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Intragenic Activating and Repressing Elements Control Transcription from the Adenovirus IVa_2 Initiator

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The downstream stimulatory segment of the adenovirus type 2 IVa_2 promoter includes a TA-rich sequence that binds recombinant TATA-binding proteins (TBP) in vitro. We now demonstrate that when placed upstream of the IVa_2 initiator, this TA-rich sequence operated as a TATA element but exhibited significantly lower transcriptional and TBP-binding activities than did the TATA box of the adenovirus major late (ML) promoter. In sharp contrast, changing the IVa_2 TA-rich sequence in its natural, intragenic context to the ML TATA sequence increased the activity of the IVa_2 promoter only slightly. In view of this discrepancy, we examined the effects of single, double, and clustered point mutations in the downstream sequence on the activity of a minimal IVa_2 promoter. Mutations between positions +21 and +29 inhibited IVa_2 transcription, in some cases to the very low level directed by the IVa_2 initiator alone. By contrast, substitutions within the TA-rich sequence increased the efficiency of IVa_2 transcription. These results indicated that the downstream, TA-rich sequence does not function as an intragenic TFIID-binding site but rather is included within a negative regulatory element. Electrophoretic mobility shift and methylation interference assays using wild-type and mutated, intragenic promoter sequences identified a HeLa cell component whose binding to the sequence +11 to +27 correlated with repression of IVa_2 transcription, suggesting that a negative regulatory element is superimposed upon the intragenic sequence required for efficient transcription from the IVa_2 initiator.

Among the several types of eukaryotic RNA polymerase II (pol II) promoters that can be defined are those that lack upstream TATA elements yet direct initiation at one or a few closely spaced sites. The subgroup C adenovirus type 2 (Ad2) IVa_2 promoter is a member of this class (4–6, 16), which also includes the promoters of the terminal deoxynucleotidyltransferase (TdT) (33), ribosomal protein (15), porphobilinogen deaminase (2), and c-*mos* (23) genes. The mechanisms by which transcription initiates from such promoters are not fully understood but must differ from those operating at prototypical pol II promoters, which contain TATA elements 20 to 35 nucleotides (nt) upstream of initiation sites: binding of the TATA-binding protein (TBP)-containing complex TFIID (10, 36) to the TATA boxes of such promoters is an early, and committing, step in the assembly of initiation complexes (26, 29, 38).

During productive adenovirus infection, the IVa_2 gene is transcribed only during the late phase of infection (11). Nevertheless, this viral late promoter can be transcribed accurately and efficiently by the cellular transcriptional machinery in vitro (16, 18, 21). Previous studies (5, 16) have established that the IVa_2 promoter, like other pol II promoters that both lack and possess TATA elements (22, 33), contains an autonomous initiator element that directs specific initiation from the major in vivo initiation site. The sequence of the IVa_2 initiator element exhibits some similarity to those of the TdT and major late (ML) promoters (4), and it can therefore be defined as a member of the class termed InR (22). A number of factors that bind specifically at or near initiator elements have been described (2, 8, 20, 27, 30, 31). Although some, such as YY-1 and TFII-1, bind specifically to initiators of the InR class (27, 30), factors interacting specifically with the IVa_2 InR have not been identified. This element has, however, been reported to be weakly recognized by pol II (5). Although InR-directed transcription is specific, it is inefficient unless stimulated via upstream binding sites for any one of several sequence-specific factors, such as TFIID or Sp1 (22, 25, 33, 34, 38). The IVa_2 promoter includes an upstream stimulatory element centered on position −47 (16, 21), but this plays a relatively minor role both in vitro (16) and in adenovirus-infected cells (11a). Rather, efficient IVa_2 transcription depends on intragenic promoter sequences that activate InR-dependent transcription by more than a factor of 10 (4–6, 16) but do not influence initiation specificity (6).

Analysis of the activities of mutated and synthetic IVa_2 promoters has established that the major activating element lies between the InR and position +31. This region contains the TA-rich, TATA-like sequence TATAGAA oriented toward the InR in the noncoding strand (see Fig. 2), which specifically binds recombinant yeast or human TBP (4, 16). It is now clear that transcription of all class II promoters requires TFIID, regardless of whether they contain upstream TATA elements to which this factor can bind (25, 34, 40). In the case of the IVa_2 promoter, however, the properties of mutant templates suggested that the TA-rich sequence represented an intragenic binding site for TFIID: certain mutations that improved the resemblance of the TA-rich sequences to canonical TATA sequences increased IVa_2 promoter activity (4), whereas substitutions altering the TA-rich sequence inhibited IVa_2 transcription (4, 16).

The hypothesis that the downstream, TA-rich element of the IVa_2 promoter serves as a TFIID-binding site implies that this sequence should function as an upstream TATA element. We now show that this prediction is correct. Nevertheless, some observations are difficult to reconcile with the notion that the TA-rich sequence comprises an intragenic TFIID-binding site in the IVa_2 promoter. In

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particular, a single-base-pair substitution that converted this element to the sequence of the ML TATA stimulated IVa_2 transcription by less than a factor of 2, despite an increase of at least fivefold in the affinity with which human TBP bound (6). Furthermore, IVa_2 promoter derivatives carrying the mutation to the ML TATA sequence supported initiation of pol II transcription from heterogeneous sites in the IVa_2 noncoding strand, 29 to 34 nt downstream of the TATA element (6). By contrast, the TA-rich sequence of the wild-type IVa_2 promoter exhibited no such activity, even when InR function was eliminated by mutation (6). Such observations prompted us to examine the functional organization of the downstream region of the IVa_2 promoter in greater detail.

MATERIALS AND METHODS

Preparation and partial fractionation of HeLa WCE. HeLa cells were grown in SMEM medium supplemented with 5% calf serum (GIBCO) and 1% glutamine and harvested at a density of 4×10^5 to 5×10^5 cells per ml. Whole cell extracts (WCE) (17) were prepared from 3×10^9 to 4×10^9 freshly harvested cells as described previously (16) and dialyzed against buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9] containing 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol [DTT], and 17% glycerol). Protein concentrations were determined by the method of Bradford (3), and extracts were stored at −85°C in small portions. To fractionate WCE proteins, extracts were loaded onto Affi-Gel heparin-agarose (Bio-Rad) columns preequilibrated with buffer A and washed with 5 column volumes of the same buffer. Bound proteins were eluted successively with buffer A containing 0.15, 0.2, 0.3, and 1 M KCl. Peak fractions were collected, pooled, and dialyzed into buffer A.

Plasmid and mutant construction. The construction of plasmids pIVInr-TC, pIVInr-TA, and pIVInr-TC12 (see Fig. 2 and 3) has been described elsewhere (6). To construct 3′ deletion mutants, successive nucleotides were removed from the 3′ end of the Ad12 IVa_2 promoter in plasmid pIVInr-TC12 (6), using T4 DNA polymerase and mung bean nuclease. All substitutions of intragenic promoter sequences were introduced into the Ad2 IVa_2 promoter by insertion of synthetic oligonucleotides carrying the desired mutations and *Ava*II and *Bam*HI sites at their 5′ and 3′ ends, respectively, into plasmid pIVInr-TC, from which the *Ava*II-*Bam*HI (positions +8 to +31) fragment had been removed. The spacing mutant pIVInr-TC5S$^+$ was constructed by ligating a synthetic oligonucleotide carrying a 5′ *Ava*II site and a 3′ *Bam*HI overhang to position +8 of IVInr-TC. The resulting clone had an impaired *Bam*HI site at the 3′ end of the inserted IVa_2 sequence (position +32) and an internal *Bam*HI site immediately 5′ to the TA-rich element. Plasmid pIVInr-TC10S$^+$ was created by insertion of an extra 5 bp into this internal *Bam*HI site of pIVInr-TC5S$^+$. All IVa_2 promoter mutants were sequenced by the dideoxy-chain termination method (28) and prepared for transcription by CsCl gradient centrifugation of plasmid DNA followed by Bio-Gel A150m gel filtration to eliminate trace amounts of RNA.

In vitro transcription reactions. In vitro transcription reactions were carried out as described previously (6) except for those shown in Fig. 1B, in which only 400 ng of template and 125 μg of WCE were used. All gels were dried and exposed to Kodak XAR film at −85°C. The relative quantities of radioactivity incorporated into specific transcripts were determined with a model 400E PhosphorImager (Molecular Dynamics). The concentrations of IVa_2 transcripts measured in this way were corrected by using the ML internal control included in all reactions and were expressed relative to the value for the wild-type control in each experiment.

Expression and purification of human TBP. Plasmid pRset-TFIID containing the complete coding sequence of human TBP (24) under T7 promoter control (35) was transformed into *Escherichia coli* BL21(DE3). Bacteria were grown in LB medium to an optical density at 600 nm of 0.5, and TBP expression was induced by addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After incubation for 1 h at 37°C, cells were harvested and resuspended in 50 ml of K100 buffer (25 mM HEPES-KOH buffer [pH 7.9] containing 100 mM KCl, 0.1 mM EDTA, 2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM DTT, 17% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium metabisulfite). Lysozyme (20 mg) was then added, and the mixture was incubated for 20 min in ice-water prior to sonication at 4°C for a total of 1 min at maximal output. The supernatant was collected following centrifugation at 15,000 rpm in a Sorvall RG-5B centrifuge for 15 min and adjusted to 300 mM KCl. Polymin-P and Nonidet P-40 (NP-40) were then added to final concentrations of 0.25% (vol/vol) and 0.1% (vol/vol), respectively. The mixture was stirred for 20 min at 4°C, and aggregated nucleic acids and cell wall debris were then removed by centrifugation for 15 min at 15,000 rpm. Solid $(NH_4)_2SO_4$ was gradually added to the supernatant to 50% saturation, and the mixture was then stirred for 30 min at 4°C. After centrifugation for 15 min at 15,000 rpm, the pellet was resuspended in 30 ml of K100 buffer containing 0.1% NP-40. The conductivity of the solution was adjusted to that of K400 buffer, and the extract was applied to a 12-ml DEAE column preequilibrated in K400 buffer. The flowthrough fraction was loaded directly onto a 15-ml phosphocellulose column preequilibrated with K400 buffer. TBP was eluted with K1000 buffer. Peak fractions were pooled and dialyzed into K100 buffer containing 0.1% (vol/vol) NP-40. TBP, which was about 60% pure, as judged by Coomassie blue staining, was stored at −85°C in small portions.

DNase I footprinting assays. Footprinting probes were prepared by *Eco*RI digestion of plasmids containing the ML TATA or IVa_2 TA-rich sequences in the context of ML TATA flanking sequences inserted 31 nt upstream of the IVa_2 InR (Fig. 1). The digested plasmids were 3′ end labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3,000 Ci/mmol; NEN-Dupont) and then digested with *Drd*I, to generate labeled fragments of some 600 bp. These fragments were purified by electrophoresis in 1% low-melting-point agarose gels. Footprinting reactions mixtures (50 μl) contained 36 mM HEPES-KOH buffer (pH 7.9), 57 mM KCl, 9 mM $MgCl_2$, 0.1% (vol/vol) NP-40, 10% (vol/vol) glycerol, 1 mM DTT, 1.2×10^4 cpm (about 0.5 ng) of probe, 50 ng of poly(dG-dC) · (dG-dC), and various concentrations of TBP. After incubation at 30°C for 30 min, reaction mixtures were incubated with 0.1 U of RQ DNase I (Promega) for 1 min at room temperature. DNase I digestion products were purified by phenol-chloroform extraction, ethanol precipitated, and analyzed by electrophoreses in 6% polyacrylamide sequencing gels.

Electrophoretic mobility shift and methylation interference assays. A synthetic, double-stranded oligonucleotide spanning the IVa_2 promoter sequence from −2 to +36 was labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; NEN-Dupont) and T4 polynucleotide kinase (New England Biolabs) and used in

electrophoretic mobility shift assays (12, 13). Binding reaction mixtures (15 μl) contained 12 mM HEPES-KOH buffer (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 3×10^4 cpm (approximately 0.5 ng) of probe, 1 μg of poly(dI-dC), and empirically determined concentrations of proteins. For competition experiments, the concentrations of double-stranded competitor oligonucleotide DNA indicated in the figures were added to reaction mixtures before addition of proteins. After incubation for 15 min at room temperature, the mixtures were loaded onto 4% polyacrylamide gels cast in 0.5× TBE (1× TBE is 0.098 M Tris-borate buffer [pH 8.3] containing 2 mM EDTA) and electrophoresed at 4°C for 2 h in the same buffer. Gels were dried and exposed to Kodak X-ray film.

For methylation interference, the IVa_2 probe was labeled at the 5′ end of either the coding or the noncoding strand and partially methylated by exposure to 1 μl of dimethyl sulfate in a total volume of 210 μl for 5 min at room temperature. The probe was purified and used in mobility shift assays as described above. Bound and free DNAs were excised from gels, eluted onto DEAE paper (1), and finally subjected to G+A cleavage (26). Cleaved DNA was precipitated and separated by electrophoresis in 16% polyacrylamide–8 M urea gels. Gels were dried and exposed to Kodak X-ray film.

RESULTS

The intragenic TA-rich sequence of the IVa_2 promoter can function as an upstream TATA element. We have previously shown that the downstream TA-rich sequence of the IVa_2 promoter, TATAGAA, is bound by bacterially synthesized, human TBP with lower affinity than the canonical ML TATA box, TATAAAA. However, IVa_2 promoters carrying the wild-type TATAGAA sequence or the canonical TATAAAA sequence of the Ad2 ML promoter are transcribed with similar efficiencies in vitro (6). One explanation for this paradox could be that the IVa_2 TATAGAA sequence, while binding TBP under in vitro conditions (see the introduction), cannot fulfill TATA element function in transcription. To investigate this possibility, we exploited the fact that introduction of a TATA element about 30 bp upstream of the IVa_2 or TdT initiator element results in a dramatic (up to approximately 50-fold) stimulation of transcription (6, 33, 34). Therefore, we built promoters in which the downstream sequences +5 to +30 of both the wild-type IVa_2 promoter and its ML TATA box-containing derivative were placed at position −31 with respect to the IVa_2 InR in an orientation consistent with the transcription direction (Fig. 1A). Typical results obtained when the activities of these two templates were analyzed in vitro are shown in Fig. 1B. The IVa_2 InR alone (plasmid pIVInr) was very poorly transcribed under the conditions used in these experiments (Fig. 1B, lane 1). The efficiency of transcription was, however, increased some 10-fold in the presence of an upstream IVa_2 TA-rich sequence and more than 50 times over control levels when an ML TATA element was placed upstream of the InR (Fig. 1B, lanes 2 and 3, respectively).

This stimulatory effect was not due to IVa_2 sequences flanking the TA-rich element, for the difference in transcriptional activities exhibited by the two templates was the result of a single-base-pair change in the TA-rich sequence (Fig. 1A). Moreover, the same difference in activity was observed when IVa_2 TA-rich and ML TATA sequences were built into analogous promoters in the context of the sequences that flank the ML TATA element (data not shown). Furthermore, the greater stimulatory activity of the ML TATA sequence (Fig. 1B) was matched by its better binding by human TBP: as illustrated in Fig. 1C, bacterially expressed, human TBP specifically protected both the IVa_2 TA-rich and the ML TATA sequences from DNase I cleavage, but a fivefold-higher concentration of TBP was required for protection of the former sequence. A similar difference in the affinity of TBP for the IVa_2 TA-rich and ML TATA sequences has been observed previously in mobility shift competition assays (6). Thus, the results shown in Fig. 1 indicated that the IVa_2 TA-rich sequence can be defined as a TATA element by virtue of its ability to stimulate InR-dependent transcription. Nevertheless, substitution of the G of the IVa_2 TATAGAA sequence at its natural, intragenic position in the IVa_2 promoter with an A stimulated IVa_2 transcription to only a very modest degree (6), a result in sharp contradiction to those obtained when the same change was made in the context of an upstream TATA sequence (Fig. 1). This difference strongly suggested that the downstream TA-rich sequence of the IVa_2 cannot be a functional TATA element, capable of stimulating transcription from the IVa_2 promoter through interaction with the TBP-containing complex TFIID.

Interaction between the InR and downstream stimulatory elements exhibits a strict spacing requirement. We previously observed that insertion of 11 bp between the InR and TA-rich sequences of the IVa_2 promoter reduced transcription to undetectable levels (16). Direct assays of RNA stability established that the reduced quantities of IVa_2 transcripts detected when transcription reaction mixtures contained this template or other templates carrying large alterations of the IVa_2 sequence transcribed into RNA were not the result of instability of the mutated, compared with wild-type, transcripts (16). Before embarking upon further mutational analysis of the downstream sequence, we therefore investigated whether this inhibitory effect were the result of altered spacing between the InR and downstream stimulatory elements. In these experiments, insertions or deletions were made at position +14 or +13, respectively, immediately adjacent to the 3′ end of the initiator element used in our previous experiments (6). Substitution of position +13 (and +12) did not alter the activity of the IVa_2 promoter (Fig. 2B).By contrast, deletion of the base pair occupying position +13 resulted in a greater than threefold reduction in promoter activity (Fig. 2C, lanes 6 and 7). Similarly, insertion of 5 or 10 bp immediately 3′ to this position severely inhibited IVa_2 transcription (Fig. 2C, lanes 3 to 5): the predicted transcripts of 86 and 92 nt, respectively, were present at low concentrations, similar to that of the 63-nt transcript generated by a promoter that contained only the IVa_2 InR (compare lanes 2, 4, and 5 in Fig. 2C). Concomitantly, utilization of upstream (presumably nonspecific) initiation sites at position −10 or −11 in the wild-type sequence was increased (Fig. 2C, lanes 3 to 5). As precise substitutions within the analogous region (i.e., +14 to +19) stimulated rather than inhibited IVa_2 transcription (see below), these results suggested that functional interactions between the IVa_2 initiator and activating downstream element(s) are very sensitive to the distance that separates the elements. All mutant promoters subsequently constructed therefore contained only precise substitutions of the wild-type sequence.

Downstream positive and negative regulatory elements determine the efficiency of IVa_2 transcription. The results described in a previous section indicated that the downstream TA-rich sequence of the IVa_2 promoter did not behave as a TATA element. To identify the element(s) located between

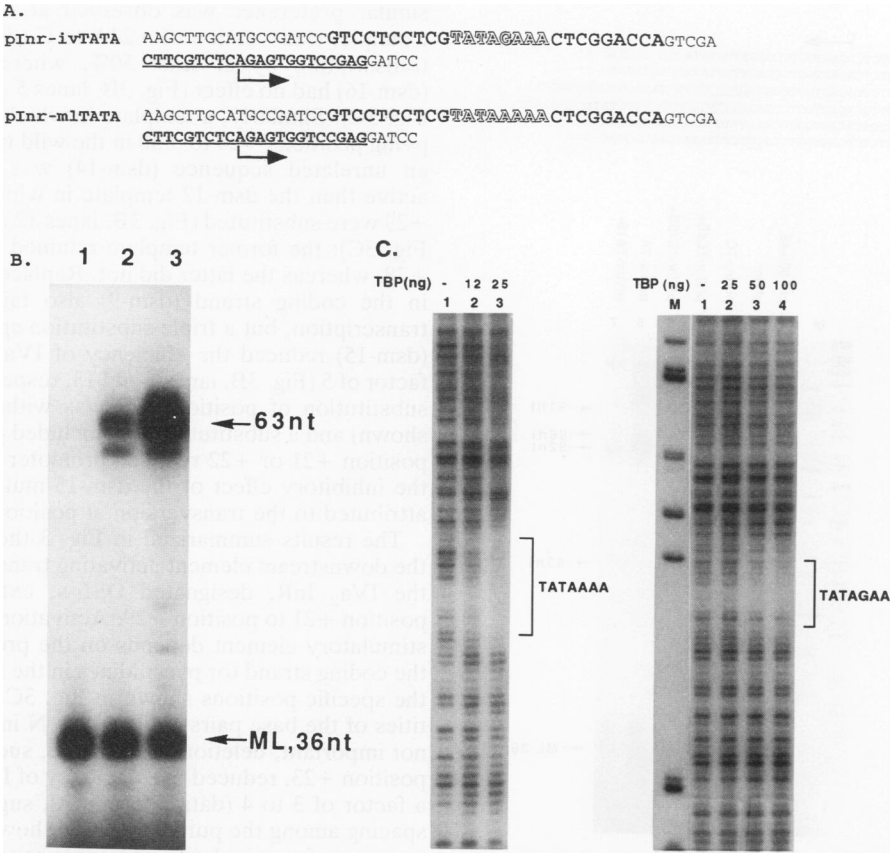

FIG. 1. The IVa$_2$ TA-rich sequence can function as TATA element. (A) Sequences of the two synthetic templates used in the experiment shown in panel B. The IVa$_2$ InR sequences are in boldface type and underlined. Sequences shown in bold and larger face are from the IVa$_2$ downstream sequence +5 to +31 inserted into position −31 with respect to the transcription initiation sites, which are indicated by arrows drawn in the direction of transcription. The TATA elements are indicated in open face. (B) In vitro transcription assays were as described in Materials and Methods. IVa$_2$ transcripts and transcripts of the ML promoter used as internal control were detected, using the Pneo and ML primers described previously (6), which yielded the cDNAs of 63 and 36 nt, respectively, indicated at the right. Transcription reaction mixtures contained pIVInr, which comprised the IVa$_2$ InR alone (6) (lane 1), or template pInR-ivTATA (lane 2) or pInR-mlTATA (lane 3), shown in panel A. (C) DNA fragments containing the upstream promoter regions were excised from pInR-ivTATA or pInR-mlTATA DNA and used in DNase I protection assays as described in Materials and Methods. Binding reaction mixtures contained 0 (lane 1 in each panel) or the quantities listed of bacterially expressed human TBP purified as described in Materials and Methods. Lane M contained end-labeled, *Msp*II fragments of pBR322 DNA.

positions +14 and +31 responsible for stimulation of InR-directed transcription (6), additional mutations were introduced into this region. To define the promoter-distal boundary of the segment required for efficient transcription from the IVa$_2$ InR, 3′ deletions were introduced into the promoter. In these experiments, the construct pIVInR-TC12, which contains the Ad12 IVa$_2$ promoter segment −9 to +32 (6) (Fig. 3A), was used, as it facilitated introduction of 3′ deletions (see Materials and Methods). This subgroup A adenovirus IVa$_2$ promoter and that of Ad2 are transcribed with efficiencies that differ by less than a factor of 2 (Fig. 3A, lanes 1 and 2) (6). Typical results obtained upon in vitro analysis of the transcriptional activities of three of these mutants are shown in Fig. 3A. Template TC12-d1, from which sequences downstream of +30 had been removed, was transcribed almost as well as the wild type (Fig. 3A, lanes 2 and 3), but deletion of an additional 2 bp (TC12-d2) reduced transcriptional activity to the basal level exhibited by the IVa$_2$ InR alone (compare lanes 2 and 4 in Fig. 3A). Removal of additional sequences did not weaken the promoter further (Fig. 3A, lane 5), although a novel primer extension product of some 60 nt was observed. This appeared to represent transcripts initiated at position +10: the 5-bp sequence centered at position +10 of the Ad12 IVa$_2$ promoter is identical to that centered on position +1 (Fig. 3A). The properties of these deleted IVa$_2$ promoters indicated that the sequence mediating transcription activation included a region downstream from the TA-rich sequence and identified positions +29G or +30A in the coding strand, conserved in the Ad12 and Ad2 promoters (6), as part of the intragenic activating element of the IVa$_2$ promoter. To define this element more precisely, we introduced the series of single-, double-, and multiple-base-pair substitutions shown in Fig. 3C into the +20 to +30 region of the Ad2 IVa$_2$ promoter. Typical results obtained when the effects of these mutations were assessed in in vitro transcription assays are shown in Fig. 3B.

Substitution of position +30 (dsm-4) did not alter IVa$_2$ promoter activity, but changing either of positions +29 and +28 (dsm-6 and dsm-7, respectively) reduced activity to less than 50% of the wild-type value (Fig. 3B, lanes 1, 3, 4, and 8; Fig. 3C). The requirement for bp +28 and +29 was

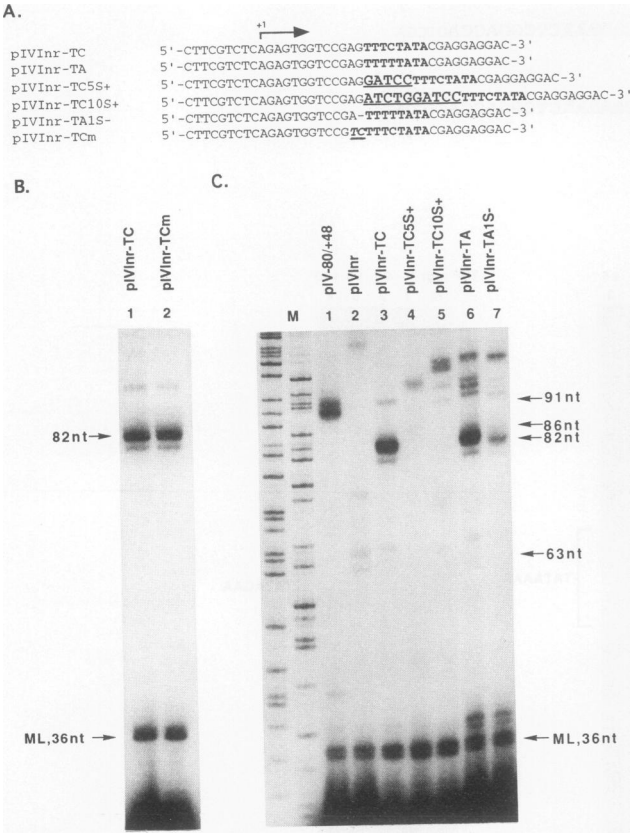

FIG. 2. Functional interaction between the IVa_2 initiator and downstream activation sequence is distance dependent. Transcription reactions were carried out as described in Materials and Methods. Reaction mixtures contained the IVa_2 templates listed above each lane and illustrated in panel A. Sequences altered by substitution (PIVInr-TCm) or insertion (pIVInr-TC5S+ and pIVInr-10S+) are underlined. The pIV−80/+48, pIVInr, and pIVInr-TA templates comprised the −80 to +48 segment of the IVa_2 promoter, the −9 to +13 initiator (6), and the −9 to +31 sequence with the TA-rich segment altered to a canonical ML TATA element (6), respectively. The 63-, 82-, and 36-nt primer extension products generated by pIVInr, pIVInr-TC, and ML transcripts are indicated at the left (B) or right (C), as is the 86-nt cDNA predicted for the 5S+ template (C). The pIV−80/+48 cDNAs, 91 and 93 nt (C, lane 1) provided markers for the predicted product of the 10S+ template. Sequencing ladders used as size markers were loaded in lane M.

confirmed by the severe inhibition of IVa_2 transcription observed when both were replaced: the resulting template, dsm-12, displayed a level of transcription comparable to that observed when transcription was directed by the InR alone (Fig. 3B, lane 10; Fig. 3C). In conjunction with the properties of deleted templates described previously, these results place the 3′ boundary of the downstream activating element at position +29. The identity of base pairs between positions +25 and +27 appeared relatively unimportant: substitution of position +27 (dsm-5) or of positions +25 to +27 (dsm-17) did not significantly alter the efficiency of IVa_2 transcription (Fig. 3B, lanes 2 and 15; Fig. 3C). However, transversion at position +26 (G in the wild-type coding strand) in dsm-10 modestly but reproducibly impaired promoter activity (Fig. 3B, lane 7; Fig. 3C). As transition at this same position was without effect (dsm-17; Fig. 3C), a purine at position +26 in the coding strand was more favorable than a pyrimidine. A similar preference was observed at other positions. For example, transversion of +24A to C (dsm-8) inhibited IVa_2 transcription by at least 50%, whereas transition to G (dsm-16) had no effect (Fig. 3B, lanes 5 and 14, respectively; Fig. 3C). Similarly, a template in which the sequence occupying positions +24 to +30 in the wild type was replaced by an unrelated sequence (dsm-14) was reproducibly more active than the dsm-12 template in which only bp +28 and +29 were substituted (Fig. 3B, lanes 12 and 10, respectively; Fig. 3C): the former template retained a purine at position +28, whereas the latter did not. Replacement of +21A by G in the coding strand (dsm-9) also failed to inhibit IVa_2 transcription, but a triple substitution spanning this position (dsm-15) reduced the efficiency of IVa_2 transcription by a factor of 5 (Fig. 3B, lanes 6 and 13, respectively; Fig. 3C). As substitution of position +22 was without effect (data not shown) and a substitution that included position +20 but not position +21 or +22 reduced promoter activity by 50% (6), the inhibitory effect of the dsm-15 mutation can largely be attributed to the transversion at position +21.

The results summarized in Fig. 3 therefore indicate that the downstream element activating transcription directed by the IVa_2 InR, designated DSE-s, extends from at least position +21 to position +29. Activation by the downstream stimulatory element depends on the presence of purines in the coding strand (or pyrimidines in the noncoding strand) at the specific positions shown in Fig. 3C. Although the identities of the base pairs indicated as N in this sequence were not important, deletion of even one, such as that occupying position +23, reduced the efficiency of IVa_2 transcription by a factor of 3 to 4 (data not shown), suggesting that correct spacing among the purine residues shown in Fig. 3C may be necessary for stimulation of transcription from the IVa_2 InR.

To investigate whether the TA-rich, intragenic sequence were partially or wholly included within the activating element, additional, precise substitutions were introduced into the Ad2 IVa_2 promoter between positions +14 and +23. When the TA-rich segment +14 to +19 was replaced with two unrelated sequences, the efficiency of IVa_2 transcription was significantly increased (templates pIVdsm-2 and pIVdsm-3; Fig. 4, lanes 1, 3, and 4). The greatest increase, five- to sevenfold, was induced by the dsm-2 mutation (Fig. 4, lanes 3 and 7). Substitution of positions +17 to +19 and +20 to +23 (template pIVdsm-18) also increased the efficiency of IVa_2 transcription (Fig. 4, lanes 5 and 6), as, by a small degree, did a single substitution, of the C in the coding strand at position +17 with a G (dsm-1; Fig. 4, lane 2). The latter increase was similar in magnitude to that observed when the same C was replaced by T, so that the resulting plasmid (pIVInr-TA) carried the canonical ML TATA element (6). The unexpected, elevated transcriptional activity displayed by these templates could be attributed to neither the creation of canonical TATA elements nor inadvertent introduction of a specific activation sequence: no TATA-like sequences were present in pIVdsm-2 or pIVdsm3, and the substitutions that they contained were unrelated (Fig. 4). The results shown in Fig. 4 therefore indicated that bp +14 to +19 of the IVa_2 promoter are included within a negative regulatory element.

A HeLa cell factor that binds specifically to an intragenic sequence of the IVa_2 promoter. The properties of the IVa_2 promoters carrying altered sequences between positions +14 and +30 described above indicated that this segment of the IVa_2 transcription unit contains closely juxtaposed, or overlapping, negative and positive regulatory elements. In a first attempt to construct a more detailed picture of the

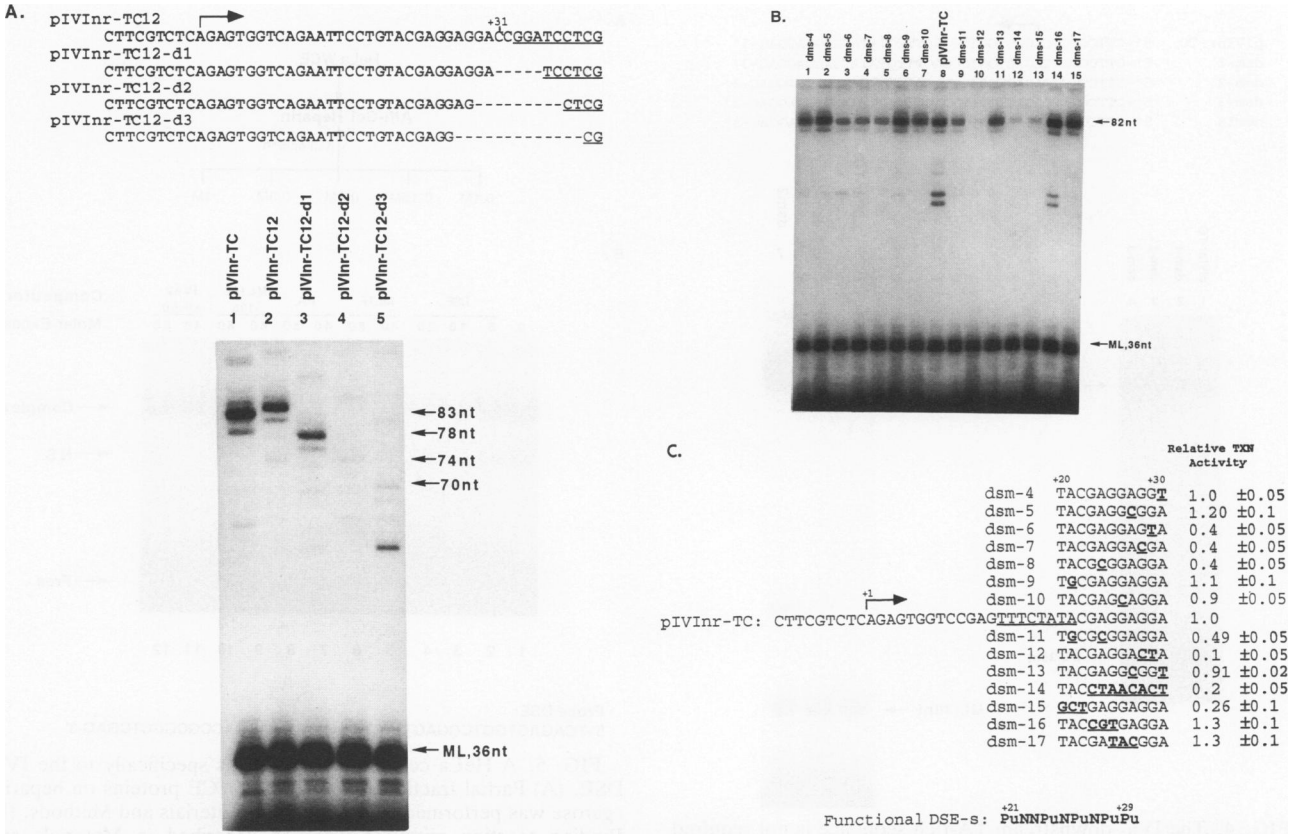

FIG. 3. The element responsible for stimulation of IVa$_2$ transcription is located between positions +20 and +30. (A) Transcription reaction mixtures were as described in Materials and Methods and contained the Ad2 or Ad12 IVa$_2$ templates listed at the top. After deletion of CCGGA from plasmid pIVInr-TC-12 (wild type; see reference 6), a new site for *Bam*HI cleavage was created in the resulting plasmid, pIVInr-TC-12-d1, which served as the parent for creation of pIVInr-TC12-d2 by removal of 4 nt by *Bam*HI cleavage followed by mung bean nuclease digestion. The resulting plasmid, pIVInr-TC-12-d2, carried a new restriction site for *Sac*II, which was used to construct plasmid pIVInr-TC-12-d3 in a similar way. The deleted nucleotides are indicated by dashes and plasmid sequences are underlined. The predicted primer extension products from each 3′ deletion mutant are indicated at the right. (B) Transcription reaction mixtures contained 750 ng of the IVa$_2$ templates carrying base substitutions indicated above the lanes. The control wild-type IVa$_2$ promoter, pIVInr-TC, is shown in larger letters. The lengths of IVa$_2$ (82 nt) and control ML (36 nt) primer extension products are indicated at the right. (C) The sequences of the mutant templates used in the experiment shown in panel B are compared with that of the wild-type IVa$_2$ promoter, drawn in the middle. Mutated positions are indicated in boldface and underlined. The transcription (TXN) activity of each template (right) was determined by direct quantitation of specific transcripts, using a Molecular Dynamics model 400E PhosphorImager as described in Materials and Methods. The relative activities shown are the means of at least two independent measurements with each template. The sequence of the functional DSEs deduced from these results (see text) is shown at the bottom.

organization of this potentially complex, intragenic control region, we investigated whether HeLa cells contained proteins that specifically bound to the IVa$_2$ intragenic promoter segment. Accordingly, a DNA fragment comprising the IVa$_2$ sequence −2 to +36 (DSE in Fig. 5B) was incubated with fractions obtained from HeLa cell WCE by chromatography on heparin-agarose (Fig. 5A), to increase the likelihood of detecting more than one factor. Specific DSE-binding activity, assessed in electrophoretic mobility shift assays, was recovered only in the 0.15 M KCl fraction: this fraction contained an activity whose binding to the DSE oligonucleotide was severely inhibited by a 10-fold molar excess of unlabeled DSE DNA (Fig. 5B, lanes 1 to 4), whereas nonspecific competitor DNAs were all unable to compete at 80-fold molar excess concentrations (Fig. 5B, lanes 5 to 12). Methylation interference assays (see Materials and Methods) were then used to define at higher resolution the intragenic IVa$_2$ promoter sequences necessary for formation of the specific complex illustrated in Fig. 5B. Methylation of guanines at positions +11, +23, and +26 in the coding strand or at positions +17 and +22 in the noncoding strand interfered with formation of the specific complex (Fig. 6; compare lanes B and F), as did methylation of A's at positions +27, +21, and +19 in the coding strand or at positions +18 and +20 in the noncoding strand. By contrast, A methylation at position +24 in the coding strand or +15 in the noncoding strand enhanced binding (Fig. 6).

The results shown in Fig. 5 and 6 established that the HeLa cell extracts used in our transcription assays contain a protein that makes extensive contact with intragenic sequences of the Ad2 IVa$_2$ promoter to form a specific complex. Purines located at specific positions within the sequence +11 to +27 of the promoter, that is, within both the promoter-proximal, negative and promoter-distal, positive regulatory regions described previously, are required for formation of this specific complex (Fig. 6). To investigate whether this HeLa cell DNA-binding activity might mediate the repression and stimulation of IVa$_2$ transcription, respectively, ascribed to these elements, we examined the effects of mutation of the two regions on the DNA-binding activity.

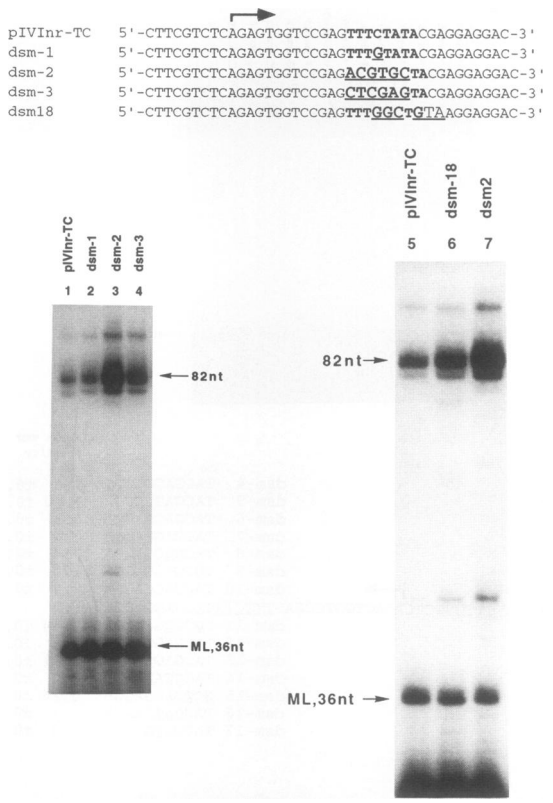

FIG. 4. The IVa_2 downstream TA-rich sequence is not required for stimulation of IVa_2 transcription. Transcription reactions were performed as described in Materials and Methods, and mixtures contained the templates indicated above the lanes. The positions of ML (36 nt) and IVa_2 (82 nt) primer extension products are indicated at the right or left. Templates carrying mutations of the TA-rich sequence (bold) of the IVa_2 promoter are shown at the top, with mutated sequences underlined and in larger face.

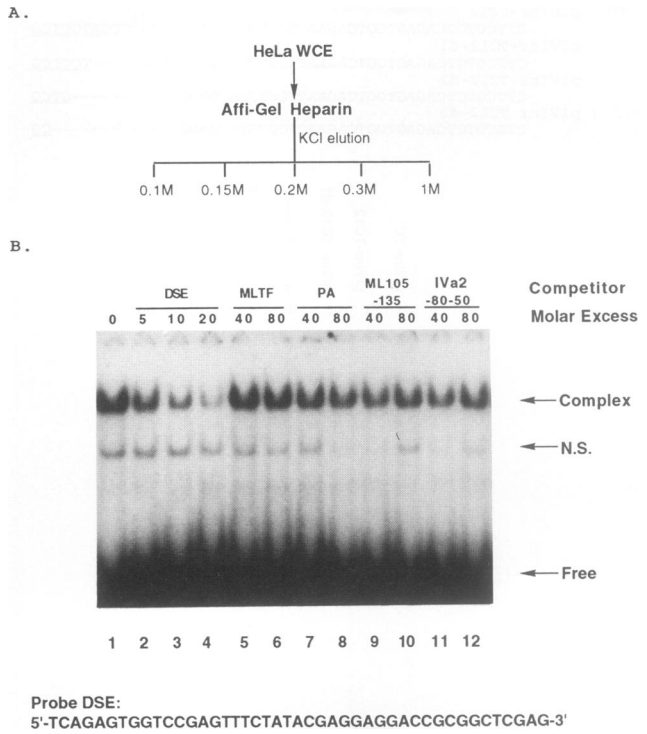

FIG. 5. A HeLa cell protein that binds specifically to the IVa_2 DSE. (A) Partial fractionation of HeLa WCE proteins on heparin-agarose was performed as described in Materials and Methods. (B) Binding reaction mixtures were as described in Materials and Methods section and contained 7 μl of 0.15 M fraction protein (approximately 1 μg) and 2×10^4 cpm (0.5 ng) of DSE DNA. Reaction mixtures contained no competitor (lane 1) or the concentrations listed at the top of the competitors indicated. The sequence of the DSE DNA fragment used as the probe is shown at the bottom. The nontranscribed strand sequences of the other (double-stranded) competitors were as follows: MLTF, 5'-AGGTGTAGGCCACGTGAC CGGGTGTTCCTG-3'; PA, 5'-CCACTATAATGTGCTGGTAAG GATCTATAAATGACA-3'; ML+105/+135, 5'-AGGAGGATTTG ATATTCACCTGGCCCGCGG-3'; and IVa2−80/−50, 5'-GTGGAC CCCTAGTGGACAACGACCGCTAC-3'. The positions of free probe and the specific complex discussed in the text are indicated at the right. N.S., nonspecific.

The 2-bp substitution of positions +28 and +29 that eliminated stimulation of InR-directed IVa_2 transcription (template dsm-12 in Fig. 3B and C) had no effect on binding of the HeLa cell protein: an oligonucleotide containing this mutation (DSE-m) inhibited formation of the specific complex at least as efficiently as did the wild-type DNA fragment (Fig. 7A). Furthermore, identical interference patterns were obtained when the DSE and DSE-m DNA fragments were compared in methylation interference assays (Fig. 6 and data not shown). Thus, binding of the HeLa cell protein did not correlate with stimulation of IVa_2 transcription by intragenic promoter sequences. By contrast, mutations of the TA-rich sequence that stimulated IVa_2 transcription resulted in reduced binding: in competition assays, the wild-type sequence, DSE, was an effective inhibitor of specific complex formation when added to binding reaction mixtures at fivefold molar excess (Fig. 7B, lanes 1 to 4). However, the dsm-2 mutation, which increased the efficiency of IVa_2 transcription some fivefold (Fig. 4), did not compete as well until added at at least a fourfold-higher concentration (Fig. 7B, lanes 5 to 7). An oligonucleotide containing the dsm-18 mutation, which also increased IVa_2 promoter activity (Fig. 4), also inhibited binding to the wild-type sequence less efficiently than did the wild-type DNA (Fig. 7B; compare lanes 8 to 10 with lanes 1 to 4).

DISCUSSION

Efficient InR-directed initiation of transcription from the Ad2 IVa_2 promoter requires intragenic sequences located between the major initiation site (+1) and position +31 (see the introduction). This region contains the TA-rich sequence TATAGAA in the noncoding strand, with which TBP interacts specifically (see the introduction). The results shown in Fig. 1 provide strong support for the conclusion that, in an appropriate promoter context, this TA-rich sequence can serve as a functional TATA box: the abilities of the IVa_2 TATAGAA and the ML TATAAAA sequences to stimulate transcription when placed upstream of the IVa_2 InR correlated quantitatively with the affinities with which they were bound by human TBP. The IVa_2 TA-rich sequence thus resembles the many variant TATA box sequences that have been shown to function effectively (14, 32, 37). Despite its activity as a TATA element when placed at an appropriate upstream position (Fig. 1), the results presented here indicate that the intragenic TA-rich sequence of the wild-type IVa_2 promoter is not responsible for activation of IVa_2

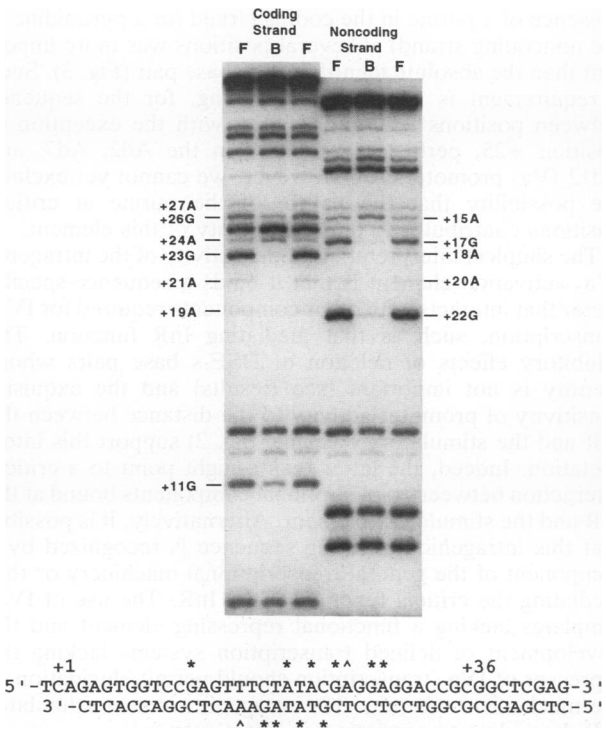

FIG. 6. Methylation interference mapping of the HeLa cell protein-binding site. (A) Methylation interference assays were performed as described in Materials and Methods. The coding strand is shown at the left, and the noncoding strand is shown at the right, with free DNA and DNA recovered from the specific complex shown in Fig. 5 run in lanes F and B, respectively. The nucleotides whose methylation prevented binding are indicated by bars at the side of each panel. (B) The bases of the wild-type IVa$_2$ sequence whose methylation interfered with formation of the specific complex are indicated by asterisks, and those whose methylation enhanced complex formation are indicated by carets.

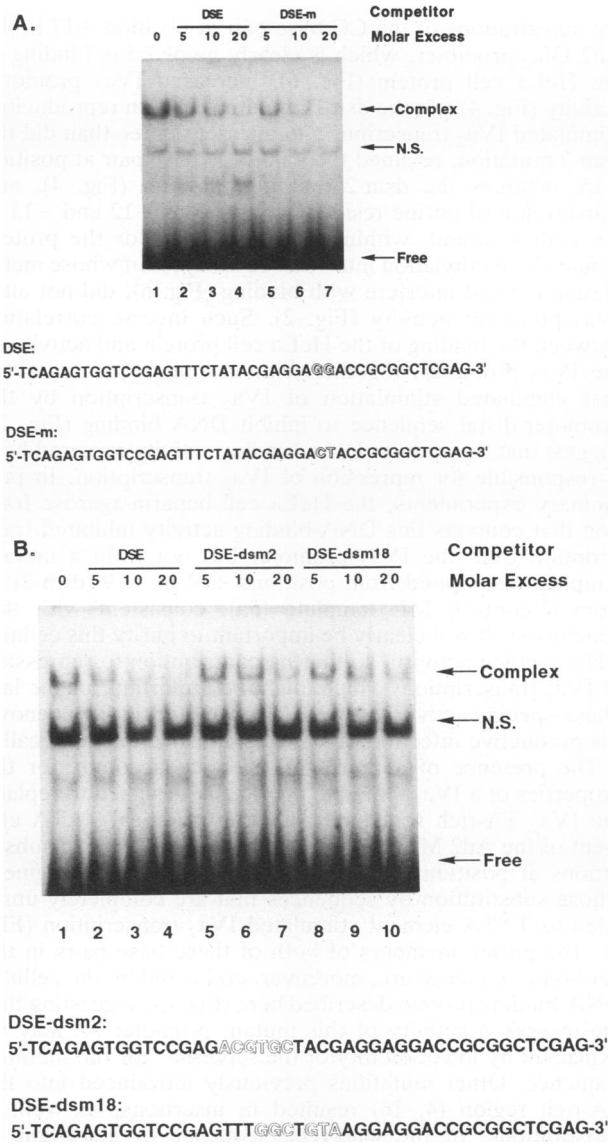

FIG. 7. Effects of mutation of IVa$_2$ intragenic promoter sequences on specific HeLa cell DNA-binding activity. Binding competition reaction mixtures were as described in the legend to Fig. 5 and contained the molar excess concentrations of the wild-type (DSE) or mutant oligonucleotide competitors listed at the top of each panel. The DSE-m oligonucleotide (A, lanes 5 to 7) contained the +28 and +29 substitutions present in the dsm-12 template whose activity is illustrated in Fig. 3B, whereas the DSE-dsm 2 and DSE-dsm 18 competitors (B) contained the mutations present in the dsm-2 and dsm-18, respectively, templates shown in Fig. 4.

transcription: substitutions within this sequence result in a stimulation of IVa$_2$ transcription (Fig. 4). Moreover, sequences essential for efficient transcription from the IVa$_2$ InR are located further downstream in the IVa$_2$ promoter (Fig. 3). The lack of conservation of the Ad2 IVa$_2$ TATA-like sequence in other adenovirus serotypes (6) and the properties of mutant IVa$_2$ promoters in which the TA-rich sequence was inverted or precisely replaced with the canonical TATA sequence of the Ad2 ML promoter (6) (see the introduction) reinforce the conclusion that this IVa$_2$ sequence does not function as an intragenic TATA element to stimulate InR-dependent transcription.

The inhibition or stimulation of IVa$_2$ transcription by mutations altering the TA-rich sequence reported previously (6, 16) can now be interpreted in terms of the unexpectedly complex, functional organization of the short intragenic region required for efficient IVa$_2$ transcription. The TA-rich sequence itself forms part of a negative regulatory element for its substitution (by at least two sequences unrelated to one other) increased the activity of the IVa$_2$ promoter (Fig. 4). Uninfected HeLa cells contain an activity that binds specifically to an intragenic region spanning the TA-rich sequence (Fig. 5 and 6). The relationship between the site to which this cellular protein binds and the repressing element of the IVa$_2$ remains to be precisely established: whether substitution of the G at +11 in the coding strand, whose methylation partially inhibited binding (Fig. 6), stimulates IVa$_2$ transcription is not yet known. Moreover, functional definition of the 3′ boundary of the repressing element is complicated by its probable overlap with the promoter-distal element required for efficient IVa$_2$ transcription, which extends from at least position +21 to position +29 (Fig. 3). Despite such uncertainties about boundaries, mutations at internal positions of the binding site for the HeLa cell protein, defined by methylation interference mapping (Fig. 6), inhibited binding and stimulated IVa$_2$ transcription to comparable degrees (Fig. 4 and 7). Furthermore, two differ-

ent substitutions of the CG base pair at position +17 in the Ad2 IVa_2 promoter, which is clearly involved in binding of the HeLa cell protein (Fig. 6), increased IVa_2 promoter activity (Fig. 4) (6); the dsm-3 mutation, which reproducibly stimulated IVa_2 transcription to a lesser degree than did the dsm-2 mutation, retained the wild-type base pair at position +15, whereas the dsm-2 mutation did not (Fig. 4), and substitution of purine residues at positions +12 and +13 in the coding strand, within the binding site for the protein defined by methylation interference assays, but whose methylation did not interfere with binding (Fig. 6), did not alter IVa_2 promoter activity (Fig. 2). Such inverse correlation between the binding of the HeLa cell protein and activity of the IVa_2 promoter, together with the failure of mutations that eliminated stimulation of IVa_2 transcription by the promoter-distal sequence to inhibit DNA binding (Fig. 7), suggest that the cellular DNA-binding activity reported here is responsible for repression of IVa_2 transcription. In preliminary experiments, the HeLa cell heparin-agarose fraction that contains this DNA-binding activity inhibited transcription from the IVa_2 promoter but not from a mutant template substituted from positions +14 to +19 (dsm-2) or from a control, ML template (5a), consistent with this conclusion. It will clearly be important to purify this cellular DNA-binding activity, so that its contribution to repression of IVa_2 transcription, which might be important for the late phase-specific activity of the IVa_2 promoter during adenovirus productive infection, may be established unequivocally.

The presence of a repressing element accounts for the properties of a IVa_2 template previously designed to replace the IVa_2 TA-rich sequence with the canonical TATA element of the Ad2 ML promoter: this template carried substitutions at positions +14 and +17 (4), within the segment whose substitution by sequences that are completely unrelated to TATA element stimulated IVa_2 transcription (Fig. 4). The purine members of both of these base pairs in the wild-type sequence are, moreover, contacted by the cellular DNA-binding protein described here (Fig. 6), suggesting that the increased activity of this mutant promoter (4) may be explained by lower affinity of the repressor for the mutated sequence. Other mutations previously introduced into the TA-rich region (4, 16) resulted in insertions, as well as substitutions, of the wild-type sequence or alteration of sequences within the promoter-distal, intragenic element essential for efficient IVa_2 transcription, alterations whose inhibitory effects (4, 16) can now be ascribed to properties of the IVa_2 promoter (Fig. 2 and 3, respectively) that have nothing to do with binding of TFIID.

The properties of IVa_2 promoters carrying 3′ deletions or single or clustered point mutations within the segment +21 to +29 (Fig. 3) mapped an activation element to this region. This element appears to account for the stimulatory activity of downstream promoter sequences (see the introduction), for certain mutations, for example, dsm-12, reduced IVa_2 transcription to the low level supported by the InR alone (Fig. 3B and C). The promoter-distal boundary of this stimulatory element has been located at position +29, but definition of the left-hand limit based on the activities of mutated promoters is not yet possible: all transcription assays performed to date included the putative repressor whose binding site extends into the stimulatory element. Thus, some of the mutations of most interest in this regard might alter the activity of both repressing and stimulatory elements. Although the importance of specific base pairs within the +21 to +30 segment, for example, those at positions +24, +28, and +29, was clearly established, the presence of a purine in the coding strand (or a pyrimidine in the noncoding strand) at several positions was more important than the absolute identify of the base pair (Fig. 3). Such a requirement is somewhat surprising, for the sequence between positions +20 and +30 is, with the exception of position +25, perfectly conserved in the Ad2, Ad7, and Ad12 IVa_2 promoters (6). However, we cannot yet exclude the possibility that the nature of the purine at critical positions contributes to optimal activity of this element.

The simplest interpretation of the activity of the intragenic IVa_2 activation element is that it binds a sequence-specific factor that interacts with other components required for IVa_2 transcription, such as that mediating InR function. The inhibitory effects of deletion of DSE-s base pairs whose identity is not important (see Results) and the exquisite sensitivity of promoter activity to the distance between the InR and the stimulatory element (Fig. 2) support this interpretation. Indeed, the latter result might point to a critical interaction between transcriptional components bound at the InR and the stimulatory element. Alternatively, it is possible that this intragenic activating sequence is recognized by a component of the general transcriptional machinery or that mediating the critical function of the InR. The use of IVa_2 templates lacking a functional repressing element and the development of defined transcription systems lacking the repressor of IVa_2 transcription should permit elucidation of the mechanism whereby this intragenic element stimulates InR-dependent transcription so effectively.

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