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Anatomy of an Unusual RNA Polymerase II Promoter Containing a Downstream TATA Element

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The adenovirus type 2 IVa_2 promoter lacks a conventional TATA element yet directs transcription from two closely spaced initiation sites. To define elements required for in vitro transcription of this promoter, IVa_2 templates carrying 5′ deletions or linker-scanning mutations were transcribed in HeLa whole-cell extracts and the transcripts were analyzed by primer extension. Mutation of the sequence centered on position −47, which is specifically recognized by a cellular factor, reduced the efficiency of IVa_2 transcription two- to threefold, whereas mutation of the sequence centered on position −30 selectively impaired utilization of the minor in vivo initiation site. Utilization of the major in vivo site was decreased no more than fivefold by deletion of all sequences upstream of position −15. By contrast, mutation of the region from +13 to +19 or of the initiation region severely impaired IVa_2 transcription. The sequence spanning the initiation sites was sufficient to direct accurate initiation by RNA polymerase II from the major in vivo site. Thus, the two initiation sites of the IVa_2 promoter are specified by independent elements, and a downstream element is the primary determinant of efficient transcription from both of these sites. The downstream element identified by mutational analysis altered the TATA element-like sequence TATAGAAA lying at positions +21 to +14 in the coding strand. Transcription from the wild-type IVa_2 promoter was severely inhibited when endogenous TFIID was inactivated by mild heat treatment. Exogenous human TATA-binding protein (TBP) synthesized in *Escherichia coli* restored specific IVa_2 transcription from both initiation sites when added to such heat-treated extracts. Although efficient IVa_2 transcription requires both the downstream TATA sequence and active TFIID, bacterially synthesized TBP also stimulated the low level of IVa_2 transcription observed when the TATA sequence was mutated to a sequence that failed to bind TBP.

Specific initiation of transcription by eukaryotic RNA polymerase II (RNApII) depends on the concerted action of several accessory factors. In addition to general factors, which apparently do not recognize specific sequences in the promoter (44, 46), initiation of transcription from the majority of RNApII promoters requires the specific binding of TFIID to a TATA element (16, 38, 45) located between positions −20 and −35 (10, 48). Such TATA elements, and hence TFIID, are important determinants of the rate of initiation of transcription and, in some cases, the site at which RNApII begins transcription (for reviews, see references 10, 15, and 34). Binding of TFIID to the TATA element is an early, and committing, step in the formation of initiation complexes in vitro (see references 34, 46, and 44), and this reaction can be facilitated by certain cellular and viral transactivating factors (for examples, see references 1, 26, 27, 41, 56, and 57). Despite the critical role played by binding of TFIID, many promoters transcribed by RNApII lack TATA elements. One class of such promoters are G-C rich, contain multiple Sp1-binding sites, and generally direct initiation of transcription from multiple, relatively dispersed sites (reviewed in references 17 and 55). Transcription of a synthetic promoter of this kind (G_6I) has recently been shown (42) to require a multiprotein TFIID complex containing the 38-kDa human TATA-binding protein (TBP) (24, 30, 39). This TFIID complex must assemble into the initiation complex by protein-protein interaction(s), for the G_6I promoter cannot bind TBP or human TFIID (41, 42).

Promoters composing a second class that also lack TATA elements do not appear to be Sp1 dependent and direct initiation of transcription from one or a few closely spaced sites (see reference 50). The initiator element of at least one such promoter, that of the terminal deoxynucleotidyl transferase gene, is sufficient to direct specific initiation by RNApII (50). The upstream sequence of the adenovirus type 2 (Ad2) IVa_2 gene also lacks a TATA element and, despite a G-C-rich element between positions −48 and −39 (35), binding sites for Sp1. On the other hand, this viral promoter contains the sequence TATAGAAA at positions +21 to +14 of the transcribed strand, which is specifically bound by yeast TBP synthesized in *Escherichia coli* (13). Moreover, efficient transcription from one of the two IVa_2 initiation sites (6) in reconstituted systems requires partially purified human TFIID or yeast TBP (12, 13). These properties suggest that the Ad2 IVa_2 transcriptional control region represents the first encountered member of a new class of RNApII promoters, those with a functional TATA element within the transcribed sequence.

In the adenovirus genome, the two IVa_2 initiation sites, which are separated by 1 bp (6), lie just over 200 bp upstream of the initiation site of the major late (ML) promoter (Fig. 1A). The divergent ML and IVa_2 promoters are both transcriptionally active during the late phase of infection; indeed, IVa_2 transcription is not activated until this phase of the infectious cycle (see reference 18). Nevertheless, it has been reported that these promoters compete in in vitro assays for one or more components of the transcription machinery (12, 36). To obtain a better understanding of the mechanisms that mediate and regulate IVa_2 transcription, we wish to define in a systematic way the elements composing this unusual promoter and to determine their functional

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roles. As a first step toward this goal, we have examined the effects of both 5′ deletion and clustered point mutations on the efficiency of transcription from the two IVa_2 initiation sites in an in vitro system. We now report the results of this mutational analysis and those of experiments to identify cellular factors required for IVa_2 transcription.

MATERIALS AND METHODS

Construction of IVa_2 promoter mutants. The parental plasmid for the construction of IVa_2 5′ deletion mutants was pIVn, which contains, at the *Hin*dIII site of pBR322, the Ad2 sequence from genomic positions 2798 to 6084, with the *Sca*I site at position 6084 replaced by a *Sal*I linker. To construct pIVn, a 762-bp *Pvu*II fragment containing coding sequences of the Neo^r gene was inserted at the IVa_2 *Xho*I site at position 5778 in the Ad2 sequence. The sequence of the Neo^r-IVa_2 junction closest to the IVa_2 initiation sites was determined by dideoxy sequencing (58). The parental plasmid for construction of an IVa_2 3′ deletion library was pHindIIIC, which contains the Ad2 sequence from 2798 to 6231 cloned into the *Hin*dIII site of pBR322. Libraries of 5′ and 3′ deletions were generated by *Bal* 31 exonuclease digestion followed by the addition of *Sal*I and *Xho*I linkers, respectively. The exact endpoints of the *Bal* 31 deletions and linker ligation were determined by dideoxy sequencing. Fifteen of the 5′ deletion mutants were subcloned to restore Ad2 sequences downstream of position 5778 and were used in the construction of linker-scanning (LS) mutants. A series of IVa_2 upstream LS mutants, pIVLS, was constructed from pairs of 5′ and 3′ deletion parents with endpoints separated by approximately 10 bp by using the compatible *Sal*I and *Xho*I sites added at the 5′ and 3′ endpoints, respectively. The resulting pIVLS series plasmids contain Ad2 sequences from positions 5362 to 6231 with clustered point mutations, together with small insertions or deletions, in the region upstream of the IVa_2 transcription initiation sites (Fig. 2A). The IVa_2 internal LS mutants, the pIVnLS series, were constructed in a similar fashion, except that pIVn 5′ deletion mutants were used as the 5′ deletion parents. This series of mutants contain Ad2 DNA sequences from positions 2798 to 6231, the Neo^r fragment insertion described previously, and LS mutations at or downstream of the IVa_2 transcription initiation sites (Fig. 4A). Construction of pIV-InR, which contains the IVa_2 sequence from −9 to +13 in pUC19, will be described elsewhere (14a).

Manipulation and cloning of DNA were by using standard procedures (4, 32). The positions of all mutations were determined by dideoxy sequencing (58) with avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.). Plasmid DNA was isolated by using standard procedures (32) and purified by centrifugation to equilibrium in $CsCl_2$ gradients containing ethidium bromide and chromatography on Bio-Gel A150m (Bio-Rad).

Cell culture and preparation of transcription extracts. HeLa cells were maintained in suspension culture at a density of 2×10^5 to 3×10^5 cells per ml in SMEM (GIBCO) supplemented with 5% calf serum (Flow Laboratories) and 1% glutamine. For the preparation of extracts, cells were harvested 6 h after dilution to 2.5×10^5 to 4×10^5 cells per ml. Whole-cell extracts were prepared as described previously (41) and dialyzed against 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9) containing 0.04 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 17% glycerol. In some experiments (see the text), KCl was replaced by 0.04 M $(NH_4)_2SO_4$. High-efficiency nuclear extracts were prepared according to the protocol of Shapiro et al. (47). Extracts were quick-frozen in small portions and stored at −80°C. Protein concentrations were determined by using the method of Bradford (9).

In vitro transcription. Unless otherwise stated, transcription reaction mixtures with IVa_2 templates contained, in a 50-μl volume, 0.012 M HEPES (pH 7.9), 0.067 M KCl, 6.7 mM $MgCl_2$, 0.06 mM EDTA, 1.2 mM dithiothreitol, 10% glycerol, 0.6 mM (each) ATP, CTP, GTP, and UTP, 10 to 20 μg of circular template DNA per ml, and 6.4 to 8.0 mg of extract protein per ml (31). Incubation was at 30°C for 60 min, and reactions were terminated by the addition of 5 μl of proteinase K (1 mg/ml) in 5% sodium dodecyl sulfate (SDS) to the mixture. Following incubation at 37°C for 20 min, 0.3 ml of urea buffer (25) and 20 μg of glycogen were added to each reaction mixture. Nucleic acids were purified by phenol-chloroform extraction and concentrated by ethanol precipitation followed by a 70% ethanol wash. The pellet was resuspended in 50 μl of 0.066 M Tris-HCl, pH 8.0, containing 10 mM NaCl, 6 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 1 mM β-mercaptoethanol, and the DNA was digested by incubation with 5 U of DNase I (RNase free; Promega) for 30 min at 37°C. The RNA was purified as described above after the addition of 0.2 ml of 0.01 M Tris-HCl, pH 7.9, containing 0.1 M NaCl and 1 mM EDTA. When oligonucleotides corresponding to sequences downstream of the IVa_2 promoter were present in transcription reaction mixtures, 50 U of exonuclease III (Boehringer Mannheim) was included in the digestion with DNase I and a second incubation was performed with 5 U of T4 DNA polymerase in 0.067 M Tris-HCl, pH 8.8, containing 0.017 M $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, and 10 mM β-mercaptoethanol. These additional nuclease digestions were included to ensure the complete degradation of oligonucleotides that might interfere with primer extension reactions.

Primer extension was performed according to the protocol of Hernandez and Keller (22) by using RNA purified from transcription reaction mixtures or 5 μg of cytoplasmic RNA purified from adenovirus 5-infected 293 cells harvested 14 to 20 h after infection. Primers of 25 to 26 nucleotides that generate primer extension products of 55 and 57 nucleotides from wild-type IVa_2 templates, of 56 and 58 nucleotides from the wild-type pIVn template, of 36 nucleotides from ML transcripts, and of 108 nucleotides from β-globin–major histocompatibility complex (MHC) D^d transcripts were synthesized as described previously (14). Primers were purified by electrophoresis in 20% polyacrylamide gels containing 7 M urea and were eluted from crushed gel slices by shaking in H_2O for 12 to 16 h at 37°C. The DNA was purified by chromatography on prepacked G-25 columns (NAP-25; Pharmacia) and 5′ end labelled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (>7,000 Ci/mmol; New England Nuclear). The labelled DNA was purified by chromatography on G-25 (NAP-10 columns; Pharmacia). In some experiments with exogenous human TBP (hTBP), transcripts were analyzed by using an S1 protection assay. An oligonucleotide complementary to the sequence from −12 to +48 of the IVa_2 promoter was purified, 5′ end labelled as described above, and hybridized to purified transcripts for 12 to 16 h in 0.01 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8) containing 0.4 M NaCl, 1 mM EDTA, and 80% formamide at 30°C. Reaction mixtures were then diluted 10-fold with 0.01 M sodium acetate, pH 4.5, containing 0.2 M NaCl, 5 mM zinc acetate, and 5% glycerol and digested with 100 to 300 U of nuclease S1 (Boehringer Mannheim) for 45 min at room temperature. Primer extension and nuclease S1

products were analyzed by electrophoresis in 6 and 8% polyacrylamide gel, respectively, containing 7 M urea (33). Gels were dried and exposed to Kodak XAR or XRP film in the presence of intensifying screens at −80°C. In all primer extensions, the products appeared as two sets of doublets (e.g., Fig. 1B) in which the upper member of each doublet represented the predicted product. The lower partner of each doublet was probably created by premature termination by avian myeloblastosis virus reverse transcriptase, for its production varied with different lots of the enzyme (see also reference 6).

Mobility shift assays. Oligonucleotides composing the two strands of the Ad2 IVa_2 promoter regions +32 to +2 and −36 to −61 were synthesized as described previously (14). Complementary oligonucleotides, in 0.01 M Tris-HCl, pH 7.9, containing 0.01 M NaCl and 1 mM EDTA, were incubated at 75°C for 10 min and then cooled by approximately 5°C/h to 40°C. Such double-stranded oligonucleotides were designed to possess a 4-bp overlap at each 5′ end. The oligonucleotides were purified by electrophoresis in 12% polyacrylamide gels and eluted as described in the previous section. They were labelled by using the Klenow fragment of *E. coli* DNA polymerase I (Promega) supplied with 250 μM (each) dATP, dGTP, and TTP and 100 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear). Unlabelled oligonucleotides used as competitors in mobility shift or transcription assays were prepared in identical fashions, except that the Klenow reaction was performed in the presence of the four unlabelled deoxynucleoside triphosphates (dNTPs).

Binding reaction mixtures with total extract proteins, in a 20-μl volume, contained approximately 0.1 ng of ^{32}P-labelled DNA (ca. 5×10^3 cpm), 1 μg of poly(dI-dC) · poly(dI-dC), and the concentrations of protein indicated in the figure legends. Otherwise, conditions were as for in vitro transcription reactions, except that rNTPs were omitted. Samples were incubated at 30°C for 30 min and electrophoresed in 4% polyacrylamide gels cast and run in 0.089 M Tris-0.089 M boric acid (pH 8.0) containing 1 mM EDTA. Binding of hTBP (see the next section) was analyzed under the conditions described by Starr and Hawley (53), except that electrophoresis was in 0.5× TGEM buffer. Gels were dried and exposed to Kodak XAR or XRP film at −80°C in the presence of intensifying screens.

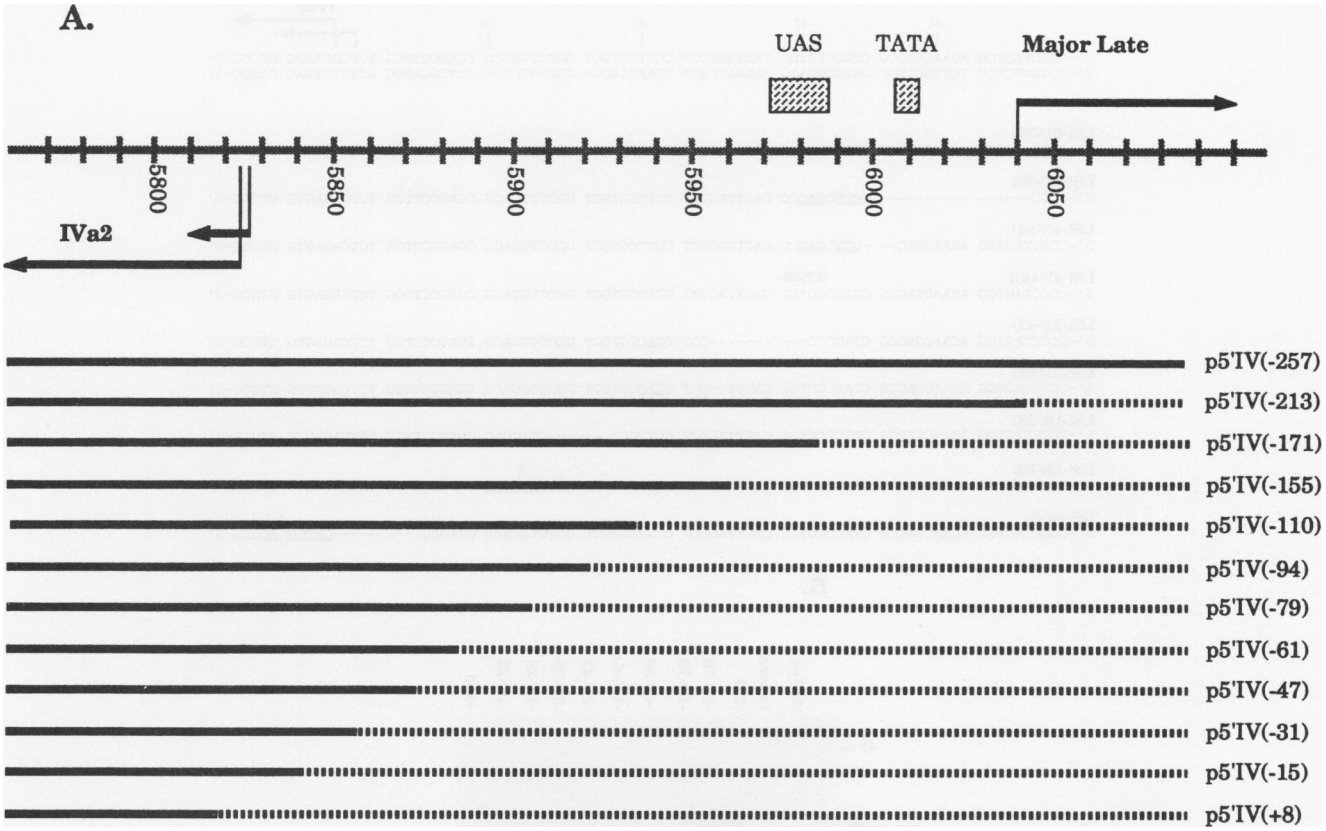
Expression and purification of hTBP. The complete coding sequence of hTBP in a cDNA clone (kindly provided by R. Tjian) was amplified and modified by using the polymerase chain reaction (38) for insertion into the pDS56/RBSII-6XHis vector obtained from R. Gentz (Hoffmann-LaRoche and Co., Basel, Switzerland). The resulting construct expressed a $(His)_6$-TBP fusion protein under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. *E. coli* containing this construct and the *lacI*^{q}-containing plasmid pDMI, also supplied by R. Gentz, were grown to mid-log phase and harvested 45 min after the addition of 1 mM IPTG. Soluble extracts were prepared by sonication in 0.01 M sodium phosphate buffer, pH 8.0, containing 0.05 M KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg (each) of pepstatin, antipain, and leupeptin per ml following digestion with 1 mg of lysozyme per ml for 20 min at 4°C. Insoluble debris was removed by centrifugation, and the $(His)_6$-TBP fusion protein was purified by nickel-chelate affinity chromatography (23) under native conditions by using nitrilotriacetic acid-agarose affinity resin (Qiagen). Purified TBP, a band with an apparent molecular mass of 40 kDa in SDS-polyacrylamide gels (data not shown), was assayed by its ability to restore ML transcription when added to heat-treated nuclear extracts (e.g., Fig. 7A). The purified protein was dialyzed against 0.01 M HEPES, pH 7.9, containing 0.10 M KCl, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 20% glycerol, 1 mM PMSF, and 0.1% (vol/vol) Nonidet P-40 (NP-40) and stored in small portions at −80°C.

RESULTS

Analysis of 5′ deletion mutants. To begin to define *cis*-acting elements of the Ad2 IVa_2 promoter, the series of 5′ deletion mutants illustrated in Fig. 1A was created by nuclease *Bal* 31 digestion from a site at position −257, with respect to the major IVa_2 initiation site (see Materials and Methods). The effects of these deletions were assessed by transcription of deleted templates in a modified HeLa whole-cell extract under conditions optimal for IVa_2 transcription (see Materials and Methods). The products of in vitro transcription were analyzed by extension from a primer hybridizing to the region from +55 to +30 of the IVa_2 transcription unit. As expected (6), two products of 55 and 57 nucleotides corresponding to IVa_2 initiation sites at nucleotides 5826 and 5828, respectively, in the Ad2 genome were observed upon primer extension of cytoplasmic RNA purified from Ad2-infected cells (Fig. 1B, lane 2). The same products were observed when IVa_2 RNA synthesized in vitro from the wild-type template was analyzed in parallel (Fig. 1B, lane 3). However, the two initiation sites were used with approximately equal efficiencies in vitro, whereas the 5826 initiation site was strongly favored in vivo (e.g., Fig. 1B, lane 2) (6). Production of the IVa_2 transcripts was inhibited by 1 μg of α-amanitin per ml (Fig. 1B, lane 15). These results established that RNApII mediated accurate initiation of transcription from the two IVa_2 start sites utilized in vivo in this in vitro system.

The majority of the IVa_2 5′ deletions removed the ML promoter (Fig. 1A). Transcription from a template comprising the human β-globin promoter fused to the MHC D^d gene was therefore used as an internal control in all analyses of IVa_2 5′ deletion templates. Results typical of those obtained with five independently prepared whole-cell extracts are shown in Fig. 1B. None of the deleted templates with endpoints between positions −257 (wild type) and −61 showed major alterations in the efficiency of IVa_2 transcription (Fig. 1B, lanes 3 to 10). However, deletion to position −47 (Fig. 1B, lanes 10 and 11) inhibited IVa_2 transcription three- to fivefold in independently prepared extracts, as judged by direct counting of bands cut from gels like that shown in Fig. 1B and normalization against the transcript of the control promoter (data not shown). Further deletion of the template to position −15 induced no additional change in production of the IVa_2 transcript initiated at the 5826 site (Fig. 1B, lanes 11 to 13). Thus, a template that contained only 15 bp of IVa_2 sequence upstream of the major initiation site directed accurate IVa_2 transcription from this site with a three- to fivefold lower efficiency than promoters containing 61 or 257 bp of 5′ flanking sequence. However, quantitation of the results shown in Fig. 1B and those of similar experiments performed with independently prepared extracts established that deletion of the sequence between positions −47 and −31 reduced initiation of IVa_2 transcription from the 5828 start site to close to background levels. The equivalent degree of inhibition of IVa_2 transcription from the 5826 site was not achieved until the promoter was deleted to position +8 (Fig. 1B, lane 14).

It has previously been reported that the strong ML pro-



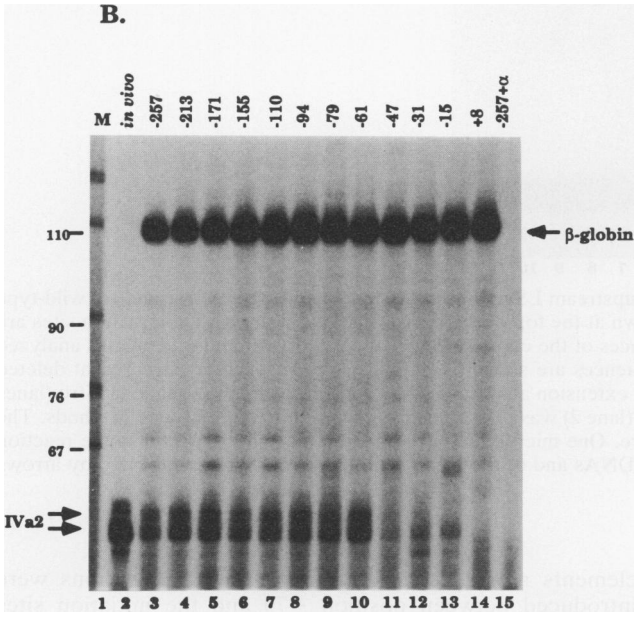


FIG. 1. Analysis of 5′ deletion mutants. (A) Summary of IVa_2 5′ deletion mutants. The region of the Ad2 genome between nucleotides 5760 and 6100 containing the IVa_2 and ML promoters is represented by the solid line at the top of the figure. The sites at which transcription initiates in these promoters are indicated by arrows. The ML promoter-binding sites for USF/MLTF (UAS) and TFIID (TATA) are indicated by shaded boxes. The structures of the IVa_2 5′ deletion templates are summarized below, with solid and dashed lines representing Ad2 DNA and pBR322 DNA sequences, respectively. (B) Transcription reaction mixtures (lanes 3 to 15) were as described in Materials and Methods and contained 10 μg of DNA of the IVa_2 templates indicated at the top of the figure per ml, 2.5 μg of internal control β-globin–MHC D^d DNA per ml, and 7.0 mg of whole-cell extract protein per ml. The reaction mixture whose products are shown in lane 15 was identical to that shown in lane 3, except that it also contained 1 μg of α-amanitin per ml. Products of in vitro transcription (lanes 3 to 15) or RNA from adenovirus 5-infected cells (lane 2) were analyzed by primer extension with both IVa_2 and D^d primers as described in Materials and Methods. End-labelled *Hpa*II fragments of pBR322 DNA were applied to lane 1. The positions of the 55- and 57-nucleotide IVa_2 cDNAs and that of the 108-nucleotide β-globin–MHC D^d cDNA are indicated at the left and right, respectively.

moter interferes with transcription from the weaker IVa_2 promoter when the two are present in the same template (12, 35, 36). For example, removal of the ML cap site and TATA element has been reported to stimulate IVa_2 transcription by as much as 15-fold (36). However, deletion of ML promoter sequences increased IVa_2 transcription in these experiments by no more than 60% (Fig. 1B, lanes 3 to 5). In an attempt to resolve this discrepancy, wild-type and mutant IVa_2 templates were transcribed in reaction mixtures containing ammonium sulfate under the conditions described by Natarajan et al. (35). Deletion of ML promoter elements again resulted in a less-than-twofold increase in the efficiency of IVa_2 transcription (data not shown). We also failed to observe significant competition between the ML and the IVa_2 promoters when basal IVa_2 transcription was reduced by decreasing the concentration of extract proteins or increasing the concentration of KCl in reaction mixtures (data not shown). It therefore appears that some component(s) of the transcription machinery essential to transcription from

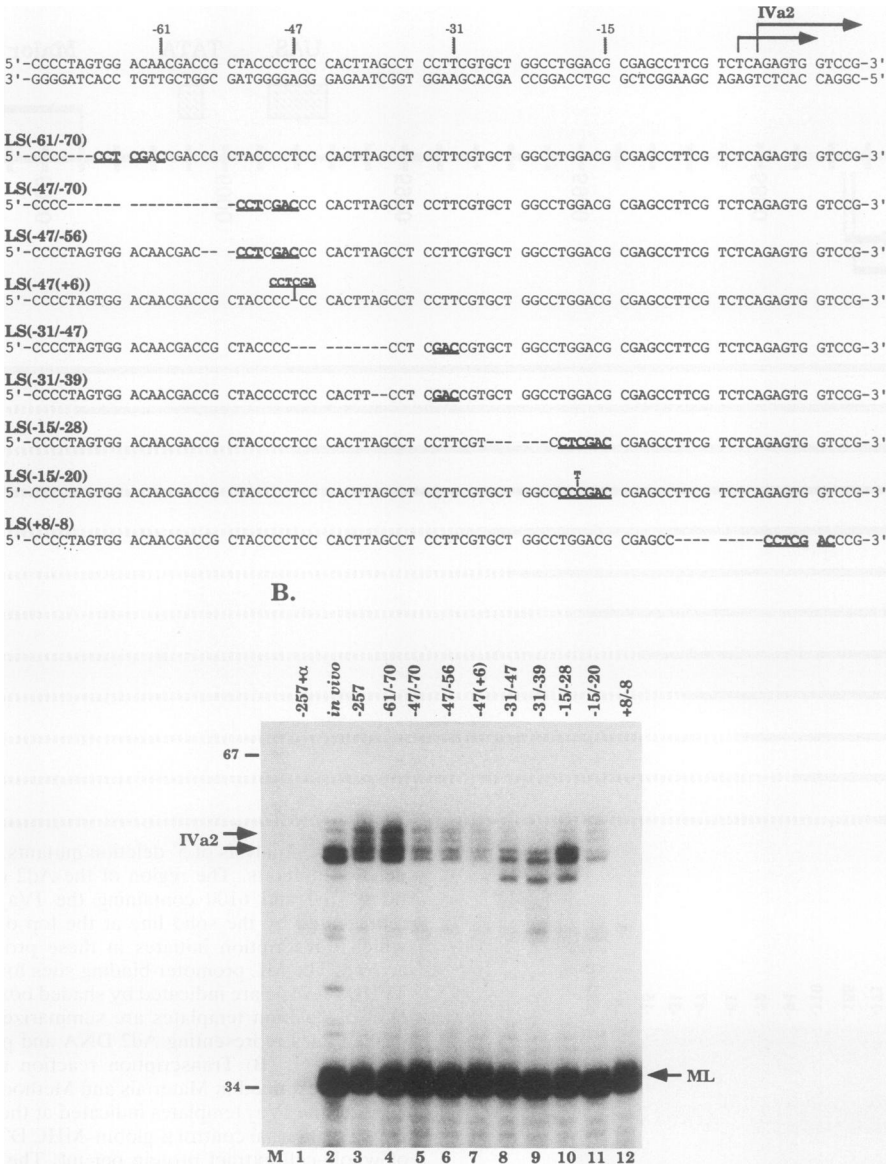


FIG. 2. Analysis of IVa_2 upstream LS mutants. (A) Summary of IVa_2 upstream LS mutations. The sequences of both strands of wild-type Ad2 DNA between positions −74 and +11 of the IVa_2 promoter are shown at the top of the figure. The two transcription initiation sites are indicated by arrows pointing in the direction of transcription. The sequences of the coding strands of each of the mutant templates analyzed are shown below the wild-type sequence. In each case, substituted sequences are shown in boldface type, dashed lines represent deleted sequences, and sequences above the line represent insertions. (B) Primer extension analysis of IVa_2 and ML transcripts made in vitro (lanes 3 to 12) under the conditions described in the legend to Fig. 1B or in vivo (lane 2) was performed as described in Materials and Methods. The DNA templates present in each reaction are listed at the top of the figure. One microgram of α-amanitin per ml was added to the reaction mixture whose products are shown in lane 1. The positions of the IVa_2 cDNAs and of the 36-nucleotide ML cDNA are indicated by arrows at the left and right, respectively.

both the IVa_2 and ML promoters can be limiting in some extracts or reconstituted systems (12, 35, 36) but not in other extracts (Fig. 1B). This view is consistent with the fact that our protocol for the preparation of whole-cell extracts included the extraction step reported by Sugden and Keller (54) to increase the efficiency of extraction of RNApII (and perhaps other factors) from mammalian cells.

Upstream elements of the IVa_2 promoter. The results of the analysis of 5′ deletion mutants (Fig. 1B) suggested that the major elements of the IVa_2 promoter were contained between positions −61 and +48. To identify 5′ promoter elements more precisely, a series of LS mutations were introduced between position −70 and the initiation sites (Fig. 2A). In these IVa_2 LS mutant templates, wild-type upstream sequences, including the ML promoter, were restored (Fig. 2A). The ML promoter was therefore used as an internal control. Results typical of those obtained with five independently prepared HeLa whole-cell extracts are shown in Fig. 2B. Alteration of the sequence between positions −70 and −61 of the IVa_2 promoter had no significant effect on IVa_2 transcription (Fig. 2B, lanes 3 and 4), in agreement with the properties of the 5′IV(−61) deletion

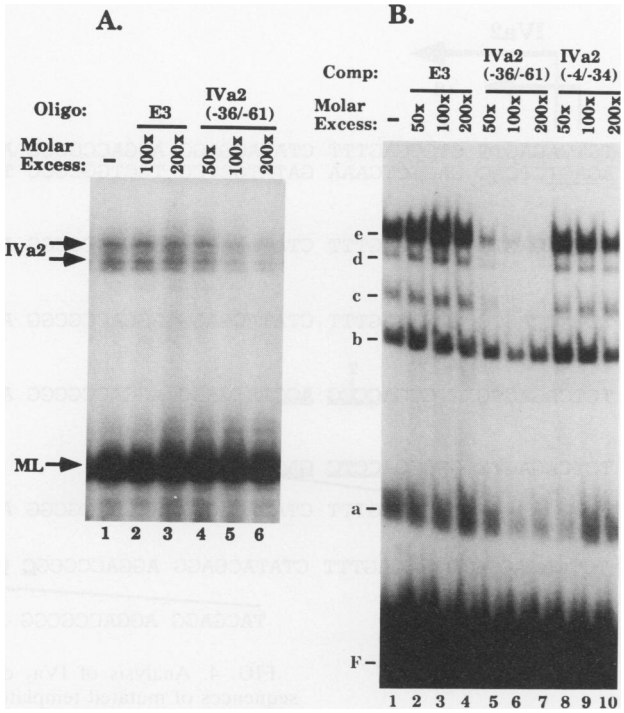
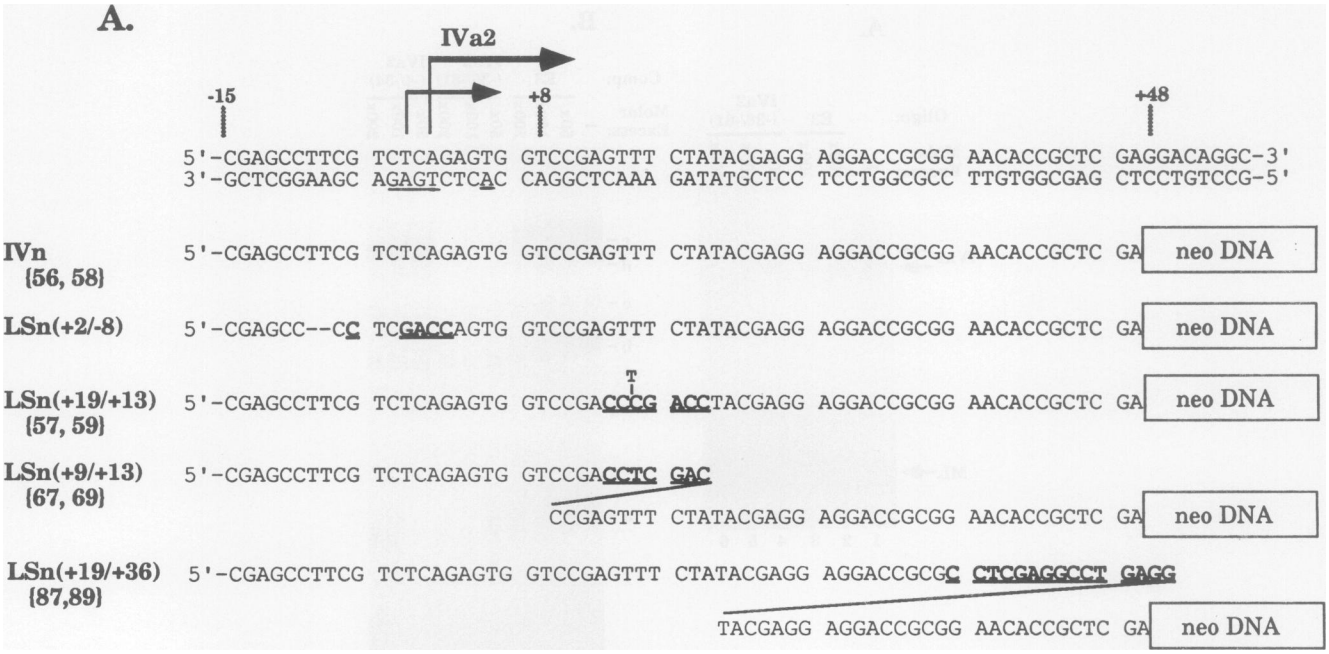


FIG. 3. A cellular factor binds to the −47 element to stimulate IVa_2 transcription. (A) To investigate transcription competition, ML and IVa_2 transcription from the p5′IV(−257) wild-type template were assayed in reaction mixtures containing 15 μg of DNA per ml and 6.4 mg of whole-cell extract protein per ml in the presence of the relative molar concentrations of the oligonucleotides listed at the top of the figure. The IVa_2 oligonucleotide contained the sequence from −36 to −61 of the IVa_2 promoter, whereas the E3 oligonucleotide corresponded to the sequence from +10 to +29 of the viral E3 gene. (B) For the electrophoretic mobility shift assay, binding reactions contained 0.1 ng of ^{32}P-labelled IVa_2 (−36/−61) oligonucleotide, 1 μg of poly(dI-dC) · poly(dI-dC), the relative molar concentrations of unlabelled oligonucleotides listed at the top of the figure, and 4 μg of whole-cell extract proteins. The positions of free DNA (F) and protein DNA complexes (a to e) are indicated at the left.

template (Fig. 1B). The effects of the other LS mutations permitted definition of at least two upstream elements of the IVa_2 promoter.

One element includes the sequence spanning position −47, for the LS(−47/−70), the LS[−47/(+6)], and the LS(−47/−56) mutations each decreased IVa_2 transcription two- to threefold, but none altered the use of the two IVa_2 initiation sites (Fig. 2B, lanes 5 to 7; Table 1). As illustrated in Fig. 3A, an oligonucleotide comprising the sequence from −36 to −61 of the IVa_2 promoter efficiently inhibited IVa_2, but not ML, transcription, whereas a heterologous oligonucleotide had no effect, even at 200-fold molar excess. As this result implied that a positive-acting transcription factor bound to the region of the IVa_2 promoter defined by the LS −47 mutations, direct evidence for the existence of such a factor(s) was sought by using an electrophoretic mobility shift assay (19, 20). The oligonucleotide used in the competition experiment described above was end labelled and incubated with HeLa whole-cell extract under the conditions employed for transcription. The oligonucleotide comprising the sequence from −36 to −61 of the IVa_2 promoter formed five more slowly migrating complexes, a to e (Fig. 3B, lane 1, and 4). Species a and b were not specifically inhibited and, moreover, were not observed when the unlabelled carrier DNA employed in these assays was preincubated with extract prior to the addition of the labelled oligonucleotide (data not shown). By contrast, formation of species c, d, and e was inhibited by the homologous oligonucleotide but not by either of two heterologous oligonucleotides (Fig. 3B), indicating that HeLa cell extracts contain a factor(s) that binds specifically within the sequence from −61 to −36 of the IVa_2 promoter. The element recognized appears to include the sequence spanning position −47, for insertion of a 6-bp linker at this site reduced IVa_2 transcription from either initiation site (Fig. 2B, lane 7; Table 1). Indeed, HeLa cell fractions containing the cellular factor PuF, which recognize the homopurine sequence 5′ GGGGAGGG 3′ (40), like that centered on position −47 of the IVa_2 promoter (Fig. 2A), are required for maximal IVa_2 transcription in a reconstituted system (39a). Moreover, an oligonucleotide containing the homopurine element of the c-*myc* promoter, whose in vitro transcription is strongly dependent on PuF (40), inhibits IVa_2 transcription (30a). In addition, an oligonucleotide containing the IVa_2 homopurine sequence listed previously, but no other IVa_2 oligonucleotides tested, inhibited the formation of specific complexes containing PuF and the c-*myc* homopurine oligonucleotide, and vice versa (39a). It therefore seems likely that the distal upstream element of the IVa_2 promoter defined by mutation serves as a binding site for the cellular factor PuF.

A second upstream element that controls IVa_2 transcription is centered on position −30, for any mutation of this region altered the frequency with which IVa_2 initiation sites were recognized: LS(−31/−47), LS(−31/−39), and LS(−15/−28) shared a phenotype characterized by inefficient utilization of the 5828 initiation site but efficient recognition of a start site at position +4 (Fig. 2B, lanes 8 to 10; Table 1). Although the LS(−31/−39) and LS(−15/−28) mutations



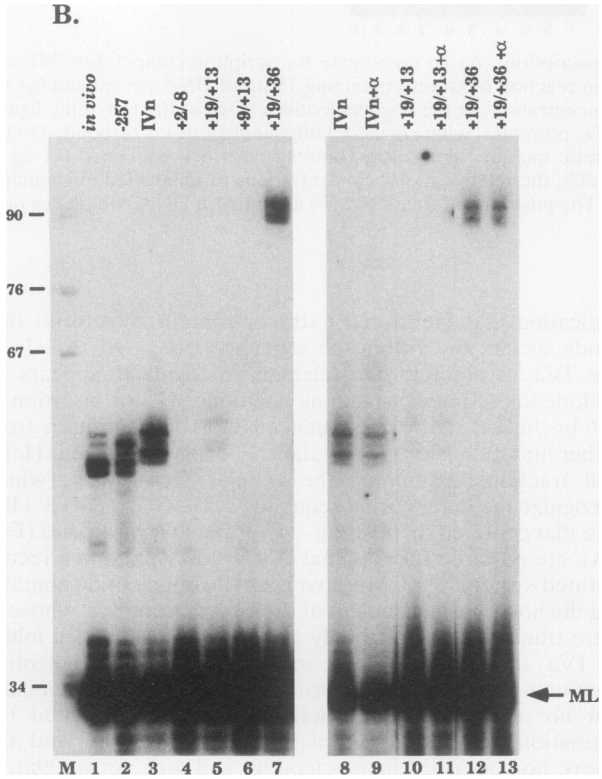


FIG. 4. Analysis of IVa_2 downstream LS mutations. (A) The sequences of mutated templates are illustrated as described in the legend to Fig. 2A. (B) Primer extension analysis of the ML and IVa_2 transcripts synthesized from the wild-type (−257) and mutated templates shown in panel A (lanes 2 to 6) or of in vivo RNA (lane 7) was performed as described in Materials and Methods. Transcription reaction mixtures contained 15 μg of template DNA per ml and 6.4 mg of whole-cell extract protein per ml. End-labelled *Hpa*II fragments of pBR322 DNA were applied to lane 1. The stabilities of wild-type and mutated IVa_2 RNA species synthesized in vitro were assessed in the experiment shown in lanes 8 to 13. Transcription from the templates listed at the top of the figure was performed under the conditions described for panel B for 30 min. At the end of the incubation period, transcription was terminated (see Materials and Methods) (lanes 8, 10, and 12) or incubation was continued for an additional 30 min in the presence of 1 μg of α-amanitin per ml (lanes 9, 11, and 13).

introduced substantial deletions (Fig. 2A), it seems unlikely that this phenotype is simply the result of alterations in the spacing among IVa_2 initiation sites and upstream promoter elements; the LS(−31/−39) template, which exhibited the most dramatic phenotype (Table 1), contained a net deletion of only 2 bp (Fig. 2A), and an identical pattern of initiation of IVa_2 transcription was observed when the template was simply deleted to position −31 (Fig. 1B). Although the data shown in Fig. 2B do not permit precise delineation of the boundaries of this element, the properties of the LS(−39/−31) mutant template indicate that it includes the sequence from −39 to −31.

IVa_2 promoter elements at and beyond the cap sites. The results described in the preceding sections indicated that, although specific sequences 5′ to the IVa_2 initiation sites can be defined as IVa_2 promoter elements, they are not sufficient to determine IVa_2 transcription in vitro. Thus, for example, deletion to position −15 of the promoter resulted in only a modest reduction (three- to fivefold) in the efficiency with which IVa_2 transcription initiated at the major in vivo site. Moreover, when an IVa_2 gene with an insertion of 11 bp at position +13 was created, we observed that IVa_2 transcription was reduced to background levels (Fig. 4B, lane 6). The roles of IVa_2 sequences at and downstream of the cap sites were therefore investigated in more detail by construction of additional LS mutant templates, as described in Materials and Methods.

A mutation disrupting the region spanning the IVa_2 cap sites, LS(−8/+8), reduced IVa_2 transcription to background levels (Fig. 2B, lane 12; Table 1), even though all upstream elements described in previous sections were retained. The LS(−8/+8) mutation introduced a deletion of the region from

TABLE 1. Effects of upstream LS mutations on IVa_2 promoter activity

Mutation[a]	Relative transcription efficiency[b] at initiation site:	
	5826	5828
−257	1.0	1.0
−61/−70	0.88 ± 0.01	0.87 ± 0.13
−47/−70	0.34 ± 0.05	0.26 ± 0.03
−47/−56	0.45 ± 0.10	0.44 ± 0.19
−47(+6)	0.32 ± 0.06	0.28 ± 0.09
−31/−47	0.35 ± 0.04	0.15 ± 0.01(+[c])
−31/−39	0.52 ± 0.05	0.15 ± 0.08 (+)
−15/−28	0.59 ± 0.03	0.36 ± 0.11 (+)
−15/−20	0.14 ± 0.01	0.17 ± 0.01
+8/−8	0.04 ± 0.01	<0.003

[a] Transcripts of the templates were quantitated and corrected relative to the ML internal control as described in Materials and Methods.
[b] The values obtained were expressed relative to that of the wild-type (−257) IVa_2 promoter and represent the mean of two experiments with independently prepared extracts.
[c] Indicates utilization of an initiation site at position 5823 (+4).

−2 to −7, which includes the 5828 initiation site, as well as substitution of the sequence (from +1 to +8) immediately surrounding the major initiation site at 5826 (Fig. 2A). We therefore next constructed the LSn(−8/+2) mutant, in which the wild-type initiation sequence 5′ CC(TTCGTCTCAG)AG 3′ was replaced by the sequence 5′ CC(CCTCGACC)AG 3′. The LSn(−8/+2) template contained potential A and T(U) start sites (underlined) placed in an altered sequence context. Nevertheless, when LSn(−8/+2) was analyzed by in vitro transcription, no specific IVa_2 transcripts could be detected (Fig. 4B, lane 4; Table 2). This result implies that the sequence spanning the wild-type start sites, altered by the LSn(−8/+2) mutation, comprises a critical element of the IVa_2 promoter.

The effects of mutations introduced further downstream of the initiation sites were also investigated. All such mutations altered the spacing between the IVa_2 initiation sites and the primer hybridization site and thus generated novel primer extension products. LSn(+19/+36), in which the sequence from +36 to +46 was mutated (Fig. 4A), directed accurate initiation of IVa_2 transcription, but with a five- to sixfold decrease in efficiency (Fig. 4B, lane 7; Table 2). The LSn(+13/+19) template, in which the sequence from +13 to +19 was substituted (Fig. 4A), was transcribed 15- to 20-fold less efficiently than the wild-type template (Fig. 4B, lane 5; Table 2). The LSn(+9/+13) mutation, which carried a similar substitution of the sequences from +13 to +19 and duplication of the wild-type sequence from +10 to +20 inserted at position +20 (Fig. 4A), reduced IVa_2 transcription to undetectable levels (Fig. 4B, lane 6). As the sequences of the RNA products of transcription from the IVa_2 initiation sites were altered by each downstream mutation (Fig. 4A), it was possible that the mutations altered the stability of IVa_2 RNA in vitro. In order to assess any such effects on RNA stability, RNA was synthesized from the wild-type template and mutant templates in duplicate reactions. After 30 min, one of each pair of reactions was stopped by the addition of SDS and proteinase K to the mixture, while α-amanitin was added to the second member of each pair of reaction mixtures to inhibit transcription, and incubation was then continued for 30 min further. No differences in the recoveries of wild-type and mutated transcripts were observed following the latter treatment (Fig. 4B, lanes 8 to 13). The mutations inserted at or downstream of the initiation site alter the sequence of the template transcribed by RNApII (Fig. 4A), raising the possibility that promoter clearance rather than the formation of initiation complexes was defective. However, a representative set of these mutations has been shown to exhibit the same promoter clearance efficiency as the wild-type IVa_2 template (31a). We can, therefore, conclude that the deleterious effects of these mutations observed in our primer extension assay are the direct result of impaired assembly of initiation complexes.

TABLE 2. Effects of downstream LS mutations on IVa_2 promoter activity

Mutation	Relative transcription efficiency[a]
IVn (+48)	1.0
+2/−8	<0.004
+19/+13	0.056 ± 0.009
+9/+13	<0.010
+19/+36	0.19 ± 0.03

[a] Relative transcription efficiencies were determined as described in Table 1, footnote *b*, and are expressed relative to the activity of the wild-type pIVn promoter.

The most parsimonious interpretation of the inhibitory effects of the downstream LS mutations on IVa_2 transcription (Fig. 4B) is that the sequences or locations of two promoter elements are altered; the substitution at positions +36 to +46 would define one such element, and the LSn(+13/+19) mutation would define a second. We would then attribute the inhibition of transcription induced by the LSn(+9/+13) mutation (Fig. 4B) to an altered position of the element at positions +13 to +19; both the LS(+9/+13) and the LS(+13/+19) mutant templates contain a linker substitution between positions +13 and +19, but in the former mutant, the linker insertion was accompanied by duplication of the wild-type sequence from +10 to +20. The greater impairment of transcription by the LSn(+9/+13) mutation than by the LSn(+13/+19) mutation, therefore suggests that the sequence between positions +13 and +19 is not only critical to IVa_2 transcription [LSn(+13/+19)] but is also inhibitory when displaced downstream by 11 bp [LSn(+9/+13)].

With the exception of mutations in which the initiation site sequence was replaced, which failed to support any detectable IVa_2 transcription, no mutations impaired the accuracy of initiation from the 5826(A) site (Fig. 2B and 4B). The sequence spanning the initiation sites exhibits some similarity to the corresponding sequence of the TdT gene (12) (Fig. 9), which is sufficient to direct specific initiation by RNApII (50). To determine whether the IVa_2 initiation sequence also possesses this property, we examined the ability of the sequence from −9 to +13 to direct specific initiation in our in vitro system. When inserted into pUC19, this sequence directed the synthesis of a specific transcript by RNApII (Fig. 5, lanes 3 and 4). However, the "initiator-only" template directed transcription from only one initiation site, the 5326(A) site, whereas transcription from both the 5826(A) and 5828(U) initiation sites described previously was observed when the template from −80 to +48 was transcribed in parallel reactions (Fig. 5; compare lanes 2 and 4). These results establish that a 23-bp sequence comprising positions −9 to +13 of the IVa_2 promoter specifies initiation from the 5326(A) site, the major in vivo initiation site.

Recognition of the initiator-proximal downstream element

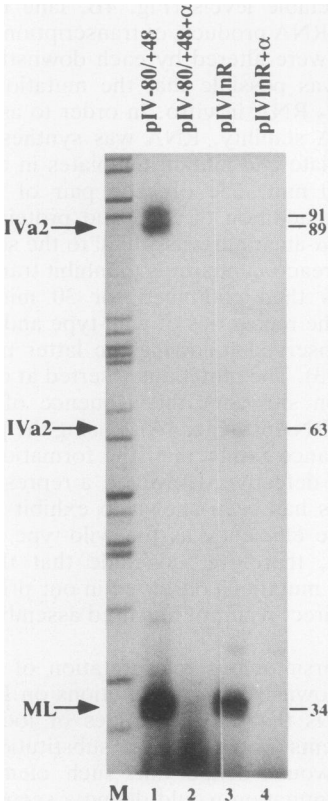


FIG. 5. The IVa_2 initiator element directs specific initiation by RNApII. Transcription reaction mixtures contained 15 μg of pIV−80/+48 (lanes 1 and 2) or pIVInR (lanes 3 and 4) DNA per ml, which comprise the sequences from −80 to +48 and −9 to +13, respectively, of the IVa_2 promoter linked to the Neo^r gene fragment described in Materials and Methods in pUC19 (14a) and 5 mg of whole-cell extract protein per ml (lanes 1 and 2) or 3.4 mg of nuclear extract protein per ml (lanes 3 and 4). Reaction mixtures whose products are shown in lanes 2 and 4 also contained 1 μg of α-amanitin per ml. The Neo^r sequence described in Materials and Methods, which is predicted to yield extension products of 89 and 63 nucleotides from the major A initiation sites in the two templates, was used in primer extension analysis. Sequencing ladders used as size markers were run in lane M.

by TBP. As the results described in the previous section suggested that the sequence from +13 to +19 of the IVa_2 gene is a critical element of the promoter, we investigated whether this element served as the binding site for a *trans*-acting transcription factor by using transcription competition experiments. In these assays, a modified procedure (see Materials and Methods) was used to ensure digestion of the downstream competitor, which interfered with extension from the IVa_2 primer when incompletely digested prior to primer extension (data not shown). As the efficiencies of primer removal did vary (data not shown), all experiments with IVa_2 downstream competitors include a control reaction mixture to which the competing oligonucleotide was added after the transcription reaction had been terminated. IVa_2 transcription was as efficient when the homologous competitor was added after transcription was completed as it was in the presence of a heterologous oligonucleotide (Fig. 6, lanes 1 and 2) and only marginally reduced compared with that of reaction mixtures to which no oligonucleotide had been added (Fig. 6, lane 3). However, the addition of the IVa_2 +32/+2 oligonucleotide at the beginning of transcription specifically and severely inhibited IVa_2 transcription (Fig. 6, lanes 1, 2, 4, and 5). This result indicates that the downstream oligonucleotide competes specifically for binding of a cellular factor to the IVa_2 promoter.

Carcamo and colleagues have reported that IVa_2 transcription in a reconstituted mammalian system depends on the TATA element-binding factor TFIID (12). Although the IVa_2 promoter contains no TATA element in the conventional position, these workers have shown that highly purified yeast TFIID and yeast TFIID synthesized in *E. coli* bind to an IVa_2 sequence centered at around position +15 (13), which contains the TATA-like sequence 5′ TATAGAAA 3′ in the noncoding strand (Fig. 4A). We therefore wished to determine whether TFIID played a role in IVa_2 transcription in the unfractionated in vitro transcription system used in these experiments via binding to the promoter element from +13 to +19 described in previous paragraphs. In these experiments, we took advantage of the observation that mild heat treatment of nuclear extracts preferentially inactivates TFIID (34). Such treatment of a HeLa cell nuclear extract severely inhibited both ML and IVa_2 transcription (e.g., Fig. 7A, lane 2, and 7B, lane 2). The addition of human TFIID partially purified by sequential chromatography on phosphocellulose, DEAE-cellulose, and ω-amino-octyl agarose according to the procedure of Nakajima et al. (34) restored both ML and IVa_2 transcription (data not shown). Although these results are consistent with the conclusion that transcription from the ML and IVa_2 promoters depends on TFIID present in the ω-amino-octyl agarose fraction, they do not rule out the possibility that a separate factor(s) present in this fraction restored IVa_2 transcription. To address this

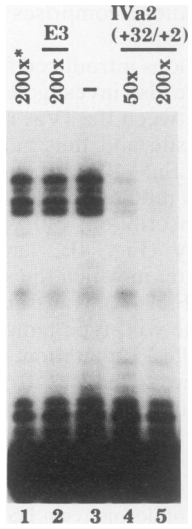
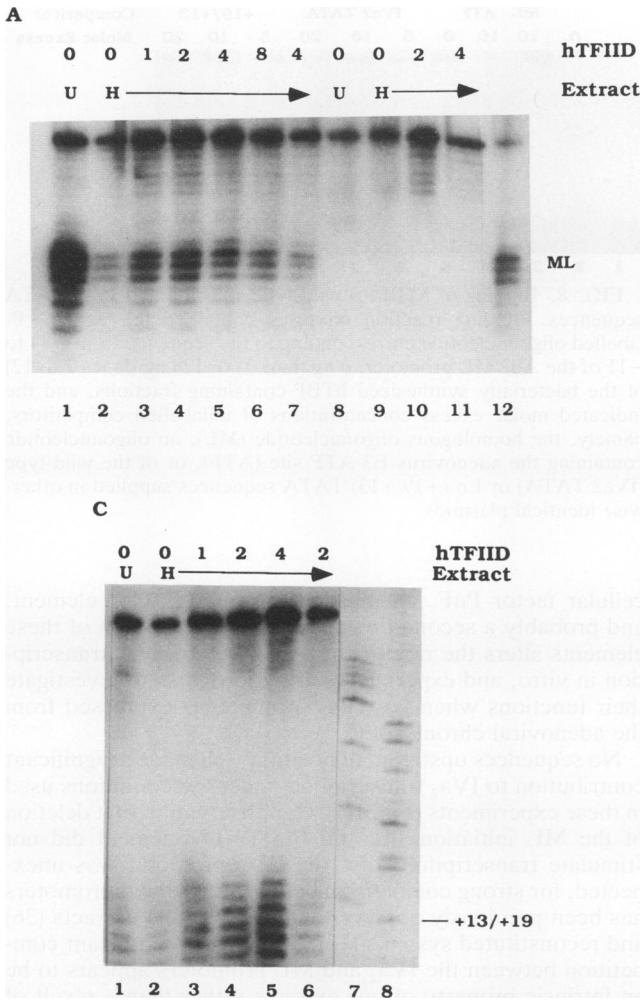


FIG. 6. The proximal downstream element of the IVa_2 promoter is recognized by a cellular transcription factor. The pIV (−257) wild-type template was transcribed in standard reaction mixtures (see Materials and Methods) containing 15 μg of DNA per ml and 6.0 mg of whole-cell extract protein per ml, and the molar concentrations of competitor oligonucleotides indicated at the top of the figure. The reaction mixture whose products are shown in lane 1 (200×*) contained no competitor during transcription, but a 200-fold molar excess of the IVa_2 +32/+2 oligonucleotide was added as soon as the reaction was stopped. Processing of transcripts for primer extension included digestion with exonuclease III and T4 DNA polymerases, as described in Materials and Methods.



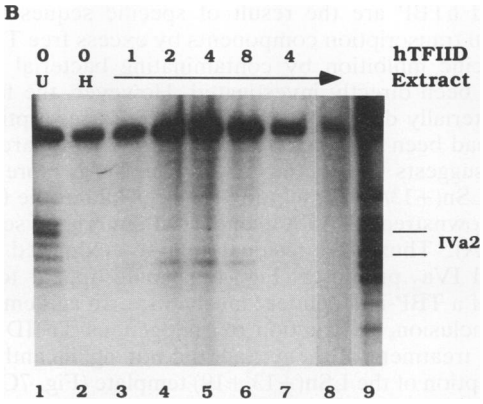


FIG. 7. Stimulation of ML and IVa_2 transcription by bacterially synthesized hTBP. Nuclear extracts (47) were preincubated at 47°C for 15 min to inactivate TFIID (34). The same preparation of heat-treated extract was used in the experiments shown in panels B and C. Transcription products were analyzed by using the oligonucleotide S1 protection assay described in Materials and Methods. (A) Reaction mixtures contained 30 μg of template per ml containing the wild-type ML promoter (lanes 1 to 7) or the ML TATA-less promoter pLS (−182/−195) (lanes 8 to 11), 1.67 mg of untreated (U) or heat-treated (H) nuclear extract per ml, and the relative concentrations of bacterially synthesized hTBP indicated. The bacterially synthesized hTBP added to the reaction mixture whose products are shown in lane 7 was incubated at 47°C for 15 min (34). The products of hybridization of the oligonucleotide probe to cytoplasmic RNA purified from Ad2-infected cells are shown in lane 12. (B) Reaction mixtures contained 80 μg of pIVn DNA per ml, 5 mg of untreated (U) or heat-treated (H) nuclear extract per ml, and the relative concentrations of bacterially synthesized hTBP indicated. Heat-treated TBP or proteins prepared from *E. coli* lacking the hTBP expression plasmid were added to the reaction mixtures whose products are shown in lanes 7 and 8, respectively. The protection products obtained with cytoplasmic RNA purified from Ad2-infected cells are shown in lane 9. Protected products predicted for precise S1 cleavage of hybrids containing the minor and major IVa_2 transcripts are indicated by the top and bottom lines, respectively, at the right. (C) Reaction mixtures were as described for panel B but contained 80 μg of pIVn (+13/+19) DNA per ml (Fig. 4A). Lanes 7 and 8 show sequencing ladders used to identify the product of hybridization of LSn(+13/+19) RNA to the wild-type IVa_2 oligonucleotide probe; the LSn(+13/+19) mutations result in mismatch, and hence S1 cleavage, at positions +13 to +19 and, consequently, in a protection product of 29 bases.

possibility, similar experiments were performed with highly purified hTBP synthesized in *E. coli* (see Materials and Methods).

Bacterially synthesized hTBP increased the low level of ML transcription observed in heat-treated nuclear extract (Fig. 7A, lanes 1 to 6). No stimulation was observed when purified hTBP was incubated at 47°C for 15 min, the conditions used for inactivation of endogenous TFIID (34) (Fig. 7A, lane 7), or when the template contained an LS mutation that eliminated the ML TATA element (Fig. 7A, lanes 8 to 11). In reaction mixtures containing such a template, nonspecific transcription (represented in this nuclease S1 protection assay as products completely protecting the oligonucleotide probe) was stimulated, confirming the presence of active factor in the reaction mixtures. These properties established that hTBP was responsible for the stimulation of ML transcription induced upon the addition of the bacterially synthesized protein.

In the nuclease protection assay, the two IVa_2 transcripts made either in vivo or in vitro generated a set of six protected fragments (Fig. 7B; compare lanes 9 and 1), a degree of heterogeneity identical to that observed when uniquely initiating ML transcripts were assayed in the same way (Fig. 7A). The two longest protected fragments can therefore be assigned to IVa_2 transcripts initiated at the minor in vivo site, and the two shortest protected fragments can be assigned to transcripts initiating at the major in vivo site, with the two intermediate fragments being generated by both transcripts. Heat inactivation of endogenous TFIID reduced IVa_2 transcription to undetectable levels (Fig. 7B, lanes 1 and 2). The addition of bacterially synthesized hTBP to the heat-treated extract restored IVa_2 transcription from the major initiation site to the level observed in untreated extracts and stimulated transcription from the minor in vivo initiation site (Fig. 7B, lanes 1 to 6). No such stimulation was observed when transcription reaction mixtures were supplemented with heat-inactivated hTBP or an analogous fraction prepared from *E. coli* cells that do not express this protein (Fig. 7B, lanes 7 and 8, respectively). We can, therefore, conclude that the human TATA-binding protein TBP is essential for efficient IVa_2 transcription in vitro and, moreover, that heat treatment did not inactivate the component that recognizes the initiator element. The degree of stimulation of both ML and IVa_2 transcription initially increased with increasing concentrations of exogenous hTBP but decreased at the highest concentrations tested. Whether such inhibitory effects of high concentrations of bacterially syn-

thesized hTBP are the result of specific sequestration of essential transcription components by excess free TBP or of nonspecific inhibition by contaminating bacterial proteins has not been directly investigated. However, the failure of the bacterially derived fraction to inhibit transcription once hTBP had been heat inactivated (Fig. 7A; compare lanes 7 and 5) suggests that the former mechanism is more likely.

The LSn(+13/+19) mutation replaces all but the first 2 bp of the downstream TATA element with unrelated sequences (Fig. 4A). Thus, the residual activity exhibited by this mutated IVa_2 promoter (Fig. 4B) would appear to be the result of a TBP-independent mechanism. In agreement with this conclusion, destruction of endogenous TFIID activity by heat treatment of the extract did not significantly impair transcription of the LSn(+13/+19) template (Fig. 7C, lanes 1 and 2). Nevertheless, transcription from this TATA-less IVa_2 promoter was stimulated some threefold by the addition of native, but not heat-inactivated, bacterially synthesized hTBP (Fig. 7C; compare lanes 3 to 5 with lane 6). The stimulation of LSn(+13/+19) transcription observed increased more or less monotropically as a function of hTBP concentration (Fig. 7C, lanes 1 to 5) and could thus be distinguished from the response of the wild-type IVa_2 or the ML promoters to exogenous hTBP (Fig. 7A and B). A multisubunit TBP-containing complex is required for transcription from a promoter to which neither TBP nor endogenous TFIID can bind (42), and the LSn(+13/+19) mutation destroys the ability of yeast TBP to footprint the IVa_2 TATA element (13a). The results shown in Fig. 7C therefore suggest that hTBP stimulates transcription from the IVa_2 template whose TATA element is mutated by a mechanism that does not require direct binding of TBP to the promoter (42). However, the LSn(+19/+13) mutant template retains the first 2 bp of the downstream TATA element (Fig. 4A), and TFIID can recognize sequences poorly matched to the canonical TATAAAA motif (21, 49), raising the possibility that the mutated sequence of LSn(+13/+19) is bound by hTBP, albeit with an affinity too low to permit DNase I footprinting. The abilities of hTBP to bind the wild-type and mutated sequences were, therefore, examined by using an electrophoretic gel mobility shift assay.

Bacterially synthesized hTBP formed one specific complex with an oligonucleotide containing the ML TATA element (Fig. 8, lanes 2 to 4). Specific binding was inhibited by the IVa_2 TATA sequence, although at higher concentrations than required for essentially complete inhibition by the homologous oligonucleotide (compare lanes 6 to 8 with lane 3 in Fig. 8). By contrast, a plasmid in which the IVa_2 TATA element was replaced by the sequence of the +19/+13 template failed to inhibit hTBP binding (Fig. 8, lanes 9 to 11). This result indicates that the stimulation of transcription from the +19/+13 template by hTBP was independent of the binding of the factor to the promoter; similar concentrations of hTBP stimulated transcription from the wild-type and +19/+13 promoters (Fig. 7), yet hTBP exhibited no binding to the mutated TATA element under conditions that permitted efficient binding to the wild-type IVa_2 TATA element (Fig. 8).

DISCUSSION

The results presented here establish that the Ad2 IVa_2 promoter, whose organization is summarized in Fig. 9, is significantly more complex than previously recognized (13, 35–37). It comprises separate elements specifying the major and minor initiation sites, an upstream binding site for the

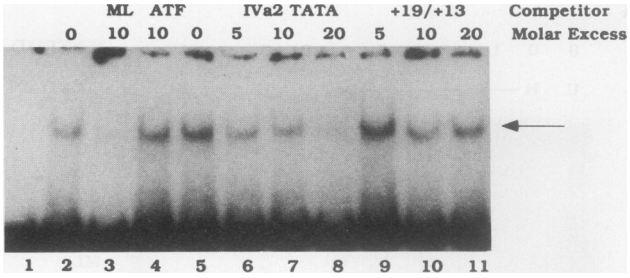


FIG. 8. Binding of hTBP to wild-type and mutated IVa_2 TATA sequences. Binding reaction mixtures contained 0.3 ng of ^{32}P-labelled oligonucleotide corresponding to the sequence from −44 to −11 of the Ad2 ML promoter, 0 ng (lane 1) or 125 ng (lanes 2 to 12) of the bacterially synthesized hTBP-containing fractions, and the indicated molar excess concentrations of unlabelled competitors, namely, the homologous oligonucleotide (ML), an oligonucleotide containing the adenovirus E3 ATF site (ATF), or of the wild-type (IVa2 TATA) or Ln (+19/+13) TATA sequences supplied in otherwise identical plasmids.

cellular factor PuF, an unusually located TATA element, and probably a second downstream element. Each of these elements alters the rate or the specificity of IVa_2 transcription in vitro, and experiments are in progress to investigate their functions when the IVa_2 promoter is expressed from the adenoviral chromosome.

No sequences upstream of position −60 made a significant contribution to IVa_2 transcription under the conditions used in these experiments (Fig. 1B). Our observation that deletion of the ML initiation site and the TATA element did not stimulate transcription from the IVa_2 promoter was unexpected, for strong competition between these two promoters has been previously observed with both crude extracts (36) and reconstituted systems (12). The lack of significant competition between the IVa_2 and ML promoters appears to be an intrinsic property of our extracts rather than a result of the conditions used to assay IVa_2 transcription (see Results). We therefore believe that these different results stem from differences in the concentrations of one or more transcriptional components that operate at both of these promoters in the various in vitro systems. A question of considerable interest is whether the relevant factor, or RNApII, is limiting in vivo, that is, whether competition between the ML and IVa_2 promoters contributes to the regulation of IVa_2 tran-

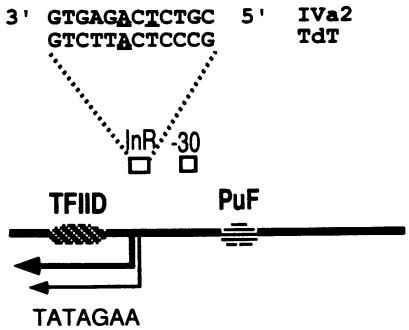


FIG. 9. Organization of the Ad2 IVa_2 promoter. Open boxes represent elements specifying initiation sites. Above the initiator element, designated InR, is a comparison of its sequence to that of the initiator element of the TdT promoter. These sequences are shown in the orientation of the IVa_2 promoter shown below.

scription in adenovirus-infected cells. Although this question has not been fully addressed, mutations of the ML promoter that reduce the efficiency of ML transcription in infected cells do not stimulate IVa_2 transcription (43).

Deletion to position −47 or the introduction of clustered point mutations at this site reduced the efficiency of IVa_2 transcription by a factor of 2 to 3 (Fig. 1B and 2B), in agreement with previous observations (35–37). The results of transcription competition and gel mobility shift assays (Fig. 3) indicated that this region of the IVa_2 promoter is specifically bound by a positive-acting cellular factor. Protection of the region from −38 to −83 of the IVa_2 promoter, with a DNase I hypersensitive site at position −55, has also been observed in vivo (2). As described in the previous section, several lines of evidence indicate that this element comprises the polypurine.polypyrimidine sequence, centered on position −47 of the IVa_2 promoter (Fig. 2A) and recognized by the cellular factor PuF (40). The upstream region of the IVa_2 promoter also contains an element that determines the site of transcriptional initiation in an unusual manner. Mutations in the vicinity of position −30, or deletion to position −31, selectively impaired transcription from the 5828 initiation site, with concomitant utilization of an initiation site at position +4 (Fig. 1B and 2B). These properties suggest that an element that governs transcription initiation primarily from the 5828 site (the minor in vivo site) is located between positions −39 and −20. Despite its role in specifying a site of initiation, this region of the IVa_2 promoter is G-C rich and exhibits no sequence similarity to the TATA element (Fig. 2A), which occupies an analogous position in many promoters and can determine the site of initiation (see the introduction).

More important elements of the promoter, at least as judged by these in vitro assays, are found spanning and downstream from the initiation sites. The sequence from +13 to +19 is essential for efficient initiation of IVa_2 transcription, for mutations that alter this region severely impaired transcription (Fig. 4B; Table 2). Such downstream mutations, which alter the transcribed sequence, affect neither the stability of transcribed RNA in in vitro reactions (Fig. 4B) nor the efficiency of promoter clearance (31a). Several lines of evidence reported here and previously (13) indicate that the TATA-binding factor TFIID plays an important role in the initiation of IVa_2 transcription by binding to this downstream site, which contains the TATA-like sequence 5′ TATAGAAA 3′ in the transcribed strand (Fig. 4A). Yeast TBP protects the IVa_2 TATA-like sequence from DNase I digestion, and such protection is inhibited by the ML TATA sequence (13), hTBP synthesized in *E. coli* binds specifically to this sequence (Fig. 8), transcription from the IVa_2 promoter in reconstituted systems is stimulated by TFIID-containing fractions or cloned TBP (12, 13), mutations that eliminate the downstream TATA sequence severely impair IVa_2 transcription (Fig. 4B), as does heat inactivation of endogenous TFIID in unfractionated extracts (Fig. 7B), these same mutations prevent TBF binding (Fig. 8), and the addition of hTBP synthesized in *E. coli* to heat-treated extracts fully restores transcription from the major IVa_2 initiation site (Fig. 7). Thus, despite its unusual location, the TATA-like element downstream of the IVa_2 initiation site meets several criteria that define the sites of action of TFIID.

These observations, in particular the inhibitory effects of mutations that impair or prevent TBP binding (13) (Fig. 4B and 8), provide strong support for the conclusion that initiation of IVa_2 transcription normally requires binding of the TBP-containing factor TFIID to the downstream TATA element. Although transcription from the LSn(+19/+13) promoter, like that from other promoters that lack functional TATA elements (for examples, see references 11, 42, and 51), is stimulated by exogenous TBP, this response appears to be the result of a different mechanism, one that is independent of direct binding of TBP to DNA. The mutated TATA element of the LSn(+19/+13) template, unlike the wild-type sequence, fails to bind TBP (Fig. 8); the wild-type and mutant promoters exhibit different responses to increasing concentrations of TBP (Fig. 7B and C), and exogenous TBP can only partially overcome the severe inhibitory effect of the +19/+13 mutation (Fig. 4B and 7C). Thus, the data presently available are most compatible with recruitment of TBP, or TFIID, to the wild-type IVa_2 promoter by direct binding to the downstream TATA element but with recruitment of TBP, or TFIID, to the LSn(+19/+13) promoter via interaction with tethering factors (see reference 42). Additional experiments will, however, be required to provide definitive evidence for the former mechanism.

Although the TATA element does not appear to determine the sites at which IVa_2 transcription initiates (Fig. 4B), comparison of the effects of the LSn(+13/+19) and LSn(+9/+13) mutations suggests that its function is position dependent; both mutant templates contain a linker substitution between positions +13 and +19 (Fig. 4A), but the latter retains the wild-type TATA element displaced downstream by 11 bp (Fig. 4A). Nevertheless, the TATA element moved 11 bp downstream from its normal location was significantly more deleterious to IVa_2 transcription than simple replacement of most of the element by an unrelated sequence (Fig. 4B). Similar phenotypes are exhibited as the results of additional spacing mutations (11, 14a). This result strongly implies that interaction between TFIID bound to the TATA element and transcriptional components recognizing one or more upstream sites mediates an important step in initiation from this promoter.

One such element appears to be the initiator region itself; substitution of the sequence spanning the IVa_2 initiation sites by unrelated sequences reduced IVa_2 transcription to background levels (Fig. 2B and 4B), and a promoter comprising only the sequence from −9 to +13 of the IVa_2 promoter, which includes the sequence identical in 7 of 13 positions to that of the initiator element of the terminal deoxynucleotidyl transferase gene (50) (Fig. 9), is sufficient to direct specific initiation by RNApII (Fig. 5). This observation, and the phenotypes resulting from the mutations altering sequences around position −30 described previously indicate that the major (+1) and minor (−2) sites of initiation of IVa_2 transcription are independently specified, although efficient utilization of both requires the downstream TFIID-binding site (Fig. 4B).

The results of the mutational analysis reported here indicate that the Ad2 IVa_2 promoter contains at least five promoter elements, the four illustrated in Fig. 9 and an additional downstream element (Fig. 4B) within the approximately 100-bp region centered on the sites of transcription initiation. Thus, this viral gene joins the growing list of eukaryotic genes transcribed by RNApII whose basal or regulated transcription is mediated by interactions among elements located relatively short distances on either side of initiation sites (for examples, see references 3, 5, 7, 8, 28, 29, 50, and 52). On the other hand, the IVa_2 promoter is, at present, uniquely distinguished by the downstream location of a TFIID-binding site and a critical initiator element. Thus, further genetic and biochemical analyses of transcription

from the IVa_2 promoter should improve our understanding of the mechanistic roles played by TFIID and the interactions among transcriptional components that result in specific initiation of transcription by RNApII.

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