Mutational Analysis of the Adenovirus 2 IVa2 Initiator and Downstream Elements*

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The initiator element of the adenovirus type 2 IVa2 promoter is sufficient to direct accurate initiation by RNA polymerase II. Analysis of the effects of substitution of specific base pairs on initiator activity in vitro transcription systems indicated that specific sequences between positions -4 and +5 were essential for initiator activity. Mutations that impaired or eliminated initiator activity altered both base pairs that are conserved in sequence-related initiators and non-conserved sequences. Neither the downstream TA-rich sequence of the IVa2 promoter, nor the adenovirus 2 major late TATA element placed at the same downstream site could overcome the severe inhibitory effects of initiator mutations, indicating that the initiator is the primary determinant of the specificity and direction of IVa2 transcription. By contrast, when the ML TATA element was placed 31 nucleotides upstream of the IVa2 initiator, the precise specificity, but neither the efficiency nor direction of transcription, depended on the presence of a functional initiator. Activity of the IVa2 promoter was relatively insensitive to changes in the orientation or nature of the TA-rich sequence. Furthermore, only a promoter containing the ML TA-TAAA sequence downstream of the IVa2 initiator was competent to direct both IVa2 transcription and transcription from the opposite strand. The implications of this functional difference for recognition of the downstream element are discussed.

The TATA element of eukaryotic RNA polymerase II promoters plays a pivotal role in initiation of transcription by this enzyme (see Refs. 1–3). Binding of the general transcription factor (TF)
1 IID to the TATA element of a typical promoter seeds an ordered assembly of additional initiation factors, such as TFII-B, -E, and -F, and RNA polymerase II itself, to form a stable preinitiation complex (reviewed in Ref. 3). When such preinitiation complexes are formed on mammalian promoters, transcription initiates, in the presence of ribonucleotide triphosphates, 25–30 nucleotides downstream of the TATA element (see Ref. 3), and the interaction of TFIIID with this sequence can govern both the efficiency and specificity of initiation (e.g. 4–8; see Refs. 1 and 9, for reviews).

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1 The abbreviations used are: TF, transcription factor; ML, major late; bp, base pair(s); TdT, terminal deoxynucleotidyl transferase; Ad2, adenovirus type 2; TBP, TATA-binding protein; hTBP, human TATA-binding protein; nt, nucleotide(s).

In promoters of Saccharomyces cerevisiae, by contrast, the distance between initiation sites and TATA elements is both greater and more variable (reviewed in Refs. 10 and 11). Moreover, sequences located near initiation sites themselves, termed initiator elements, are the primary determinants of the specificity with which yeast RNA polymerase II begins transcription (see Refs. 10 and 11). Although sequences near initiation sites were initially implicated in specificity determination in certain mammalian and viral promoters (e.g. 12–18), initiator elements of higher eukaryotes have been subject to detailed scrutiny only since the demonstration (19) that a 17-bp sequence of the terminal deoxynucleotidyl transferase (TdT) promoter is sufficient to direct accurate initiation by RNA polymerase II.

Initiator elements have been identified in a variety of mammalian or viral promoters, by the criterion of functional autonomy developed by Smale and Baltimore (19) or by the effects of mutations (e.g. 20–31). Although such elements were initially identified in promoters that lack TATA elements (19–23), they have now been shown to operate in several viral promoters that contain typical TATA sequences, including those of the Ad2 ML (24, 25, 27, 28) and E1B (30) and the AAV p5 cap (26) transcription units. Moreover, an initiator element from a promoter that contains no TATA element, the TdT initiator, can cooperate with heterologous TATA elements placed distant sequences upstream of the initiation site (19, 24, 30, 32). Such observations have prompted a reevaluation of the mechanisms that determine the direction and specificity of transcription by RNA polymerase II (e.g. 30, 32).

Our previous mutational analysis of the IVa2 promoter indicated that its two initiation sites, A at position +1 and U at position -2 (34), are specified by separate elements, spanning the sites at which transcription begins and centered at position -30, respectively (31). Although a short initiator sequence is sufficient to direct accurate initiation at the +1 site (25, 31), efficient transcription from both sites requires downstream sequences of the promoter (31, 33). This downstream segment includes the TA-rich sequence TAGA, located in the transcribed strand some 20 bp downstream of the initiator (see Fig. 1A). This sequence differs from a canonical TATA element only by the G at position 5. In reconstituted transcription systems, or following heat inactivation of endogenous TFIIID in unfracionated extracts, recombinant TATA-binding protein (TBP) is required for efficient IVa2 transcription (31, 33). Downstream sequence mutations that reduce the ability of TBP to bind to the TA-rich sequence impair IVa2 transcription (31, 33). Such observations are consistent with the conclusion that TFIIID can bind to the TA-rich element to stimulate IVa2 transcription but do not rule out other explanations of the TFIIID requirement. This factor is necessary for transcription from promoters that do not contain its binding sites, including promoters tran-
scribed by RNA polymerase I and II (35-40), and can enter RNAP II initiation complexes via constituent “tethering factors” (41).

The ability of the isolated IVa₂ initiator sequence to direct accurate transcription is consistent with the suggestion (33) that it is primarily responsible for setting the direction and specificity of IVa₂ transcription. The downstream sequence would, then, be expected to be limited to increasing the efficiency with which the IVa₂ initiator operated. To investigate the functions of these elements of the IVa₂ promoter in more detail, and interactions between an initiator and a downstream element, we have examined the effects of alteration of the position, nature, or orientation of the TA-rich element on transcription directed by the IVa₂ initiator or its mutated derivatives.

MATERIALS AND METHODS

Cell Culture and Extract Preparation—Hela cells were grown in suspension minimal essential medium supplemented with 5% calf serum (GIBCO) and 1% glutamine and harvested at a density of 4 x 10⁵ to 5 x 10⁶ cells/ml. Whole cell extracts (42) were routinely prepared from 3-4 x 10⁶ freshly harvested cells as described (43) and nuclear extracts (44) from 10⁶ cells. Protein concentrations were determined by the method of Bradford (45). Extracts were divided into small portions and stored at −80 °C.

Plasmids and Mutant Construction—The plasmid pIVneo was constructed by inserting a SalI- EcoRI fragment comprising the sequence −257 to +48 of the Ad2 IVaz gene linked to a 760-bp fragment of the TATA box-containing oligonucleotide into SalI-digested pIVlnr and its derivative in which the downstream, TA-rich element was placed. A relatively small difference in efficiency was observed when the activities of the pIVlnr template and its derivative containing the ML TATA sequence (position −44 to −11 of the Ad2 ML promoter) was end-labeled using polynucleotide kinase and [γ-³²P]ATP and used as probe in nTBP binding assays. Binding reactions contained 100 ng of TBP-containing fraction, 0.2 ng of [³²P]-labeled oligonucleotide, the concentrations of competitors indicated in figure legends, 10 mM Hepes, pH 7.9, 0.05 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10% (v/v) glycerol, and 0.1% (v/v) Nonidet P-40. After incubation at 30 °C for 1 h, Sarkosyl was added to a final concentration of 0.02% and reactions incubated for a further 2 min at room temperature (48). Electrophoresis was done in 4% polyacrylamide (60:1 acrylamide/bisacrylamide) gels cast and run in TGEM (1 x TGEM is 0.02 M Tris, 0.19 M glycine, 5 mM MgCl₂, and 1 mM EDTA) (48) buffer for 90-120 min at room temperature.

RESULTS

To facilitate identification of functional residues of the IVa₂ initiator (subsequently termed the InR based on its sequence similarity to the TdT InR (19, 30)) and investigation of its interaction with downstream sequences, we constructed minimal IVa₂ promoters, comprising one or both of these elements, but lacking any upstream elements (Fig. 1A). The accuracy and efficiency of in vitro transcription from such synthetic promoters was first examined under various conditions. All transcription reactions were performed under conditions empirically determined to be optimal for IVa₂ transcription (see “Materials and Methods”). Some pertinent comparisons of the activities of synthetic and control templates in whole cell and nuclear extracts prepared from HeLa cells are shown in Fig. 1B. When transcribed by nuclear extracts, a template comprising only the sequence spanning the IVa₂ initiation site, which would yield a primer extension product of 63 nucleotides from transcription initiated at the IVa₂ +1 site (pIVlnr, Fig. 1A), directed specific transcription by RNA polymerase II, as we have reported previously (31). The major primer extension product of RNA polymerase II transcripts was the predicted 63 nt C DNA (Fig. 1B, lanes 9 and 10). As expected (19), the analogous sequence from the TdT promoter also directed the synthesis of specific RNA polymerase II transcripts (Fig. 1B, lanes 11 and 12). The accuracy of initiation from the IVa₂ InR was confirmed by the one nucleotide difference in the transcripts of the IVa₂ and TdT InRs (Fig. 1B, lanes 9 and 11; see Fig. 1A).

Although nuclear extracts supported specific transcription from the IVa₂ InR, this assay system was not optimal for transcription of IVa₂ promoters that also contained a TA-rich element. A relatively small difference in efficiency was observed when the activities of the pIVlnr template and its derivative in which the downstream, TA-rich element was restored at its correct position were compared in nuclear extracts (data not shown). Transcription from these synthetic promoters was, therefore, compared in whole cell extracts, the transcription system used in our previous experiments (31). Although both the IVa₂ and the TdT InRs supported RNA polymerase II transcription from their +1 sites (Fig. 1B, lanes 3–6), initiation was, in both cases, more heterogeneous than when transcription was by nuclear extract components (Fig. 1B, compare lanes 3 and 5 to 9 and 11, respectively). Moreover, the TdT InR was more active in the whole cell extract (Fig. 2 A, lanes 7 and 8; see Fig. 1B).
initiation from the minor in vivo site, the U at position -2 (Fig. 1B, compare lanes 7 and 1), as discussed previously (31). As nuclear extracts permitted unambiguous assay of specific transcription from the IVa2 InR in the absence of additional promoter elements, but whole cell extracts supported a more dramatic response to the presence of downstream sequences, the former were used in subsequent analyses of promoters comprising only an InR, whereas all other templates were transcribed in the latter.

**Mutational Analysis of the IVa2 Initiator Element**—The experiments described in the previous section established that the sequence -9 to +13 is sufficient to specify accurate initiation by RNA polymerase II at the +1 site of the IVa2 promoter. In order to identify functionally important sequences within the initiator, various single and double base pair substitutions were introduced into the IVa2 InR (see "Materials and Methods"). The effects of these mutations, which are summarized in Fig. 2A, on the accuracy and efficiency of transcription were first assessed in the absence of any other IVa2 promoter element. Typical results of such

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<tr>
<th>A</th>
<th>5' CTTCCTCCTCAGACTCCTCCCAC 3'</th>
<th>5' CITCCTGIGACAGTGGTCCCAG 3'</th>
<th>5' CITCCTCTCAGACGCGTCCCAG 3'</th>
<th>5' CITCCTCTCACAGTCCTCCCAC 3'</th>
<th>5' CITCCTGTCACAGTCCTCCGAC 3'</th>
<th>5' CITCCTCTCACAGTCCTCCCAC 3'</th>
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<tr>
<td>B</td>
<td>5' CTTCCTGIGACAGTGGTCCCAG 3'</td>
<td>5' CITCCTGTCACAGTCCTCCGAC 3'</td>
<td>5' CITCCTCTCACAGTCCTCCCAC 3'</td>
<td>5' CITCCTGTCACAGTCCTCCCAC 3'</td>
<td>5' CITCCTCTCACAGTCCTCCCAC 3'</td>
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**Fig. 2. Effects of InR base substitutions on the activity of the IVa2 initiator element.** A, the sequence of the wild-type IVa2 InR is shown on the first line of the left hand column and those of base pair substitution mutations below. Base pairs substituted or inserted in the individual mutants are indicated in bold face. The wild-type IVa2 initiation site is indicated by the arrow above the first line and the novel initiation site observed with plIVnr-m9 by an arrow below the line. The sequences of the TdT and ML initiator elements are shown for comparison at the right. The arrow indicates the +1 site of each initiator, and the boxes show sequences conserved among all three. B and C, transcription reactions contained 180 μg of nuclear extract proteins, the ML internal control template described in the legend to Fig. 1, and 750 ng of the wild-type IVa2 initiator element promoter (lanes 1) or the substitution mutants indicated (B, lanes 2–8; C, lanes 2–9). The lengths of the predicted ML and IVa2 primer extension products are indicated at the right and left of panels B and C, respectively. Sequencing ladders used as markers were run in the lanes marked M.

### Additional Text and Diagrams

**Fig. 1. Activities of the IVa2 and TdT initiators in whole cell and nuclear extracts.** A, sequences of the synthetic IVa2 and TdT promoters used in the experiment shown in B are illustrated, with plasmid sequences indicated by the solid lines. Transcription initiation sites are indicated by the arrows, drawn in the direction of transcription, above each sequence and the position and direction of extension of the Pneo primer, used with all templates, by the presence of the downstream TATA element was confirmed. Initiation sites are indicated by the solid portions of each arrow. The downstream TA-rich element in the pIVlnr-TC template is underlined. B, transcription was performed as described under "Materials and Methods." Reactions contained 280 μg of whole cell extract (WCE) or 180 μg of nuclear extract (NE), as indicated, the Ad2 ML plasmid described under “Materials and Methods” and the wild-type IVa2 promoter (pLV-80/+48), the IVa2 InR (pIVnr), the TdT InR (pTdTInr), or a synthetic IVa2 promoter comprising its InR and downstream TA-rich element (pIVnr-TC), as indicated. The reactions whose products are shown in the lanes marked + also contained 1 μg/ml of α-amanitin. The predicted lengths of primer extension products of ML (36 nt) and the various IVa2 and TdT (see A) transcripts are indicated at the right. Sequencing ladders used as size markers were loaded in the lanes marked M.

1B, lanes 3 and 5), although it was transcribed with half the efficiency of the IVa2 InR in nuclear extracts (Fig. 1B, lanes 9 and 11). Such differences in InR-directed transcription in whole cell and nuclear extracts are presumably the result of differences in the complement, or concentrations, of factors present in the extracts.

When the downstream TA-rich element was built into the synthetic IVa2 promoter at the location it occupies in the wild-type promoter (template pIVnr-TC, Fig. 1A), the efficiency of transcription in whole cell extracts from the +1 (A) site, which yields a primer extension product of 82 nucleotides, was increased 12-fold (Fig. 1B, compare lanes 3 and 7). That identical initiation sites were utilized in the absence and presence of the downstream TATA element was confirmed using a primer designed to generate extension products of identical length from transcripts initiated at the +1 sites in two templates (data not shown). Thus, in whole cell extracts, the synthetic IVa2 promoter comprising the InR and downstream TA-rich element supported efficient transcription from the minor in vivo initiation site, the A at position +1. However, this promoter, unlike the wild-type, cannot direct
experiments are shown in Fig. 2, B and C. Several of these mutations, including the C to G mutation at position -1 (m2) and the A to G transition at position +1 (m4), reduced InR-directed transcription to undetectable levels, whereas others were less severely inhibitory. A T to G transition at position +5 (m5) resulted in a 3-fold decrease in InR activity, whereas a C to G transition at position -3 (m1) or insertion of an additional C at position -4 (m8) resulted in no significant change in transcriptional activity, as judged by quantitation of InR activity and correction using the internal control. Replacement of the G at position +2 with C (m9) increased the overall efficiency of transcription by a factor of 2, but this mutation also altered initiation specificity. The majority of transcripts synthesized from the m9 template initiated at position +3 (Fig. 2C, lane 3). The properties of these mutant templates therefore indicate that specific base pairs neighboring and just downstream of the initiation site are important for accurate or efficient transcription from the IVa2 InR.

The effects of the same InR mutations upon IVa2 transcription from promoters that contained the downstream TA-rich sequence were also examined to investigate the contribution of the two elements to the specificity and direction of transcription. With one exception, the phenotypes exhibited by these mutant templates were the same as those observed when the promoters comprised only the IVa2 InR. Thus, for example, in both promoter contexts, the m4, m6, and m7 mutant InRs failed to support detectable IVa2 transcription, whereas the m8 and m1 mutants exhibited little change in activity (Fig. 2B, and Fig. 3, lanes 2–6). No transcription from mutant templates containing non-functional initiators could be detected even upon gross overexposure of the autoradiograms, indicating that the downstream, TA-rich element was incapable of directing RNA polymerase II to initiate transcription from the IVa2 promoter in the absence of a functional initiator. The only mutated InR that appeared to exhibit a different phenotype when tested in the presence of the downstream TA-rich element was m9. In this context, the strong initiation from position +3 observed when the promoter comprised only the initiator was not detected, although transcription initiated only inefficiently from the +1 site in both types of promoter (compare Fig. 2C, lane 3 and Fig. 3, lane 7). We believe that the failure of the m9 template containing a downstream TA-rich element to direct transcription from the +3 site is the result of the altered spacing (equivalent to a loss of 2 bp) between this site and the downstream element (25, 31).

The IVa2 InR Can Operate in Conjunction with Different TATA-related Sequences at Various Locations—An unusual feature of the Ad2 IVa2 promoter is the downstream location of a site that can bind TFIID (see Introduction), the non-canonical sequence TATAGAAA in the transcribed strand, that is TTTC/TATA in the non-transcribed strand (Fig. 4A). To begin to assess the significance of this arrangement, we constructed promoters in which the nature, orientation, or location relative to the InR were altered. The efficiency of IVa2 transcription was reduced by no more than 60% when the InR was deleted from the +3 site (Fig. 4B, compare lanes 3 and 5). Nor was a synthetic promoter in which the IVa2 TATAGAAA sequence was inverted (Fig. 4B, compare lanes 3 and 5) a better template for transcription from the +1 site (Fig. 4B, compare lanes 3 and 4). The same mutation has previously been introduced into a IVa2 promoter that retained upstream promoter elements, along with a second G for A substitution that converts a flanking position of the IVa2 TATAGAAA sequence to its ML equivalent (33).

FIG. 3. The downstream TA-rich element of the IVa2 promoter cannot function in the absence of an active initiator element. Transcription reactions contained 280 μg of whole cell extract protein, the ML internal control template described in the legend to Fig. 1, and the synthetic, IVa2 promoter comprising the wild-type initiator and downstream TA-rich element (lane 1, pIVInr-TC, see Fig. 1A) or the InR substitution mutations indicated, in this same promoter background (lanes 2–7). The positions of the predicted ML and IVa2 primer extension products are indicated at the right.

In order to investigate the contributions of the upstream

\[ \text{Ad2 IVa2 Initiator and Downstream Elements} \]

\[ \text{2 H. Chen, unpublished observations.} \]
TATA sequence and the InR to the specificity of initiation from this hybrid promoter, several of the initiator mutations described in previous sections were tested in the context of the upstream ML TATA element. In general, these mutations were far less deleterious in this background. For example, the m4 and m6 mutant initiators, which both contain substitution of the normal initiating A at +1 by G and fail to direct detectable transcription alone or in the presence of the IVa2 downstream sequence (Figs. 2B and 3), exhibited no change in initiation specificity and very little change in activity when the promoter included an upstream TATA element (Fig. 4C, lanes 1–3), which are illustrated in Fig. 1A. Synthetic promoters carrying upstream TATA elements, and the IVa2 InR shown in A (lanes 4 and 5) or derivatives comprising an upstream ML TATA element and InR mutations (lanes 6–10).

Two-way Transcription from Synthetic IVa2 Promoters—The failure of replacement of the downstream TA-rich element with the canonical ML TATA sequence to increase promoter activity (Fig. 5B) was surprising, for the latter sequence has been reported to bind yeast TFIID with greater affinity than the former (33) and is some 20-fold more active in reconstituted systems (55). We therefore wished to determine whether transcribed-strand TATAGAA and TATAAAA sequences exhibited any functional differences when placed downstream of the IVa2 InR. In one approach to this question, we investigated whether either element could direct transcription from not only the IVa2 initiator, but also the opposite template strand. The orientation of the downstream TA-rich element is such that it should support transcription from one or more sites between approximately positions −5 and −20 of the non-transcribed IVa2 strand (Fig. 5A) if it functioned like typical TATA elements. A primer from this strand, primer IV-1 (Fig. 5A), was therefore used to assay for such transcripts among the products of transcription of the plVInr-TC and plVInr-TA templates. The former promoter, which directs efficient transcription from the +1 site of the IVa2 initiator, yielded no transcripts that hybridized to the IV-1 primer (Fig. 4).
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FIG. 5. Bidirectional transcription from a IVa₂ promoter containing the ML TATA sequence downstream of the initiator. A, the sequence of the plVlnr-TA promoter is shown in bold face with flanking pUC19 sequences in normal face. The position of the IV-1 primer, designed to detect sequences in normal face. The position of IVa₂ initiation sites, and the extension direction are indicated by the arrow below the sequence. The major novel initiation sites on the non-transcribed strand and the normal IVa₂ initiation site are indicated by the arrows below and above, respectively, the sequence. B, transcription reactions were as described in the legend to Fig. 3 and contained the promoters indicated at the top of the figure. The plVlnr-TC/-22 and plVlnr-TA/-22 are identical to plVlnr-TC and plVlnr-TA (see Fig. 5A), respectively, except that they contain IVa₂ sequences to position -22, rather than to position -9. The plVneo plasmid (lane 7) contains the wild-type Ad2 ML whose transcripts would yield an extension product of 105 nucleotides from the IV-1 primer. Transcripts were analyzed by primer extension using both the Pneo primer described in the legend to Fig. 3A or the IV-1 primer, from an upstream sequence of the non-transcribed strand of the IVa₂ promoter, shown in A. The reaction whose products are shown in the lane marked + contained 1 μg/ml of α-amanitin. The positions of predicted primer extension products are indicated at the right.

5B, lane 1). This negative result was not due to a technical failure of the primer extension assay, for the IV-1 primer generated the predicted, 105 nucleotide extension products of transcripts of a template in which the ML promoter was placed upstream of the IV-1 hybridization site (Fig. 5B, lane 7). Nor can the failure to detect specific transcripts from the wild-type IVa₂ promoter with the IV-1 primer be ascribed to lack of a suitable initiation site in the non-transcribed strand. When products of transcription of the template containing the ML TATA element in place of the wild-type IVa₂ sequence were assayed in similar fashion, α-amanitin-sensitive primer-extension products were readily detected (Fig. 5B, lanes 2 and 3). The major species were 57 and 58 nucleotides in length, corresponding to initiation at the C and A residues at positions -9 and -10, respectively, of the opposite strand of the IVa₂ promoter. More minor species of 53 and 54 nucleotides, which result from initiation at positions -13 and -14, respectively, were also observed (Fig. 5B, lane 2). The sequences spanning these sites of opposite strand initiation display, at best, very limited similarity to the IVa₂ InR sequence or those of related initiators, and none contained the "core" sequence (Fig. 2A) immediately preceding the initiating nucleotide. It therefore seemed unlikely that the opposite strand transcription directed by the downstream ML TATA sequence was dependent on the presence of initiator elements in this region of the template. Indeed, a template in which this region of the promoter was replaced by sequences unrelated to those present in plVlnr-TA also supported initiation of α-amanitin-sensitive transcription from the opposite strand, although with an altered pattern of initiation specificity (Fig. 5B, lanes 5 and 6). This result confirms that initiation is directed by the TATAAAA element itself.

The IVa₂ TA-rich sequence flanked by normal IVa₂ promoter sequences is bound with lower affinity by yeast TFIID, or TBP, than the ML TATA element flanked by ML promoter sequences (33). As the difference in the abilities of the two promoters to direct transcription from the non-IVa₂ strand could be a trivial result of such differences in affinity of the TATA-binding factor for the two sequences, we compared the relative affinities of recombinant, human TBP for the two sequences. Bacterially synthesized hTBP formed one specific complex when bound to the ML TATA sequence in its natural context (Fig. 6, lanes 1–4). Although binding was competed by both the ML TATA sequence and the IVa₂ TA-rich sequences, both placed in IVa₂-flanking sequences, the IVa₂ TATA-like sequence was a significantly weaker competitor (Fig. 6, lanes 5–11). Comparison of competitor activity as a function of competitor concentration indicated that the single
A to G substitution at position 5 of the TATA sequence reduced the affinity with which hTBP binds by a factor of at least 5.

If TFIIID were supporting transcription from the two promoters, a greater number of TFIIID-containing complexes committed to initiation would therefore be expected to form on the template containing the ML TATA sequence downstream of the IVa2 InR. If the concentration of such preinitiation complexes exceeded the concentration of the factor(s) mediating function of the InR, two classes of TFIIID-containing complex would be formed. The first would contain both TFIIID and the InR factor and thus support IVa2 transcription, whereas the second would lack the InR factor(s). In such circumstances, transcription would, perforce, initiate 20–35 bp 3′ to the ML TATA element. Thus, the apparent bidirectional transcription would, in fact, represent the sum of IVa2 transcription and opposite strand transcription from different template molecules. This model of apparent bidirectional transcription from templates containing the ML TATA element provides an explanation for the failure of this high affinity TATA factor-binding site to increase the efficiency of IVa2 transcription (Fig. 4B) and predicts that elimination of InR function (see Fig. 2) would increase the efficiency of such transcription. Moreover, if this model were correct, InR mutations should also permit a TATAGAA sequence to direct transcription from the opposite strand. We therefore used the IV-1 primer to assess the efficiency of opposite strand transcription from templates carrying mutations previously found to reduce or eliminate IVa2 transcription (Figs. 2B and 3). In no case, did introduction of such IVa2 mutations induce opposite strand transcription from templates carrying mutations previously found to reduce or eliminate IVa2 transcription (Figs. 2B and 3). In no case, did introduction of such IVa2 mutations induce opposite strand transcription from templates carrying mutations previously found to reduce or eliminate IVa2 transcription (Figs. 2B and 3). In no case, did introduction of such IVa2 mutations induce opposite strand transcription from templates carrying mutations previously found to reduce or eliminate IVa2 transcription (Figs. 2B and 3). In no case, did introduction of such IVa2 mutations induce opposite strand transcription from templates carrying mutations previously found to reduce or eliminate IVa2 transcription (Figs. 2B and 3).

The ability of promoters carrying the ML TATA sequence downstream of the IVa2 InR to direct opposite strand transcription (Figs. 5 and 7) could not, therefore, be readily explained by models based on a greater affinity of TFIIID for this sequence than for the IVa2 TATAGAA sequence. An alternative explanation might be that the downstream element that stimulates transcription from the InR is normally recognized by a factor other than TFIIID. As a first step to assess this hypothesis, we compared IVa2 sequences among adenovirus serotypes representative of the A, B, and C subgroups, serotypes 12, 7, and 2, respectively (51–53). As illustrated in Fig. 8A, significant sequence conservation is observed from positions −8 to +31, including perfect conservation of the InR (−2 to +7) and of two blocks of sequence in the region +20 to +31. Although certain positions in the TA-rich sequence of the Ad2 IVa2 gene are conserved among all three serotypes (Fig. 8A), neither the Ad7 nor the Ad12 equivalents, TAGAGGA and TACAGGA in the non-transcribed strand, respectively, resemble sites that can bind TFIIID or mediate TFIIID function (e.g. 49, 50, 54, 55). Nevertheless, a minimal IVa2 promoter comprising Ad12 sequences downstream of position −2 was transcribed almost as efficiently as the corresponding promoter built from Ad2 sequences (Fig. 8B).

**DISCUSSION**

The molecular mechanisms by which initiator elements, which have been identified in a large number of mammalian promoters, direct specific transcription by RNA polymerase II are not yet understood. The initiator of the Ad2 IVa2 promoter is likely to be particularly valuable in investigations of initiator recognition and function, for its integrity is essential to initiation of transcription from this promoter. Several specific base pair substitutions reduced InR activity to undetectable levels in vitro (Fig. 2), even when promoter strength was boosted by inclusion of the downstream, TA-rich element of the wild-type promoter, or the ML TATA element (Figs. 3 and 5).
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Fig. 8. Efficient transcription from an Ad12 IVa2 promoter. A, the sequences of the 5' ends of the Ad2, Ad7, and Ad12 IVa2 genes (51-53) are shown aligned at position +1 of the IVa2 transcription unit. Conserved sequences are indicated by the vertical lines between bases. The Ad7 sequence is that reported by Engler et al. (53), whereas that of Ad12 is from the sequence reported by Shu et al. (51), but chosen to maximize conservation of IVa2 initiator elements and the spacing between ML and IVa2 initiation sites. B, transcription reactions were as described in the legend to Fig. 3 and contained the Ad2 IVa2 template plV InR-TC (lane 1) or a template comprising Ad12 sequences downstream of position −2 (lane 2). The primer extension products of transcripts of the Ad12 template are longer by 1 nucleotide as 1 base pair was inserted between the Ad12 sequence and the primer binding site during construction of the template (see "Materials and Methods").

and 7B). The failure of a bona fide, high affinity (50) TFIID-binding site, that of the Ad2 ML promoter, to reinitiate transcription from such mutated initiators (Fig. 7B) emphasizing the essential function of this element. It should, however, be noted that the presence of downstream sequences in the promoter does improve initiation specificity, at least in whole cell extracts. In this system, transcription initiated with approximately equal frequency from the −1, +1, and +3 sites in the absence of downstream sequences (Fig. 1B, lane 3), but in the presence of these sequences the +1 site was greatly favored (e.g. Fig. 3, lane 1).

The IVa2, ML, and TdT initiators share a pyrimidine-rich sequence and the trinucleotide CTC immediately preceding the initiating A residue (Fig. 2A) and have therefore been defined as members of the class termed InR by Smale and colleagues (19). In nuclear extracts in which both the IVa2 and TdT InRs support specific transcription (Fig. 1B), substitution of certain conserved sequences, notably of positions −1 (m2) and +1 (m4), eliminated detectable activity of the IVa2 InR (Fig. 2). On the other hand, substitution of positions −2 (m1) or +5 (m5), which are also conserved, were less deleterious or had no effect on the activity of the IVa2 InR. As the TdT and IVa2 InR exhibited different levels of activity when compared in either nuclear or whole cell extracts (Fig. 1B), these results suggest that the two initiators are recognized by different transcriptional components. Thus, although several factors that bind specifically to the TdT InR have been described (26, 56), it is not yet possible to infer a role for any in recognition of the IVa2 InR.

In order to make a more detailed assessment of the properties of the downstream element of the IVa2 promoter, and its functional interaction with the InR, we constructed synthetic promoters in which the nature or orientation of the downstream element were altered. In several respects, the IVa2 TA-rich sequence behaved identically to a canonical TATA element, that of the Ad2 ML promoter. When placed downstream of the IVa2 InR, for example, neither sequence could overcome the severe inhibitory effects of InR mutations (Figs. 3 and 7). These results indicate that, in the IVa2 promoter, the InR is the primary determinant of the direction and specificity of transcription. However, when placed downstream of the canonical ML TATA sequence, the IVa2 InR merely improved the specificity of initiation (Fig. 4C). The IVa2 TATAGAA sequence could, nevertheless, be functionally distinguished from a canonical TATA element placed in the identical position of the IVa2 promoter. The latter, but not the former, possesses the ability to direct transcription from the opposite strand of the template (Fig. 5). Such TATAAA-dependent initiation occurs at multiple sites 30–35 base pairs "downstream" of the TFIID-binding site and does not depend on the identity of the sequence spanning the sites at which transcription begins (Fig. 5). Thus, such opposite strand transcription exhibits the same properties as transcription from a promoter that comprises the same TATA element placed upstream of a non-functional IVa2 InR (Fig. 4C), and initiation specificity must be determined, in these circumstances, by the TATA element itself, presumably in a distance-dependent manner (see Refs 1–9).

The difference in the abilities of the TATAGAA and TATAAAA sequences to support opposite strand transcription suggested some fundamental difference in the interactions of these two sequences with transcription components or in the consequences of those interactions. IVa2 transcription in reconstituted systems requires TFIID (33, 57), and the TBP component of the factor can bind specifically to the IVa2 TA-rich sequence (31, 33) (Fig. 6). We, therefore, first tested predictions of a model to account for the different properties of the two templates based on the different affinities (Fig. 6) of TBP/TFIID for the TATAAA and TATAGAA sequences. However, the failure of InR mutations to increase the efficiency of, or induce, opposite strand transcription from templates containing the ML TATA and the IVa2 TA-rich sequence, respectively (Fig. 7), suggests that the latter sequence is recognized by a factor other than TFIID. The efficient transcription (Fig. 8B) of a minimal Ad12 IVa2 promoter, which has no sequence that could bind TFIID between positions +14 and +21 (Fig. 8A), provides strong support for this conclusion. A single base pair substitution that converts the IVa2 TA-rich sequence to the TATAAA sequence of the ML promoter significantly increases the affinity with which TBP binds (Fig. 6) (33). Thus, the failure of this substitution to increase the efficiency of IVa2 transcription (Fig. 8B) can also be more readily reconciled with recognition of the downstream element by a factor other than TFIID. This result also implies that this base pair is not essential for sequence-specific recognition of the downstream element, and thus that the binding site for the putative downstream sequence-specific factor does not coincide precisely with the TA-rich sequence. Further experiments are in progress to define this element precisely and to identify the factor(s) with which it interacts.

Although these components of the IVa2 transcription ma-
chinery remain to be identified, the results presented here establish that one or more downstream sequence factors is required for efficient IVa₂ transcription. To account for the difference in properties of templates containing the IVa₂ TA-rich sequence or the ML TATA element downstream of the IVa₂ InR (Figs. 5 and 7), we would propose that this factor is limiting under the conditions used in these experiments. In such a circumstance, an equivalent number of complexes containing the downstream sequence-specific and the InR factors committed to IVa₂ transcription would assemble on the two templates, resulting in the same levels of IVa₂ transcription (Figs 4B and 5). Excess ML TATA element-containing templates could then bind TFIID to direct opposite strand transcription, whereas the IVa₂ promoter with its low affinity TBP-binding site (Fig. 6) would not be successful in this reaction. InR mutations of either template would have no effect, as this model assumes that it is reactions preceding any in which the InR is involved that commit to IVa₂ or opposite strand transcription. An alternative explanation might be that the putative downstream sequence factor and TFIID compete for binding to downstream promoter sequences, when the ML TATA element would be more successful than the IVa₂ TA-rich sequence. Although we cannot rule out this explanation, which implies that binding of the downstream sequence factor and TFIID are mutually exclusive, it can accommodate the equally efficient IVa₂ transcription directed by the two templates only if further assumptions are made, and therefore, seems less reasonable. Irrespective of these details, both explanations postulate that when TFIID does bind downstream of the IVa₂ InR it effects not IVa₂ transcription, but transcription from the opposite strand of the template. Whether such downstream binding of TFIID can stimulate IVa₂ transcription in any circumstance, for example, when the downstream element is absent, or non-functional, therefore remains to be determined.

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