension product which maps to the adenosine residue at position 1699 of the adenovirus type 2 sequence (Fig. 2A). Thus HeLa cell nuclear extracts accurately initiate transcription in vitro at the same site utilized in vivo (1).

Accurate initiation of E1B transcription was also reconstituted with transcription factors separated by chromatography on phosphocellulose as described by Dignam et al. (9). The phosphocellulose B and C fractions supported E1B transcription only when a source of the IID factor was provided (Fig. 2B). Accurate initiation at nucleotide 1699 occurred regardless of whether the IID factor was isolated from HeLa or yeast cells. Transcription of plasmids containing the E1B promoter with linker scan mutations through the promoter region gave results very similar to those observed in vivo (32, 33). The TATA box sequence was essential for in vitro transcription, whereas in vivo the LS−30/−23 TATA mutation reduced transcription to about 2% of that observed from the wild-type promoter. The LS−48/−39 mutation of the GC box reduced in vivo transcription by a factor of 5 to 10 (33), and it had a similar effect on in vitro transcription (Fig. 2B). The LS−38/−31 mutation was reduced in transcriptional activity in vitro and in vivo due to reduced affinity for Sp1 (32; see below). One might expect that the human Sp1 factor would only activate transcription when a completely homologous transcription system was used. However, Sp1 activation of transcription was detected in reactions with both the HIID and YIID factors. In fact, transcription with YIID appeared even more dependent on the Sp1 factor than reactions with the HIID factor. From these data it appeared that YIID was functionally indistinguishable in vitro from HIID. We think it likely that Sp1 activates transcription of the E1B promoter via the same mechanism regardless of the source of the IID factor.

DNA-binding properties of Sp1 and YIID. To study the mechanism by which Sp1 stimulates E1B transcription, the binding of Sp1 and YIID to the E1B promoter was analyzed by DNase I protection assays. Sp1 protected ~16 bp of DNA, centered over the GC box, from DNase I digestion (33; Fig. 3). As first noted by Parks et al. (25), Sp1 also bound to a distal GC box, which they designated as site I, located ~245 bp upstream of the E1B start site (Fig. 3). The YIID factor bound with highest affinity to the E1B TATA box and protected a ~16-bp region of DNA. In the presence of excess YIID, an additional sequence upstream of the GC box was weakly protected from DNase I cleavage. This

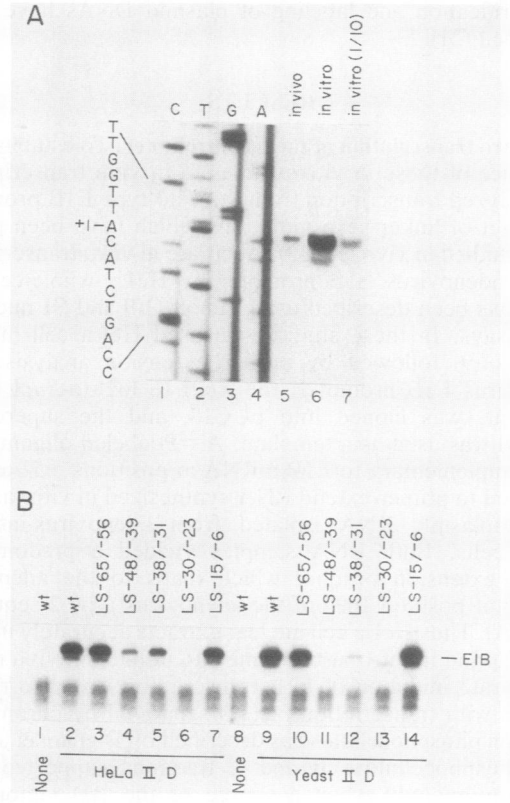

FIG. 2. Primer extension analysis of E1B RNA. (A) RNA synthesized in vitro with HeLa cell nuclear extract and pAd1B template (lanes 6 and 7) and cytoplasmic RNA isolated from adenovirus-infected HeLa cells (lane 5) were analyzed by primer extension of oligo B_{1750}. Lane 7 contains the primer extension products of 1/10 the amount of in vitro RNA as was analyzed in lane 6. Lanes 1 through 4 show dideoxynucleotide sequencing markers generated with oligo B_{1750} and a single-stranded M13 clone of E1B top-strand DNA. Samples were resolved on an 8% polyacrylamide–8 M urea sequencing gel, and a portion of the autoradiogram is shown. (B) Wild-type and mutant E1B promoters were transcribed in vitro with HIID and YIID. All reactions contained phosphocellulose B and C fractions and 2 μg of the plasmid DNA template indicated above each lane. HIID was added to reactions in lanes 2 through 7, and YIID was added to the reactions in lanes 9 through 14. No IID was added to reactions in lanes 1 and 8. The mobility of the primer extension products of accurately initiated E1B RNA is indicated on the right. Samples were resolved on a 10% polyacrylamide–8 M urea gel, and a portion of the autoradiogram is shown.

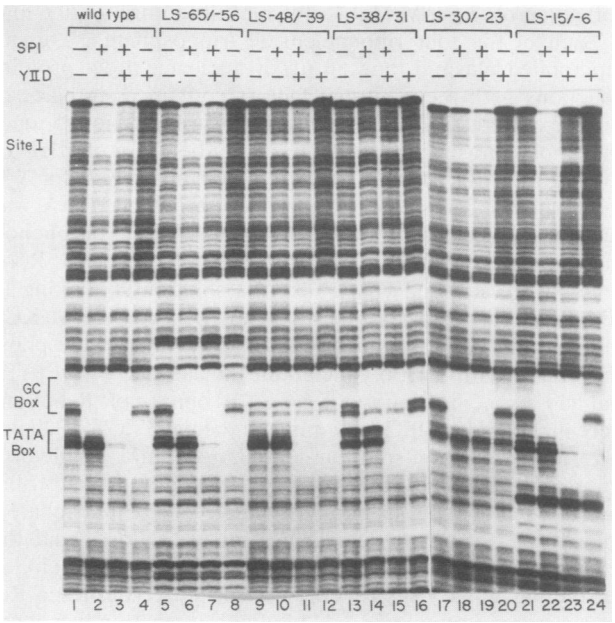

FIG. 3. DNase I footprint analysis of YIID and Sp1 on wild-type and mutant E1B promoters. Each of six DNA probes (indicated above brackets) was used in four reactions with either no protein, YIID, Sp1, or both, as indicated (+ or −) above each lane. The positions of the TATA box, the GC box, and the distal GC box, site I, are indicated on the left. Samples were resolved on 6% polyacrylamide–8 M urea sequencing gels, and the autoradiograms are shown.

sequence contains the A_2UA_3 poly(A) addition signal for the E1A mRNA (TTAATAAA; −94 to −86). When both Sp1 and YIID were present, simultaneous binding was observed and a region of ~34 bp (−52 to −18) was protected from DNase I digestion. The presence of the Sp1 factor did not alter or extend the YIID footprint.

To confirm that the GC and TATA boxes were the recognition sequences of these factors, DNAs with linker scan mutations through the promoter region were end labeled and used as footprinting probes (Fig. 3). As expected, mutations upstream (LS−65/−56) or downstream (LS−15/−6) of the recognition sequences had no effect on Sp1 or YIID binding. Mutation of the GC box (LS−48/−39) eliminated Sp1 binding to the promoter-proximal site but had no effect on Sp1 binding to the distal site I (Fig. 3, lane 10). Mutation of the E1B TATA box (LS−30/−23) eliminated YIID binding to the TATA box but not to the lower-affinity upstream site (Fig. 3, lane 20).

The transcription activity of the linker scan mutants correlated well with the factor-binding results. The linker scan mutations which interfered with the binding of Sp1 (LS−48/−39) or YIID (LS−30/−23) showed reduced levels of in vitro transcription. Mutations which did not affect Sp1 or YIID binding (LS−65/−56 and LS−15/−6) had wild-type levels of in vitro transcription. The LS−38/−31 mutation did show decreased transcriptional activity but did not interfere with the binding of either factor under the conditions used for Fig. 3. This is because the footprinting reactions in Fig. 3 were carried out with excess Sp1 and YIID. When SP1 binding was assayed at low concentrations of Sp1, LS−38/−31 showed a four- to eightfold reduced affinity for the SP1 protein (32) even though the sequences changed are outside the consensus GC box sequence (Fig. 1). The reduced affinity of LS−38/−31 for Sp1 can account for its decreased transcriptional activity, a level intermediate between those of the wild-type template and LS−48/−39, which lacks the GC box entirely.

To characterize the DNA-binding reaction, two experimental parameters were examined. The time and temperature of the binding reaction affected Sp1 and YIID DNA binding quite differently. Sp1 bound to its recognition sequence extremely rapidly. When Sp1 was mixed with the DNA probe at 0°C and the DNase I was added as quickly as possible (less than 30 s after Sp1 addition), a complete footprint was already detected (Fig. 4A). Thus the half-time for Sp1 binding ($t_{1/2}$) was less than 1 min at 0°C and 60 mM KCl. On the other hand, YIID bound to the E1B TATA box much more slowly. No footprinting activity at all was detected when YIID was added and processed as rapidly as possible (0 min; Fig. 4, lane 2), and only a very faint footprint

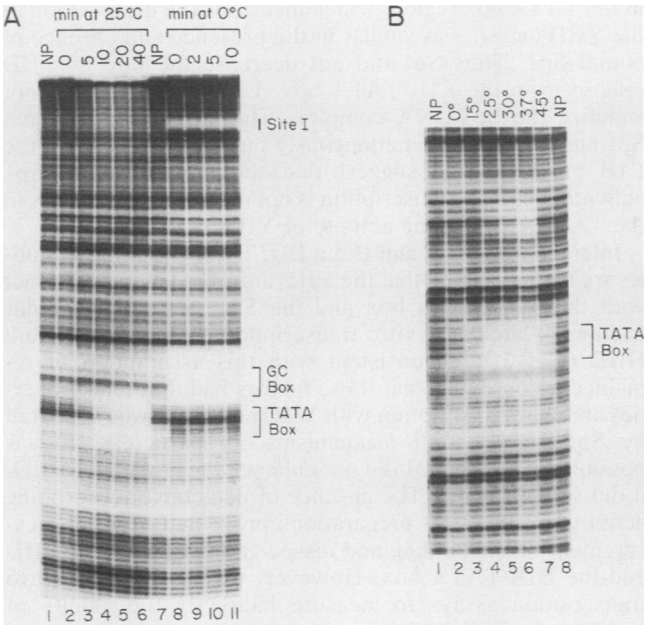

FIG. 4. Effect of time and temperature on YIID and Sp1 binding. (A) YIID (lanes 2 through 6) and Sp1 (lanes 8 through 11) were incubated with wild-type E1B probe at 25 or 0°C, respectively. The time of the binding reactions in minutes is indicated above each lane. 0 min indicates that reactions were treated with DNase I as rapidly as possible after the addition of protein factor. The actual elapsed time between the addition of protein factor and DNase I was no more than 30 s. NP indicates control reactions with no protein added. The DNA probe contained the wild-type E1B promoter. The positions of the GC box, TATA box, and site I are indicated on the right. Samples were resolved on a 6% polyacrylamide–8 M urea gel, and the autoradiogram is shown. (B) YIID was incubated with DNA probe containing the adenovirus major late promoter for 1 h at the temperatures shown above each lane. NP indicates control reactions with no protein added. The position of the TATA box is indicated on the right. The samples were resolved on a 6% polyacrylamide–8 M urea sequencing gel, and the autoradiogram is shown.

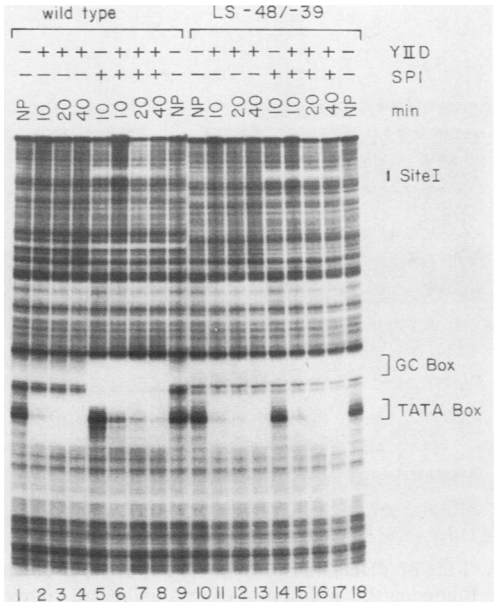

FIG. 5. Rate of YIID binding in the presence and absence of Sp1. Sp1 factor was prebound to the DNA probe for 5 min at 0°C (+) or was omitted (−) as indicated above each lane. YIID was then added, and reactions were incubated at 25°C for 10, 20, or 40 min, as indicated, before treatment with DNase I. Lanes 1 through 9 contained wild-type E1B promoter probe, and lanes 10 through 18 contained LS−48/−39 probe. NP indicates control reactions with no protein added. Samples were resolved on a 6% polyacrylamide–8 M urea sequencing gel, and an autoradiogram is shown.

was detected after 5 min at 25°C. Based on quantitative densitometry of the autoradiogram shown in Fig. 4A, under these conditions (25°C and 60 mM KCl) YIID bound to the E1B TATA box with a $t_{1/2}$ of 6 min.

The temperature of the binding reaction was also examined. Sp1 bound rapidly to its recognition sequence independent of the temperature of incubation in the range of 0 to 37°C. In contrast, YIID showed no sequence-specific DNA-binding activity at 0°C (Fig. 4B). YIID binding occurred only partially when incubated for 1 h at 15°C and was most efficient between the temperatures of 25 and 37°C. The probe used for Fig. 3B contains the major late promoter TATA box, but the results were similar with the E1B promoter. In titration experiments with the major later promoter, YIID bound first to the TATA box (data not shown). In the presence of excess YIID, an additional sequence upstream of the upstream stimulatory factor (USF) binding site was also weakly protected (Fig. 4B, lane 6). This sequence (−70 to −76 relative to the major late promoter cap site at nucleotide 6039 [16]) shares a 6-of-7-nucleotide identity with the major late promoter TATA box (TATAAAA versus TATAAAC) but is not required for optimal transcription rates in deletion analyses of the major late promoter (2, 20).

Effect of Sp1 on YIID DNA binding. One possible mechanism of Sp1-mediated activation of transcription would be to enhance the DNA-binding activity of the IID factor. Cooperative binding has been reported between the upstream factor and the human IID factor on the adenovirus major late promoter (27, 28). The association constant of YIID and the E1B TATA box could be affected by either increasing the rate of binding or decreasing the rate of dissociation. The rate of YIID binding was measured in the presence and absence of Sp1 protein (Fig. 5). The YIID factor was added to E1B probe which either had been prebound by excess Sp1 protein or not. After various times, DNase I was added to assay for YIID protection of the TATA box. It is clear that Sp1 did not increase the rate of YIID binding. In fact, the presence of Sp1 slightly reduced the rate of YIID binding. Thus Sp1 activation of transcription is not mediated by an increase in the rate of YIID binding.

To confirm that the effect of Sp1 on the rate of YIID binding was due to the presence of the Sp1 protein bound to the proximal GC box, YIID binding was also measured on the LS−48/−39 promoter, which lacks the proximal GC box (Fig. 5). The rate of YIID binding to this template was not affected by the presence of Sp1 even though the Sp1 protein bound to the DNA at the distal GC box. Thus the slight reduction of the YIID rate of binding caused by Sp1 was due to the presence of Sp1 at the proximal GC box and was not due to the presence of an inhibitor in our Sp1 preparation.

Effect of Sp1 on the dissociation of YIID. The rate of dissociation of YIID and Sp1 was measured by addition of unlabeled competitor DNA to reactions containing preformed DNA-protein complexes. After various times, DNase I was added to assay for the release of the bound factor from the labeled probe. Synthetic oligonucleotides containing the E1B TATA or GC box sequences were efficient and specific competitors of binding since they

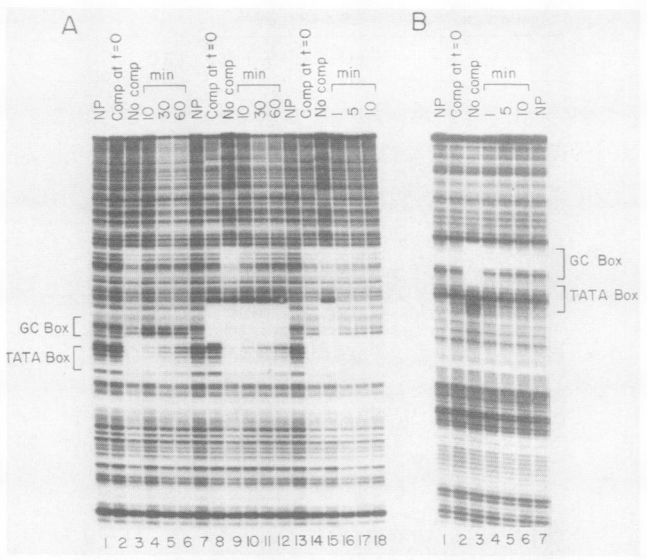

FIG. 6. Rate of YIID and Sp1 dissociation. (A) Dissociation was measured following the addition of excess unlabeled competitor to preformed DNA-protein complexes. Proteins were preincubated at 25°C for 1 h to allow complete binding. Reactions contained YIID alone (lanes 2 through 6) or both SP1 and YIID (lanes 8 through 12 and 14 through 18). TATA oligonucleotide (50 ng) was added to the reactions in lanes 2 and 8 at time 0 or after the preincubation step (at t = 60 min) in lanes 4 through 6 and 10 through 12. GC box oligonucleotide (50 ng) was added at time 0 to the reaction in lane 14 or after the preincubation step to the reactions in lanes 16 through 18. Dissociation of YIID was assayed after 10, 30, and 60 min of incubation in the presence of the TATA oligonucleotide as indicated at the top of the lanes. Sp1 dissociation was measured after incubation for 1, 5, and 10 min in the presence of the GC box oligonucleotide. No competitor DNA was added to the reactions in lanes 3, 9, and 15. NP indicates control reactions with no protein added. The DNA probe contained the wild-type E1B promoter labeled at the *Eco*RI site by treatment with T4 kinase and [γ-^{32}P]ATP. Samples were resolved on a 6% polyacrylamide–8 M urea sequencing gel, and the autoradiogram is shown. (B) Dissociation of the Sp1 protein was measured by the addition of excess unlabeled competitor to preformed DNA-protein complexes. GC box oligonucleotide (50 ng) was added at time 0 to the reaction in lane 2 and after the preincubation step to the reactions in lanes 4 through 6. No competitor was added to the reaction in lane 3. Reactions were incubated with the competitor for 1, 5, and 10 min, as shown above the lanes, before treatment with DNase I. NP indicates control reactions with no protein added. The DNA probe contained the wild-type E1B promoter labeled at the *Eco*RI site by end filling. The fact that the probes in panels A and B were labeled at the same site but on different strands accounts for the different DNase I cleavage patterns.

completely blocked Sp1 and YIID binding to the probe when added to the reaction at time 0 (Fig. 6A, lanes 2, 8, and 14; Fig. 6B, lane 2).

The dissociation rates of YIID and Sp1 were also strikingly different from each other. Quantitative densitometry of the autoradiogram in Fig. 6A showed that YIID dissociated at 60 mM KCl and 25°C with a $t_{1/2}$ of 1 h. In contrast, Sp1 dissociated from the DNA at a rate faster than could be measured by DNase I footprinting ($t_{1/2} < 1$ min). When the two factors were prebound together, their dissociation rates were indistinguishable from those measured alone. The pattern of DNase I cleavage in the TATA box region is altered by the presence of Sp1 due to the presence of an Sp1-enhanced cleavage site in that region. While the patterns are qualitatively different, the rate of appearance of cleavage in the TATA box region, which measures the dissociation of the YIID factor, was similar in the presence and absence of bound Sp1. Thus Sp1 did not decrease the rate of YIID release from the E1B TATA box. Likewise, YIID did not stabilize the Sp1-DNA complex. These data indicate that Sp1 and YIID bind simultaneously but independently to the E1B promoter and suggest that the mechanism of Sp1 activation of E1B transcription is not mediated by changes in the TATA box-binding activity of YIID.

Interactions of Sp1 and HeLa IID. Throughout these studies we have assumed that the YIID and HIID factors interact with the E1B TATA box and the Sp1 factor in a similar fashion. While the in vitro transcription data with YIID and HIID (Fig. 2) are consistent with this assumption, it remained a possibility that these factors had different properties and that transcription with YIID and HIID was activated by Sp1 via different mechanisms. In particular, it was possible that while Sp1 did not enhance the binding of YIID, it did so with HIID. The absence of detectable footprinting activity in our HIID preparations prevented a direct measurement of the binding and dissociation reactions of HIID and the E1B TATA box. However, we have used in vitro transcription assays to measure indirectly the affinity of HIID for the E1B TATA box in the presence and absence of the Sp1 factor.

Plasmids containing the wild-type E1B core promoter and the LS−48/−39 mutation were constructed to have distinguishable primer extension products. The LS−48/−39 plasmid yielded a 105-base primer extension product when a primer complementary to pUC18 sequences downstream of the *Eco*RI site was used (Fig. 7, lane 1). The wild-type promoter construct contained a 10-bp deletion in the pUC18 polylinker region and yielded a 95-base primer extension product (Fig. 7, lane 2). When both plasmids were present during transcription, both primer extension products were detected and the Sp1-mediated activation (sixfold) of the wild-type plasmid was apparent. The binding of HIID to these E1B promoters was challenged by the addition of a synthetic oligonucleotide containing the E1B TATA box sequence. If the affinity of the HIID factor for the TATA box was also unaffected by the presence of the Sp1 factor, then one would predict that the wild-type and LS−48/−39 promoters would be equally sensitive to the oligonucleotide challenge. However, if Sp1 enhanced the binding of HIID, then the wild-type promoter would be more resistant than the LS−48/−39 promoter to the TATA oligonucleotide challenge. The results of this experiment (Fig. 7) showed that both templates were inhibited equally by the TATA oligonucleotide, indicating that the TATA box-binding activity of HIID, like that of YIID, was not enhanced by the presence of bound Sp1.

Sp1 activates transcription by increasing the number of transcription complexes formed. Transcription reactions with both wild-type and LS−48/−39 templates resulted in the synthesis of five- to tenfold more transcripts from the wild-type template than from the GC box mutant. This increased yield of transcripts from the wild-type template could be the result of either a greater number of transcription complexes formed on the wild-type template, more initiation events per individual complex, or some mixture of these two possibilities. To distinguish between these mechanisms, we examined the transcription of the wild-type and GC box mutant templates under conditions that limited the reinitiation reaction either by the addition of Sarkosyl (13) or by restricting the time of transcription to 2 min. If the wild-type template retained the increased yield of transcripts under

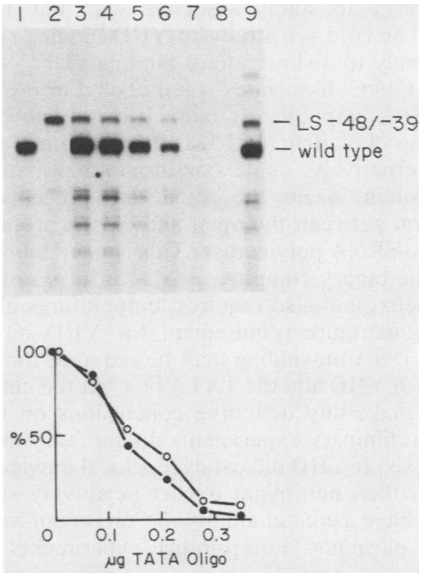

FIG. 7. Oligonucleotide challenge of in vitro transcription reactions. In vitro transcription reactions contained HeLa nuclear extract and 0.6 μg of pAd1BΔRS (lane 1), 0.6 μg of pAd1BΔLS−48/−39 (lane 2), or 0.6 μg of both plasmids (lanes 3 through 9). TATA box oligonucleotide competitor was added at time 0, and reactions were incubated for 1 h at 30°C, followed by primer extension analysis with oligo UC_{475}. The mobilities of the primer extension products from the wild-type and LS−48/−39 templates are indicated on the right. Samples of 0.07, 0.14, 0.21, 0.28, or 0.35 μg of the TATA box oligonucleotide were added to the reactions in lanes 4 through 8, respectively. No competitor DNA was added to the reactions in lanes 1, 2, and 3. A 0.26-μg sample of a control oligonucleotide containing sequences from the *Xenopus* 5S gene promoter was added to the reaction in lane 9. Primer extension products from the wild-type and LS−48/−39 promoters were excised from lanes 3 through 8 and were quantified by liquid scintillation counting. The radioactivity in the primer extension products from the wild-type template (closed circles) and from the LS−48/−39 template (open circles) is shown as the percent of the value obtained in the absence of added competitor and plotted against the micrograms of added TATA oligonucleotide competitor. The values determined for the wild-type and LS−48/−39 primer extension products in the absence of added competitor (lane 3) were 1,457 and 250 cpm, respectively.

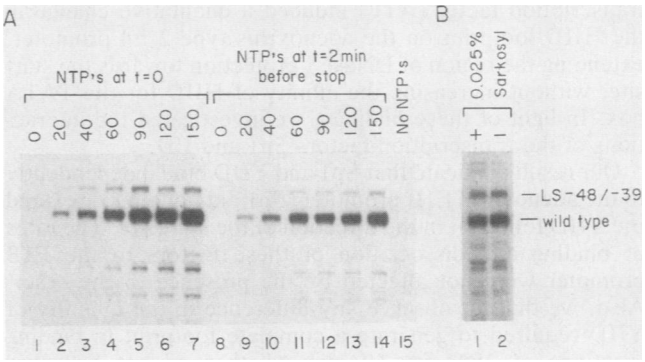

FIG. 8. E1B transcription with limited reinitiation. (A) Plasmids pAd1BΔRS and pAd1BΔLS−48/−39 (0.6 μg of each) were transcribed by HeLa nuclear extract followed by primer extension with oligo UC_{475}. The mobility of the primer extension product from the wild-type and LS−48/−39 templates is indicated on the right. Reactions were incubated at 30°C for the time shown above each lane. Nucleoside triphosphates were added either at time 0 (lanes 1 through 7) or for only the final 2 min of the incubation at 30°C (lanes 8 through 14). In the reaction in lane 15, both plasmids were incubated at 30°C for 1 h in the absence of added nucleoside triphosphates. (B) Reactions containing both plasmids were preincubated with nuclear extract for 60 min at 30°C in the presence of only ATP. Sarkosyl was added or omitted, as indicated above the lanes, to a final concentration of 0.025%. All four nucleoside triphosphates were then added and the reactions were incubated for an additional 20 min at 30°C

these limiting conditions, then Sp1 mediates the activation of E1B transcription by increasing the number of transcription complexes formed. Conversely, if Sp1 mediates an increased rate of initiation events per complex, then we would expect there to be many more transcripts synthesized in reactions that did not limit reinitiation than in reactions that limited reinitiation. Our results support the hypothesis that Sp1 increases the number of transcription complexes formed.

Transcription reactions with both the wild-type and LS−48/−39 templates were incubated at 30°C for increasing lengths of time. Nucleoside triphosphates were added either at time 0 (nonrestricting conditions) or at 2 min before the termination of transcription (restricting conditions). The yield of transcripts from both the wild-type and LS−48/−39 templates was no more than twofold higher under nonrestricting conditions (Fig. 8A, lanes 1 through 7) than under restricting conditions (Fig. 8A, lanes 8 through 14), as measured by quantitative densitometry of the autoradiogram. We conclude that either multiple initiation events from a single complex must be extremely rapid and limited primarily to the first 2 min of transcription or, more likely, that multiple initiation events from a single complex account for only a small portion of the total yield of transcripts under these in vitro transcription conditions. Similarly, reactions in which reinitiation was blocked by the addition of 0.025% Sarkosyl before the addition of nucleoside triphosphates (13) contained almost the same yield of transcripts at reactions which were not treated with Sarkosyl (Fig. 8B). These data also support the conclusion that multiple initiation events from a single transcription complex contribute only a small portion of the total yield of E1B transcripts under these conditions of in vitro transcription. Since reinitiation is only a minor contributor to the yield of transcripts and since the wild-type template produces many more transcripts than the GC box mutant template even under conditions that limit reinitiation, we conclude that Sp1 activates transcription of the E1B gene by increasing the number of productive transcription complexes formed.

DISCUSSION

How do transcriptional activators work? It is now clear that the act of DNA binding is not in and of itself sufficient to activate transcription (26). Truncated forms of Sp1 (8) and GCN4 (14) retain the ability to bind to DNA in a sequence-specific manner but are unable to activate transcription. These factors require additional protein sequence, the "activation domain," to activate transcription. The function of the DNA-binding domain is to discriminate between promoters and to position the activation domain at the promoter. One mechanism for the action of the activation domain is to interact directly with the TATA box factor. Sawadogo and Roeder (28) first noted an effect of the HIID factor on the dissociation rate of the USF from the adenovirus major late promoter. Subsequently, Sawadogo confirmed the existence of a direct interaction between HIID and highly purified USF (27). Horikoshi et al. (15) have demonstrated that activating

transcription factor (ATF) induced a qualitative change in the HIID footprint on the adenovirus type 2 E4 promoter, extending the region of DNase I protection towards the start site, without increasing the affinity of HIID for the TATA box. In light of these findings, we investigated the interactions of the transcription factors Sp1 and IID.

Our results indicate that Sp1 and YIID bind independently to the adenovirus E1B promoter. Sp1 did not alter or extend the YIID footprint in the direction of the start site. The rates of binding and dissociation of these factors to the E1B promoter were not affected by the presence of the other. Also, we did not observe any difference in the quantity of YIID required to generate a complete footprint in binding reactions at 25°C for 60 min whether or not Sp1 was prebound to the GC box (M. C. Schmidt and A. J. Berk, unpublished results). It could be argued that HIID is affected by Sp1 differently than is the YIID factor. However, the oligonucleotide competition experiments with HIID indicated that Sp1 did not enhance the binding of the HIID factor to the E1B TATA box. Thus the mechanism by which Sp1 activated transcription in vitro did not involve a direct interaction with the TATA box factor in a way that affects the DNA-binding activity of the TATA factor. Instead, it appears that Sp1 facilitates the formation of active transcription complexes by increasing the binding or activity of another factor (IIA, IIB, IIE, or IIF) or the RNA polymerase II molecule, a mechanism of transcription activation also proposed for the ATF protein (12, 15).

The finding that some transcriptional activators interact with the TATA box factor, while Sp1 does not, need not be viewed as a contradiction. The generation of complex networks of gene expression may require the ability to regulate different steps in the transcriptional initiation process. Courey and Tjian proposed that transcriptional activators whose activation domains are rich in acidic amino acids may act at a different step or with different factors than do those activators, such as Sp1, which contain glutamine-rich activation domains (8). Our work and that of Sawadogo and Roeder (27, 28) lend support to the proposal that the initiation reaction may be regulated at different steps or through different factors.

One of the interesting findings to come out of this study is the great difference in the DNA-binding properties of the Sp1 and YIID factors. Sp1 binds to its recognition sequence extremely rapidly ($t_{1/2} < 1$ min) even at 0°C. Rapid sequence-specific DNA binding has been noted for the mammalian factor ATF (15) and has been well documented for other sequence-specific DNA-binding proteins such as the procaryotic *lac* repressor. One-dimensional diffusion or sliding allows proteins to locate their recognition sequences extremely rapidly. In the case of the *lac* repressor, DNA sequences are searched at an approximate rate of 10^3 bp/s at 24°C (29). The rapid rates of binding of Sp1 and ATF make it seem likely that they use a one-dimensional diffusion mechanism for the location of their recognition sequences, although further studies of the kinetics of binding will be needed to establish this point. In addition to rapid DNA binding, Sp1 also dissociates from its binding site rapidly following addition of a high concentration of unlabeled binding sites. The rapid dissociation of Sp1 from its binding site in the presence of competitor binding sites may be accounted for by intersegment transfer (30). The USF factor dissociates rapidly via intersegment transfer in the presence of competitor DNA but much more slowly in the absence of competitor DNA (27).

YIID, on the other hand, requires more time as well as thermal energy to stably associate with the TATA box sequence. The cold sensitivity of YIID binding (Fig. 4B) is not due merely to a slow rate of binding at 0°C since YIID prebound at 30°C dissociates when cooled to 0°C (Q. Zhou and A. J. Berk, unpublished data). It is not known whether the slow step of binding at 25°C is the location of the TATA box or, alternatively, some conformational change in the DNA or protein. Again, the procaryotic precedent may be the transition between the open and closed promoter complex of *E. coli* RNA polymerase. This isomerization reaction stabilizes the binary complex, produces local unwinding of the DNA helix, and also requires temperatures above 20°C (7). The temperature requirement for YIID binding suggested that DNA unwinding may be required for the stable association of YIID and the TATA box. At the current time, we cannot make any definitive conclusions on this point; however, preliminary experiments do not favor the unwinding hypothesis. If YIID did catalyze a local unwinding of the DNA helix, then one might expect negatively supercoiled plasmids to have a greater affinity for YIID than would linear or relaxed plasmids. Footprinting experiments in which supercoiled or linear DNAs were used as competitors showed no significant differences in affinity for YIID (Schmidt and Berk, unpublished results).

The YIID factor also has a somewhat relaxed sequence specificity. YIID bound with highest affinity to functional TATA box elements, but it also bound to other AT-rich sequences present on the E1B and major late promoter probes. The exact sequence requirements for YIID binding are not well defined since there were other AT-rich sequences present in the E1B and major late probes which were not bound by YIID. In addition, the synthetic polynucleotide poly(dA-dT)-poly(dA-dT) was a potent competitor of YIID binding (unpublished results). This relaxed sequence specificity may not be too surprising since there is no strict sequence identity between TATA box elements of different genes (5). The availability of highly purified yeast TATA factor will allow detailed analysis of its DNA-binding specificity and the role it plays in the initiation of transcription.

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