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Positive and Negative Control Sequences Within the Distal Domain of the Adenovirus IVa_2 Promoter Overlap with the Major Late Promoter

VENKATACHALA NATARAJAN, MARY J. MADDEN, AND NORMAN P. SALZMAN*

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205

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The RNA initiation sites of the adenovirus IVa_2 and major late promoters (MLP) are separated by 210 base pairs and transcribed from opposite DNA strands. We had previously shown that they contained overlapping promoter sequences (V. Natarajan, M. J. Madden, and N. P. Salzman, Proc. Natl. Acad. Sci. U.S.A. 81:6290–6294, 1984). The transcription efficiencies of these two promoters were studied in vitro with templates of covalently closed circular DNAs that contained various deletion and point mutants. The distal control region of the IVa_2 promoter that is located at nucleotide position (np) −152 to −242 from the RNA initiation site consists of at least two domains. The first distal domain, present between np −152 and −179, is necessary for efficient transcription of the IVa_2 promoter, and it overlaps with sequences that have been shown to be necessary for efficient transcription of MLP. This region may serve as the entry site for the transcription machinery. The second distal domain consists of sequences present between np −211 and −242. These sequences are contained at the 5′ end in the MLP transcript, and they inhibit transcription from the IVa_2 promoter. However, these sequences are not necessary for transcription of the MLP with a covalently closed template but are needed for transcription with a linear template. The TATA box that is located at np −180 to −186 between these two domains has a critical role for efficient transcription of the MLP. A point mutation that reduces transcription from MLP by more than 80% stimulates transcription from IVa_2 promoter by 10-fold. This finding is consistent with the proposal that MLP and IVa_2 promoters share an entry site for transcription machinery and compete for its use.

The majority of eucaryotic genes transcribed by RNA polymerase II contain a TATA box sequence 30 base pairs (bp) upstream of the RNA initiation site (2, 19, 22). Promoters that do not have this sequence include those for the simian virus 40 late gene, hepatitis B virus surface antigen gene, and the adenovirus E2 and IVa_2 genes (1, 6, 11, 17). Recently, a number of cellular genes have also been characterized that lack a TATA sequence at the −30 region of the RNA initiation site (10, 24). A common alternate transcriptional control sequence has not been identified for TATA boxless promoters. We had previously reported that the promoter for the adenovirus IVa_2 gene has proximal and distal domain sequences which control the in vitro transcription of this gene (21). The distal control domain that extends from −152 to −242 bp upstream of RNA initiation site overlaps with the RNA initiation site of the major late promoter (MLP) which is located at nucleotide position (np) −210 from the IVa_2 RNA initiation site and is transcribed from the opposite strand of DNA. We had proposed a model in which IVa_2 and MLPs share an entry site for RNA polymerase II and compete with each other for its use (21).

In the present study, the upstream control region was examined further with additional deletion and point mutants. The data demonstrate that there is competition for transcription between the MLP and the IVa_2. In addition, this upstream control region contains sequences that are not necessary for transcription of the MLP but which inhibit transcription of the IVa_2 gene.

* Corresponding author.

MATERIALS AND METHODS

Plasmids. The construction of adenovirus IVa_2 promoter deletion mutants (pAd series) was described earlier (21). Additional deletion mutants, pHB217, pHB307, and pHB222, were obtained from J. Manley (14) and are referenced in this paper by the same names, but for clarity, the number of nucleotides present upstream of IVa_2 RNA initiation sites is given in parentheses [e.g., pHB217 (−211)].

The point mutants in the TATA box of MLP were obtained from R. Weinmann and have been described previously (4); the wild-type and the two mutants were referred to as MLP, MLP AC-30, and MLP AC-28, respectively. The mutants and the wild type were recloned from M13 mp8 into the *Eco*RI and *Hin*dIII sites of pBR322. The wild type and the mutants have 402 bp of adenovirus DNA upstream of the IVa_2 RNA initiation site. In this study, we refer to the wild type and two mutants as pAd402, pAd402 T → G 183, and pAd402 T → G 181, where T → G indicates the mutation and the number after it designates the position of the point mutation (see Fig. 3).

In vitro transcription and RNA isolation. In vitro transcription with HeLa cell extracts and isolation of RNA were carried out as described earlier (21). Covalently closed form I DNA was purified by two cycles of cesium chloride-ethidium bromide gradient centrifugations (18) and added as the template for transcription.

S1 nuclease mapping of RNA. The different probes used for S1 nuclease mapping of RNAs synthesized with pAd plasmids are illustrated in Fig. 1. The probe used to map the RNAs transcribed from pHB plasmids was 5′ end labeled with ^{32}P at the *Eco*RI site and extended up to the *Bam*HI site

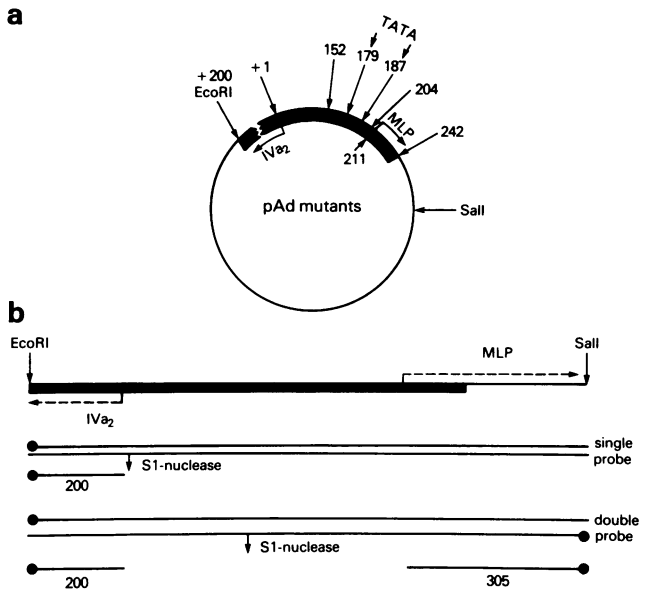

FIG. 1. (a) Structure of pAd plasmids. The adenoviral DNA is shown as a thick line and pBR322 as a thin line. The arrows indicate the direction of transcription from IVa_2 and MLP. The position of the IVa_2 RNA initiation site as well as the endpoints of different pAd deletion mutants are indicated. The TATA box of MLP present at np −180 to −186 relative to the IVa_2 promoter is also shown. (b) Strategy used to estimate the RNA synthesized from IVa_2 and MLP of different pAd mutants by the S1 nuclease method. The DNA from the *Eco*RI to the *Sal*I sites of pAd242 is shown. The adenoviral DNA sequences are indicated as a thick line, and pBR322 is shown as a thin line. The probe 5′ end labeled only at the *Eco*RI site was used to estimate the RNA synthesis from the IVa_2 promoter. To estimate the RNA synthesized from both IVa_2 and MLP, the probe was end labeled at both the *Eco*RI and the *Sal*I sites. The lengths of DNAs which will be protected from S1 nuclease digestion by RNAs synthesized from IVa_2 and MLP are also indicated.

of plasmid pϕ4 (20). With this probe, 198- and 200-nucleotide-length DNAs will be protected with the RNA synthesized from the IVa_2 promoter.

To estimate the RNA synthesized from plasmids pAd402, pAd402 T → G 181, and pAd402 T → G 183, a probe was prepared by labeling both ends of the *Eco*RI to *Hin*dIII fragment of the plasmid pAd402 (see Fig. 3). With this probe, RNA from the IVa_2 promoter protects 64- and 66-nucleotide-length DNAs, whereas RNA from MLP protects a 191-nucleotide DNA. The S1 nuclease mapping and quantitation of RNA were done as already described (20, 21).

RESULTS

The effect of deletions in the distal control region on the transcription efficiency of IVa_2 and MLP. The transcription initiation sites of IVa_2 and MLP are separated by 210 bp of DNA, and they are transcribed in opposite directions. To determine the quantity of RNA transcribed from each of the two promoters, the S1 nuclease mapping method was used. Since deletion mutants each have different sequences in the MLP region (which includes the MLP RNA initiation site), a single S1 probe cannot be used to estimate the MLP RNA synthesized from different deletion mutants, and the use of separate probes with different specific activities would preclude comparing the results in any quantitative way. The approach that we used was to determine the amount of RNA synthesized from the IVa_2 promoter of the different deletion mutants with a common single 5′ end-labeled probe (see Fig. 1b). This provides a measure of RNA synthesized from IVa_2 promoters of different deletion mutants. Then, the amount of RNA synthesized from both the MLP and the IVa_2 promoters present in different deletion mutants was also determined with the same RNA sample by using probes that were end labeled at both 5′ ends (Fig. 1b). These probes were made from each of the different deletion mutants from which RNA was transcribed. The efficiency of end labeling of both ends in each double probe was the same, as determined after restriction enzyme cleavage of the S1 probes into two fragments and comparing the two band intensities. Differences in specific activities of the doubly labeled probes can be determined based on the comparison of the S1 analysis of the IVa_2 transcripts with a common probe and the double-labeled probe. We could then validly compare the amount of RNA from the IVa_2 and the MLP from each of the deletion mutants.

Data presented in Fig. 2a and b show the results obtained with five different deletion mutants. Covalently closed DNAs of different mutants were used as templates for transcription, and the amount of RNA synthesized was estimated by the S1 nuclease mapping method. Figure 2a shows the results obtained with a probe that only measured RNA from the IVa_2 promoter. The pattern of RNA synthesized by mutants pAd242, pAd204, pAd179, and pAd152 is similar to the one reported earlier (21). The amount of RNA synthesized from pAd204 and pAd179 was 10- to 15-fold more than that synthesized from pAd242. However, the transcriptional level of pAd187, which was not previously tested, was reduced almost to the level of pAd242 (Fig. 2a, lane 3).

Results presented in Fig. 2b were obtained with the same RNA used in the above experiment, but RNA from both MLP and IVa_2 promoters was estimated with double end-labeled probes. The transcription pattern of different mutants for IVa_2 RNA was similar to the one obtained with a single end-labeled probe. These data were used to compare different mutants for the transcriptional efficiency of MLP.

Efficient RNA synthesis was observed from the MLP with pAd242, as expected, since it contained the MLP 5′ start site (at np −211 relative to the IVa_2 start site at np 1 [Fig. 2b]). In pAd204, the MLP RNA initiation site and 6 nucleotides upstream of its 5′ start site were deleted. This mutant carried out RNA synthesis efficiently from the MLP, and the reduction in length of the S1-protected fragment was that expected, based on the size of the deletion it contained. The RNA initiation site was not essential for efficient transcription with covalently closed DNA templates, a finding that is in contrast to the results obtained with a linear template of this mutant in which transcription efficiency was reduced by more than 90% (21). With pAd187, the MLP initiation site and 23 nucleotides upstream from its 5′ start site were deleted, but the TATA box was retained (Fig. 1a). Even though the MLP of this mutant was transcribed with a 90%-reduced efficiency with linear templates (data not shown), efficient RNA synthesis was seen when covalently closed DNA was used as template (Fig. 2b, lane 3). In pAd179, the MLP TATA box sequence and sequences downstream of it were deleted (Fig. 1a). Results presented in Fig. 2b (lane 4) show that no RNA was transcribed in the direction of MLP, showing that deletion of the TATA box led to abolition of the MLP transcription. This result is similar to the one obtained when linear DNA is used as the template (21).

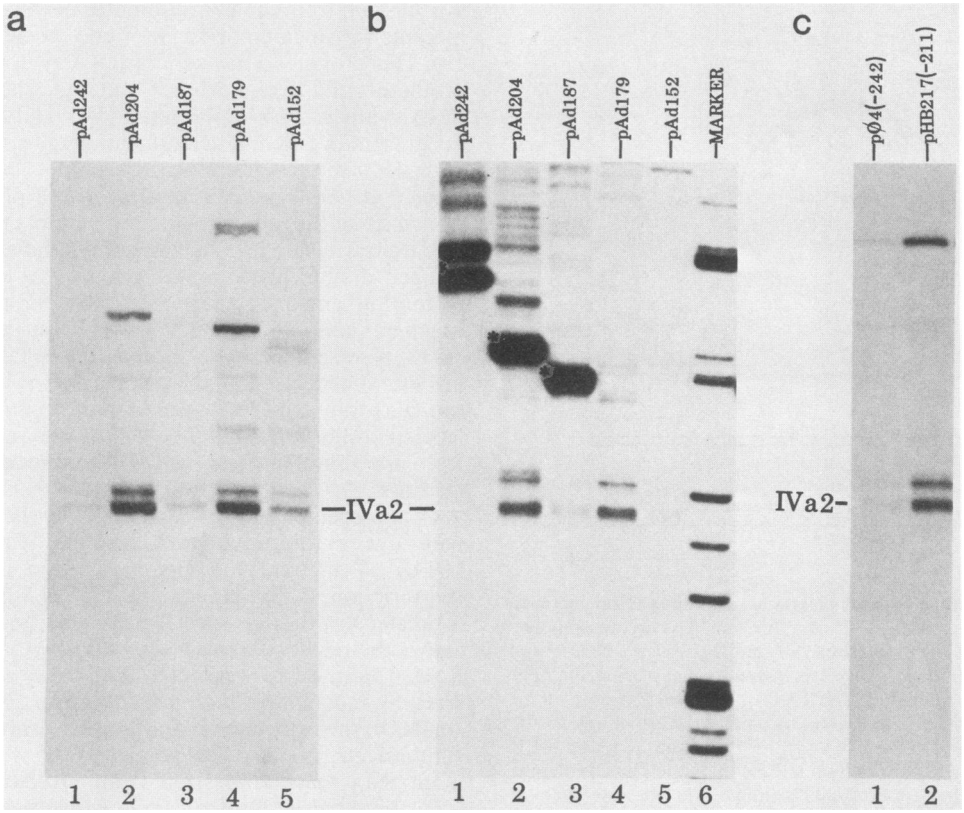

FIG. 2. S1 nuclease mapping of RNAs synthesized from the IVa_2 and the MLP of different pAd mutants. The in vitro transcriptions were carried out with form I DNA (12.5 μg/ml) of different mutants. The RNA was isolated, and the S1 nuclease mapped as described in the text. (a and b) The RNA was synthesized from the IVa_2 and the MLP of different pAd mutants, and the purified RNA was divided and S1 nuclease mapped with different probes. The results presented in panel a were obtained by using a common probe 5′ end labeled only at the *Eco*RI site of pAd242. The results presented in panel b were obtained with probes labeled at both the *Eco*RI and the *Sal*I ends of different pAd mutants. These probes were prepared from the pAd mutants from which the RNA was transcribed. The pAd mutants used for transcription are shown on the top of each lane. The position of 5′ end-labeled DNA fragment protected by IVa_2 transcript is indicated. The position of 5′ end-labeled DNA fragment protected by RNA transcribed from MLP of different pAd mutants is shown by an asterisk. Because of deletions in the MLP region, the length of DNA protected by the MLP transcript is shorter with pAd204 and pAd187 as compared with pAd242. (c) Estimation of RNA synthesized from IVa_2 promoter of pϕ4 and pHB217. The probe used was obtained by 5′ end labeling the *Eco*RI site of plasmid pϕ4. With this probe, a DNA of 200 nucleotides in length will be protected (20). The plasmids used for transcription are shown at the top of each lane. The length of adenovirus DNA present upstream of IVa_2 RNA initiation site is given in parentheses.

In earlier studies, based on the comparison of the transcription pattern of the IVa_2 promoter with pAd242 and pAd204, we had proposed that RNA polymerase II bound at an entry site that was shared by both the IVa_2 and the MLP. Subsequently, the genes competed for its use, and when transcription could occur from the strong MLP, IVa_2 transcription would be blocked. However, the present results show that with pAd204, both genes can be efficiently transcribed at the same time. To confirm these findings and to define the control regions more precisely, transcription of a mutant that has 211 bp upstream of the IVa_2 RNA initiation site was examined. This mutant (pHB217) has been described earlier by Hu and Manley (14) and includes the MLP RNA initiation site. Transcription of this mutant and the corresponding wild-type plasmid pϕ4 that contains 242 bp upstream of the IVa_2 RNA initiation site were compared (Fig. 2c).

As expected with pϕ4, which contains the MLP 5′ start site at np −211, very little RNA was transcribed from the IVa_2 promoter, but efficient IVa_2 RNA synthesis was seen with pHB217 (i.e., −211). The presence of the MLP initiation site was not sufficient to block IVa_2 transcription. The most direct interpretation of these findings is that sequences present between np −242 and −211 of the IVa_2 promoter inhibit IVa_2 transcription. Efficient IVa_2 RNA synthesis was also seen with mutants pHB307 (−208) and pHB222 (−198) (data not shown).

Transcriptional analysis of mutants in the TATA box sequence of MLP. The presence of DNA sequences that inhibit transcription of the IVa_2 gene is one factor that controls IVa_2 gene expression. To determine whether competition between the MLP and IVa_2 was a second regulatory factor, transcription patterns of IVa_2 and MLP were examined with point mutations in the TATA sequence of MLP that have been constructed and described by Concino et al. (4). MLP AC-28 and MLP AC-30 each have a single point mutation (nucleotide A to C) at positions −28 and −30, respectively, from the MLP RNA initiation site. The two mutants and corresponding wild-type plasmid contain 451 bp of adenovirus type 2 DNA cloned in the multiple cloning sites of M13 mp8 DNA. Each plasmid has 402 bp of adenovirus DNA upstream of the IVa_2 RNA initiation site. When these DNAs were transcribed in vitro, with linear DNA templates, MLP RNA synthesis from MLP AC-28 and MLP AC-30 was

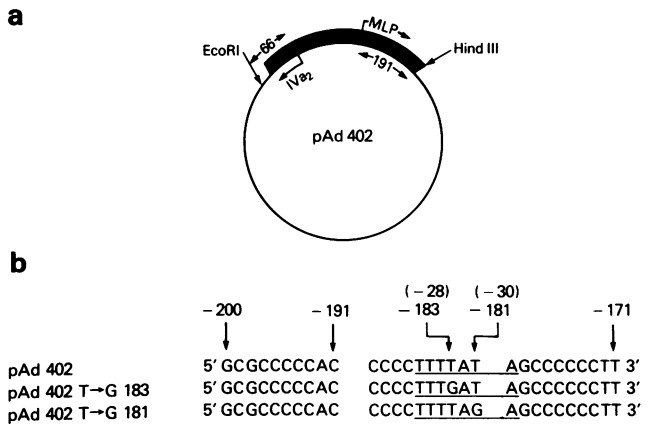

FIG. 3. (a) Structure of the pAd402 plasmid. The details of construction of this plasmid are given in the text. This plasmid has 402 bp of adenovirus DNA upstream of the IVa$_2$ initiation site. The adenovirus DNA is shown as a thick bar and the pBR322 DNA as a thin line. The direction of transcription from the IVa$_2$ and the MLP are indicated. The length of DNA which will be protected from S1 nuclease digestion by transcripts from IVa$_2$ and MLP when a probe end labeled at *Eco*RI and *Hin*dIII sites is shown. (b) The DNA sequence of the IVa$_2$ promoter. The DNA sequence from the −170 to −200 region of IVa$_2$ promoter is given. The single base pair changes in pAd402 T → G 183 and pAd402 T → G 181 are shown. The numbers in parentheses show the positions of mutations with respect to MLP RNA initiation site. The TATA box of the MLP is underlined.

reduced by about 75 and 50%, respectively, compared with the wild type (4). These mutant DNAs and the wild-type DNA were cloned between the *Eco*RI and *Hin*dIII sites of pBR322. The wild type and the two mutant plasmids are referred to as pAd402, pAd402 T → G 181, and pAD402 T → G 183, with respect to the IVa$_2$ RNA initiation site. This designation indicates the base substitution and the position of the mutation. The structures of the plasmids are illustrated in Fig. 3. The DNA sequence of the IVa$_2$ promoter in which the point mutants were located is given for comparison (Fig. 3b).

In this study, in vitro transcription was carried out with covalently closed DNA as a template, and the amount of RNA synthesized from IVa$_2$ and MLP was estimated with a double end-labeled probe made from wild-type DNA. With this probe, RNA from IVa$_2$ promoter protects 64- and 66-nucleotide lengths of DNA, whereas RNA from MLP protects a 191-nucleotide DNA. The DNAs protected by the RNA from IVa$_2$ promoter showed a group of clustered bands with the major one 66 nucleotides in length (Fig. 4). The heterogeneity that was observed was due to S1 nuclease nibbling at the 3' end of DNA. Very little IVa$_2$ RNA was synthesized from pAd402. This result was expected because pAd402 has all the control sequences present in pAd242, and with pAd242 RNA synthesis from IVa$_2$ promoter is inhibited (Fig. 2a and b). The RNA synthesis from MLP of the pAd 402 T → G 183 was reduced by more than 80% as compared with that of pAd402. At the same time, transcription from IVa$_2$ promoter was stimulated by about 10-fold compared with wild type (Fig. 4). The mutation at np −30 of the major late promoter TATA box (pAd402 T→ G 181) did not affect the RNA synthesis either from MLP or from IVa$_2$ promoter. Similar results were obtained when a probe specific for only IVa$_2$ RNA was used (data not shown).

DISCUSSION

The adenovirus IVa$_2$ gene transcription start site is separated from the adenovirus MLP 5' start site by 210 bp, and it is transcribed from the opposite strand. It is expressed at an intermediate time in the viral infectious cycle, while the MLP is expressed throughout the cycle (7, 8). In contrast to MLP, the IVa$_2$ gene does not have a TATA box upstream from its 5' start site (11, 17). In the present study, we examined the interactions that occur between these two overlapping promoter domains.

By using a series of deletion mutants, we previously

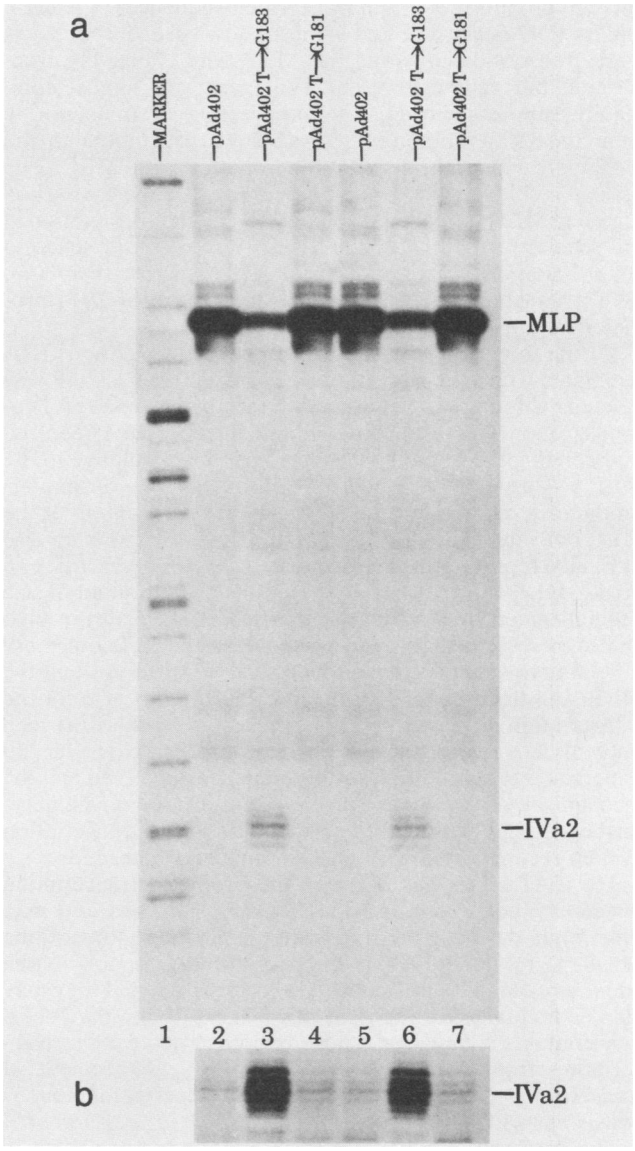

FIG. 4. S1 nuclease mapping of RNA synthesized from the MLP and the IVa$_2$ promoters of different point mutants in the TATA box of MLP. (a) The in vitro transcription and S1 nuclease mapping were carried out as described in the text. The probe used was 5' end labeled at both the *Eco*RI and the *Hin*dIII sites of pAd402 as shown in Fig. 3. The plasmids used for transcription are shown at the top of each lane. The positions of 5' end-labeled DNA fragments protected by the IVa$_2$ and MLP are indicated. In lanes 2 to 4, 16.6 μg of DNA per ml was used as templates, and in lanes 5 to 7, 25 μg of DNA per ml was used as templates. Panel b shows the autoradiogram of the IVa$_2$ promoter region exposed for a longer time.

identified two upstream regions, one proximal to the cap site at nucleotide position −38 to −49 and a distal domain between nucleotide position −152 and −242, that are essential for IVa_2 transcription (the IVa_2 start site is at np 1). The distal sequences functioned as a transcriptional control domain only with covalently closed DNA templates, and based on this observation, we proposed that this region could act as an entry site for RNA polymerase II or transcriptional factors or both. Because of the proximity of this region to the MLP RNA initiation site, this entry site also would be used by MLP (21).

When plasmid pAd242 that contains the IVa_2 promoter and 200 bp of downstream transcribed sequences, the MLP and its RNA start site, and 33 bp of downstream sequences were transcribed in vitro, the MLP was effectively transcribed, but synthesis of the IVa_2 gene was almost completely suppressed (21). The suppression of transcription from the IVa_2 promoter in pAd242 was attributed to the ineffective competition of IVa_2 promoter with MLP. The present results obtained with point mutants in the TATA box region of MLP support the competition model. A mutation in the second nucleotide of the TATA box (A → C) leads to suppression of transcription from MLP by more than 80%, with a concomitant increase in transcription of IVa_2 promoter by 8- to 10-fold.

During in vitro transcription with covalently closed DNA templates when the region between np −152 and −179 was deleted, a four- to fivefold reduction in the level of IVa_2 transcription was observed. Hen et al. (12) have reported that deleting the region between −33 and −97 relative to the MLP 5′ start site (np −177 to −133 of the IVa_2 promoter) produced a decrease in the efficiency of transcription of the MLP both in vivo and in vitro, and Jove and Manley (16) have also reported that the sequences between −51 and −66 of the MLP (np −144 to −159 of the IVa_2 promoter) are needed for enhanced transcription from MLP both in vivo and in vitro. Thus, this region between np −152 and −179 functions to support transcription for both the IVa_2 and the MLP, and we propose that it serves as an entry site for the transcription machinery since it is required by the IVa_2 gene only for covalently closed DNA templates. However, to understand the exact nucleotide sequences involved and the mechanism of the entry of the transcription machinery, further studies with additional mutants and a transcription system reconstituted with purified factors are needed.

The TATA box lies between the proposed transcription machinery entry site and the MLP 5′ start site and may function in directing the transcription machinery toward the MLP (4, 5). Factors that bind to the TATA box in the adenovirus MLP template have been reported, and they may also be involved in such a mechanism (9). When the TATA box contains a mutation, there is both a decrease in transcription from the MLP and a 10-fold enhancement of transcription from the IVa_2 gene, an observation that is consistent with this proposal.

The data obtained with deletion mutations reveal that in addition to the competition between the two promoters, other factors are also involved in the control of IVa_2 promoter. For efficient transcription from the IVa_2 promoter, the sequences between np −242 and −211 have to be deleted. When they are present, they inhibit IVa_2 expression by greater than 90%. Inhibitory sequences have also been shown to be present upstream for a number of tRNA genes transcribed by RNA polymerase III. A general feature of these inhibitory sequences is the presence of many alternating purine and pyrimidine residues which can form Z DNA structures (13, 25). The sequences present at −242 to −211 of IVa_2 promoter do not have more than one or two alternating purines and pyrimidines, suggesting that this possibility is unlikely. The other region which might have a role in the negative control of IVa_2 transcription is the inverted repeat present at −185 to −218 in the IVa_2 promoter (29). DNA sequences with dyad symmetry have the potential to interact with protein factors. This is true for many procaryotic regulatory sequences, and recently it has been shown that a dyad sequence present in the *Drosophila* heat-shock gene promoter interacts with a protein factor (23, 28). The sequence present between np −185 and −218 in the IVa_2 promoter contains two 15-bp sequences that are almost perfect inverted repeats, and it is possible that this dyad sequence by itself or after interacting with a factor negatively controls transcription from the IVa_2 promoters. Any perturbation to this sequence will reduce or eliminate the negative control. Stimulation of IVa_2 RNA synthesis, when either a part of or with complete deletion of one of these repeats, supports this possibility (pAd204, pHB217). However, it is not clear why transcription from the IVa_2 promoter is again suppressed after deletion of almost both inverted repeats (pAd187).

Another interesting finding of this study is the observation regarding the transcription from MLP with covalently closed DNA as template. Hu and Manley (14) have reported that the deletion of the MLP RNA initiation site and sequences upstream of it reduced transcription from MLP. We have also observed similar results with pAd204 and pAd187, in which the MLP RNA initiation site and 6 and 23 nucleotides upstream of it, respectively, were deleted. When linear DNAs of these plasmids are transcribed, the RNA synthesis from MLP is reduced by more than 90% (21; unpublished observations). In contrast, when covalently closed DNAs of these plasmids were used as a template, efficient RNA synthesis from MLP is seen (Fig. 2b). These results suggest that in defining the control region of promoters, both linear and circular templates have to be assayed for transcription.

Results presented in this report and in a previous study of the distal control region were obtained with supercoiled DNAs as templates that are added to the transcription mixture (21). Supercoiled DNA is converted to a relaxed covalently closed structure within 5 min after addition to a whole-cell transcription extract (12; unpublished observations). When pAd242 and pAd179 plasmids were relaxed before their addition to the transcription reaction, we obtained results similar to those that we obtained with supercoiled DNA templates (data not shown). Thus, the secondary structure present in the supercoiled DNA is not required, but a covalently closed template is necessary in determining the properties of the IVa_2 distal domain.

A number of divergently transcribed eucaryotic promoters have been characterized (3, 15, 26, 27). In a few cases that have been studied in detail, the results indicate that both promoters are regulated by a common control sequence (15, 27). During adenovirus lytic infection, RNA is synthesized from MLP at both early and late times after infection, whereas IVa_2-specific RNA is synthesized only during late times (7, 8). The clustering of control sequences of these two promoters that we observed in vitro may have some role in the regulation of these two promoters in vivo.

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