MOLECULAR AND CELLULAR BIOLOGY, Mar. 1987, p. 1021–1031
0270-7306/87/031021-11$02.00/0

Vol. 7, No. 3

Multiple Proteins Bind to VA RNA Genes of Adenovirus Type 2

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Received 26 September 1986/Accepted 10 December 1986

Using fractionated HeLa cell nuclear extracts and both nuclease (DNase I) cleavage and chemical cleavage (methidiumpropyl-EDTA · Fe(II) protection methodologies, we demonstrated the presence of three proteins which interacted specifically, yet differentially, with the two VA genes of adenovirus type 2. One, previously identified as transcription initiation factor TFIIIC, bound to a site centered on the transcriptionally essential B-block concensus element of the VAI gene and, with a lower affinity, to the analogous site in the VAII gene. Another, identified as the cellular protein involved in adenovirus replication, nuclear factor I, bound to sites immediately downstream from the two VAI terminators (at approximately +160 and +200). The third, a previously unrecognized VA gene binding protein termed VBP, bound immediately upstream of the B-block element in the VAI gene but showed no binding to VAII. Possible roles for these proteins in VA gene transcription were investigated in in vitro assay systems reconstituted with partially purified transcription factors (RNA polymerase III, TFIIIB, and TFIIIC). Although TFIIIC activity was present predominantly in fractions containing B-block binding activity, there was not complete correspondence between functional and DNA binding activities. The nuclear factor I-like protein had no effect when added to a complete transcription reaction. The presence of VBP appeared to depress the intrinsic ratio of VAI-VAII synthesis, thereby simulating the relative transcription levels observed early in adenovirus infection of HeLa cells. These observations suggest a model, involving both intragenic binding factors (VBP and TFIIIC) and variable template concentrations, for the differential regulation of VA transcription during the course of adenovirus infection.

One consequence of adenovirus type 2 (Ad2) infection is cytoplasmic accumulation of high concentrations of two low-molecular-weight RNAs (34, 38) designated VAI and VAII. These virally encoded RNAs are approximately 160 nucleotides long (1, 9), have a high degree of secondary structure (27), and are products of RNA polymerase III transcription (33, 43). Some fraction of the VA RNA molecules exist as ribonucleoprotein particles with a cellular protein, the La antigen (14, 24). Recent work has demonstrated a role for VAI RNA in activating translation during the late phase of infection (31, 40).

The genes encoding the VA RNAs are tandemly located between 29.0 and 31.0 map units on the Ad2 genome (32). A 98-base-pair (bp) spacer separates the two genes. Transcription from both genes initiates at the purine residue and terminates downstream at a stretch of thymines (5, 39). Both VAI and VAII have internal promoters characteristic of class III genes (13). Mutational analyses have divided these promoters into two domains, the A and B blocks, which share sequence homologies with their equivalents in tRNA genes (3, 13, 18). Spacing between the A and B blocks is important for maintaining transcriptional activity (3, 7). At least three cellular proteins are necessary to reconstitute transcription from the VA genes in vitro (37). These include the well-characterized RNA polymerase III and two subcellular fractions which have been functionally designated TFIIIB and TFIIIC. A component in TFIIIC has been shown by physical methods to interact directly with DNA sequences around the B-block consensus element (8, 15, 22). TFIIIC binding alone is sufficient for template commitment of transcription on VA genes, as determined by template competition assays (22). Studies with chromatographically purified transcription complexes have shown that there is a stoichiometric interaction of TFIIIB with the TFIIIC-VA gene complex, followed by rapid association of RNA polymerase III to form a complete preinitiation complex (8, 20).

During the course of adenovirus infection, the relative rates of synthesis of the two VA RNAs differ markedly (38). Transcription from both genes commences shortly after infection, with both RNAs being produced at roughly equivalent rates. This pattern continues until the onset of viral DNA replication, approximately 8 to 10 h postinfection, at which time VAII synthesis levels off while VAI synthesis accelerates. Thus, at late times the ratio of VAI to VAII RNA present may be as high as 40:1 (40). The transcriptional control underlying the differential expression of the two VA genes is not fully understood. Evidence points toward competition between the two VA promoters for a transcription factor present in limiting quantities (4).

To understand the mechanistic basis for differential regulation of the VA genes, as well as the roles of sequence and factors in the mechanism of RNA polymerase III-mediated specific transcription, we undertook a study of those proteins which interact with the VA genes and their effects on transcription. With fractionated HeLa cell nuclear extracts and both DNase I (16) and methidiumpropyl-EDTA · Fe(II) (MPE) (35, 41) cleavage protection methodologies, three proteins were shown to interact specifically, yet differentially, with the two VA genes. Two of these proteins were identified as the polymerase III transcription factor TFIIIC and the DNA replication protein nuclear factor I (NFI), whereas the third, a VA gene binding protein designated VBP, was previously unrecognized. The possible functions of these proteins in VA transcription were analyzed in reconstituted systems. Whereas NFI exhibited no effect, VBP appeared to depress the intrinsic ratio of VAI-VAII transcription, thus simulating the levels observed early in infection. A model for the differential regulation of VA

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transcription during the course of adenovirus infection is proposed.

MATERIALS AND METHODS

Protein fractions. Ion-exchange resins, including phosphocellulose (P-11; Whatman, Inc.), DEAE-Sephadex (A-25; Pharmacia, Inc.), and DEAE-Sephacel (Pharmacia), were prepared as directed by the manufacturers. The buffers used in chromatography contained 20 mM Tris (pH 7.9 @ 20°C), 20% glycerol, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol in addition to either KCl or $(NH_4)_2SO_4$ at the concentrations described below. All chromatography was performed at 4°C; fractions were frozen in liquid N_2 and stored at −80°C. Protein concentrations were measured by the method of Bradford (6).

Partially purified RNA polymerase III was obtained as follows. A HeLa S-100 extract (1,300 ml, 14 g of protein) was loaded onto a 1,000-ml phosphocellulose column equilibrated with 0.1 M KCl. Upon step elution with 0.6 M KCl, the RNA polymerase III activity was found in the leading half of the protein peak (combined fractions contained 540 mg of protein in 200 ml). A sample (50 ml, 130 mg of protein) was dialyzed to 0.12 M $(NH_4)_2SO_4$ and loaded onto a 50-ml DEAE-Sephadex column equilibrated with 0.1 M $(NH_4)_2SO_4$. The column was eluted with a five-column volume gradient of $(NH_4)_2SO_4$ (0.1 to 0.5 M). Active fractions (50 ml, 7.5 mg of protein) were pooled, adjusted to ≥0.5 mg/ml in protein with the addition of RNase-free bovine serum albumin (Pentax; Miles Laboratories, Inc.), and dialyzed until the ionic strength was ≤55 mM $(NH_4)_2SO_4$. This material was then loaded onto a small (2.5 ml) phosphocellulose column, and the bound activity was step eluted with 0.3 M $(NH_4)_2SO_4$. The final preparation incorporated 143 pmol of UMP/20′ reaction per μg of protein in a nonspecific transcription assay with poly(dA-dT) as a template (19). This material contained no detectable TFIIIB or TFIIIC activity.

Partially purified TFIIIB (hydroxyapatite gradient fraction; P. L. Martin and R. G. Roeder, unpublished data) was kindly provided by Paul Martin. This material incorporated 41 pmol of GMP into VA RNA/60′ reaction per μg of protein in a specific transcription assay with the VA genes of Ad2 (plasmid pAD2-wt, provided by T. Shenk) as a template (13, 42) and was essentially free from any TFIIIC or RNA polymerase III.

VA gene binding proteins were partially purified by the following procedure. A HeLa cell nuclear extract (75 ml, 420 mg of protein) prepared by standard procedures (11) was loaded onto a 20-ml phosphocellulose column. The column was eluted with a 10-column volume KCl gradient (0.1 to 1.0 M), and 4-ml fractions were collected. Those fractions demonstrating protein binding to the VA genes in protected regions Pr1, Pr3, and Pr4 (see Fig. 1, gradient fractions 12 to 14) were pooled (12 ml, 34 mg of protein), dialyzed to 0.12 M KCl, and loaded onto a 2-ml DEAE-Sephacel column. After being washed with buffer containing 0.12 M KCl, the column was eluted with a 4-column volume of KCl gradient (0.12 to 0.5 M), and 0.4-ml fractions were collected.

DNase I and MPE footprinting. (i) Labeled DNA fragments. Plasmid pAd2-wt was first digested with the restriction endonuclease *Nco*I and either 3′ end labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I or treated with bacterial alkaline phosphatase and then 5′ end labeled with [γ-^{32}P]ATP and T4 kinase (25). A second restriction digest with *Apa*I yielded a singly labeled 672-bp fragment containing both VA genes. Substitution of *Bam*HI for *Nco*I in the initial restriction digest yielded a 523-bp fragment containing only the 3′ half of VAI and the entire VAII gene. Fragments were isolated after electrophoresis on 1% agarose gels and elution from DEAE membranes (NA-45; Schleicher & Schuell). Purified fragments were obtained after exhaustive phenol extractions and ethanol precipitations. The intactness of each fragment preparation was gauged by high-resolution gel electrophoresis before further use.

(ii) Cleavage protection reactions. Footprinting reactions (20 μl) were performed in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 8% glycerol, 60 to 70 mM KCl, 3.5 mM $MgCl_2$, and 2 mM dithiothreitol. Each included 1 to 2 ng of labeled DNA fragment (10,000 to 20,000 cpm), 100 ng of pUC12, and 8 μl (or less) of protein-containing fractions. The volumes of fractions added were maximized to permit the highest protein concentration in each reaction while maintaining constant ionic strength. Under these conditions the difference in added protein between any two adjacent reactions was always less than 14%. Footprint competition reactions contained various amounts of other DNA fragments in place of pUC12 (for details, see the legend to Fig. 8). Reactions were assembled on ice and then incubated at 30°C for 15 min. DNase I cleavage was initiated by addition of 2 ng of DNase I (Sigma Chemical Co.) and 2 nmol of $CaCl_2$ and allowed to proceed for 30 s at room temperature. MPE cleavage was initiated by addition of 0.2 nmol of MPE (a generous gift of P. B. Dervan, Caltech) and allowed to continue for 4 min at 30°C. Both DNase I and MPE cleavage were stopped by addition of 3 μl of stop solution (3 M ammonium acetate, 0.25 M EDTA, 1 mg of *Saccharomyces cerevisiae* RNA per ml). Further processing included phenol-chloroform extraction, ethanol precipitation, and suspension in 5 μl of 90% formamide–10 mM NaOH loading buffer. Samples were heated for 5 min at 90°C to effect denaturation and then loaded onto either 10-cm-long, 1-mm-wide (minifootprint) or 40-cm-long, 0.4-mm-wide (high resolution) 8% acrylamide–0.4% bisacrylamide–50% urea sequencing gels. G+A chemical sequencing reactions were used as markers (26). Autoradiography was carried out at −80°C without intensifying screens. Quantitation was performed by densitometry.

In vitro transcription reactions. Transcription reactions (20 μl) contained 20 mM HEPES (pH 7.9); 8% glycerol, 60 to 70 mM KCl; 3.5 mM $MgCl_2$; 0.6 mM each ATP, CTP, and UTP; 0.025 mM [α-^{32}P]GTP (4,000 to 10,000 cpm/pmol); 2 mM dithiothreitol; 20 ng of pAd2-wt DNA; and 80 ng of pUC-12 DNA. Proteins present in each reaction included HeLa cell RNA polymerase III (0.16 μg, 23 poly(dA-dT) units), TFIIIB (0.11 μg, 4.6 VA units), and 8 μg of bovine serum albumin (Pentax; Miles). Complementing transcription factors (RNA polymerase III and TFIIIB) were in excess for all reactions. Protein fractions constituted 8 μl or less of the total reaction. Reactions were assembled on ice with protein fractions added last. After 60 min at 30°C, reactions were stopped by addition of 20 μl of transcription stop solution (100 mM sodium acetate [pH 5.5], 30 mM EDTA, 0.4% sodium dodecyl sulfate [SDS], 1 mg of *S. cerevisiae* RNA per ml), phenol-chloroform extracted, and ethanol precipitated. Dried samples were suspended in 10 μl of loading buffer (25% formamide, 10% glycerol, 0.5× Tris-borate-EDTA [25]) and loaded onto a 10-cm-long, 1-mm-wide 8% acrylamide–0.4% bisacrylamide–50% urea sequencing gel run at a sufficiently low voltage (200 V) to allow for resolution of the VAI and VAII transcripts. Autoradiography was performed

at room temperature without intensifying screens. Quantitation was performed by scintillation counting.

RESULTS

Multiple protein interactions observed on VA genes. Using a singly end-labeled DNA fragment containing both of the VA genes of Ad2, we investigated those regions protected by DNase I digestion when proteins from a fractionated HeLa cell nuclear extract were present. A total of five protected regions were observed when gradient fractions from a phosphocellulose column were assayed (Fig. 1A). These protected regions, labled Pr1 through Pr5, were numbered in the order in which they appeared on the VA gene cluster, reading from 5′ to 3′. The exact locations of these regions were determined by comparisons with DNA sequencing reactions (data to be shown later). Pr1 (gradient fractions 12 to 16) was mapped to a region within the internal control region of the VAI gene, Pr2 (fractions 18 to 30) was mapped to the B-block concensus element of the VAI gene, Pr3 and Pr4 (fractions 12 to 16) were mapped to the two terminator regions of the VAI gene, and Pr5 (fractions 20 to 28) was mapped to the B-block concensus element of the VAII gene. Those proteins responsible for Pr1, Pr3, and Pr4 coeluted in one set of fractions at low ionic strength (250 to 350 mM KCl), while the protein(s) responsible for Pr2 and Pr5 eluted at a higher ionic strength (400 to 700 mM KCl). This coelution behavior could indicate either identical proteins protecting multiple sites on the DNA or different proteins exhibiting similar chromatographic properties on this particular matrix.

We also monitored the phosphocellulose gradient fractions for TFIIIC activity by analyzing their ability to complement a reconstituted system lacking TFIIIC. Assays were carried out with sufficient amounts of TFIIIB and RNA polymerase III such that the amounts of VA transcription would respond linearly to TFIIIC concentration until a level of 2.5 pmol of GMP incorporated was achieved. The amount of transcription was quantitated as described in Materials and Methods, with the data presented in a graph as shown in Fig. 1B. TFIIIC activity was maximal in assays reconstituted with fractions 18 to 22, although some activity was detectable in fractions 8 to 34. Since transcription and footprinting reactions were performed under identical conditions, it was possible to compare TFIIIC activity versus the various DNA binding activities in each fraction directly. Comparison of Fig. 1A and B shows that TFIIIC activity correlated best with Pr2 and Pr5. However, it should be noted that this correlation was not an exact one, especially when comparing fractions possessing modest yet equivalent transcriptional activities. This was best exemplified by the much higher ratio of TFIIIC activity to DNA binding (Pr 2) as seen in fraction 16 when compared with that seen in fraction 24 (Fig. 1). This noncoincidence might reflect either transcriptional inhibitors present in the higher salt-eluted TFIIIC-containing fractions or DNA binding inhibitors in the lower salt-eluted fractions, but no evidence for this was found in mixing experiments (data not shown). Thus, whereas prior investigators, using partially purified fractions, have equated TFIIIC activity with the DNA binding activity of a protein which protects the internal control regions of the VAI and $tRNA^{Met}$ genes from DNase I digestion, our observations cast some doubt on this conclusion.

High-resolution footprinting analyses define protein binding sites. To locate various protected regions more precisely, we analyzed representative fractions from the phosphocellulose gradient by either DNase I or MPE cleavage coupled with high-resolution gel electrophoresis. Both DNA strands, nontranscribed and transcribed, were investigated. DNase I digestion provided a general delineation of the protected region, whereas MPE provided more precise identification of the significant protein-DNA interactions. Together these methods allowed for the definition of DNA sequences in-

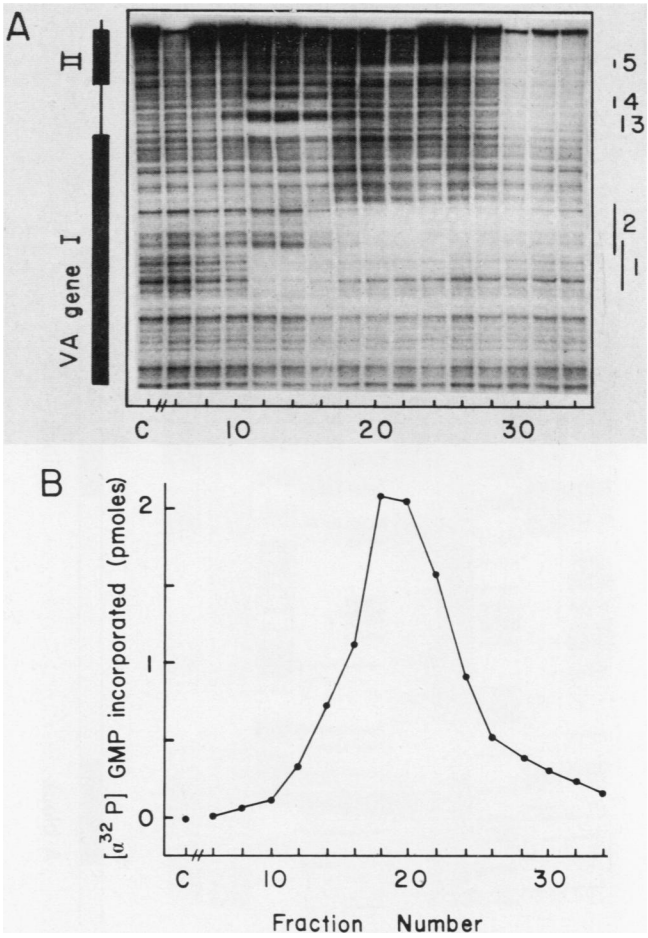

FIG. 1. DNase I footprinting and transcriptional analysis of cellular proteins interacting with the VA genes of Ad2. HeLa cell nuclear extract was chromatographed on a phosphocellulose column as described in Materials and Methods. Fractions were analyzed (A) for sequence-specific DNA binding proteins interacting with the VA genes by DNase I footprinting and (B) for TFIIIC activity by an in vitro reconstituted transcription system. (A) Footprinting reactions were carried out with a 3′ singly labeled 672-bp fragment and a 4.1- to 1.6-μl samples of even-numbered phosphocellulose gradient fractions 6 to 34 (120 to 174 mM KCl). The first lane demonstrates a control reaction containing DNase I alone. The locations of the different protected regions observed in some of the other lanes are indicated by numbered bars 1 to 5 on the right. A scale representation of the two tandem VA genes is provided on the left. (B) The amount of TFIIIC-dependent transcriptional activity present in each of the above fractions is shown. Transcription reactions were performed under conditions (template and carrier DNA concentrations, ionic strength, amount of fraction added) identical to those used in the footprinting reactions. The results are plotted in picomoles of GMP incorporated into VA transcripts per microliter of fraction. The control lane (C) contained only the complementary transcription factors (TFIIIB and RNA polymerase II).

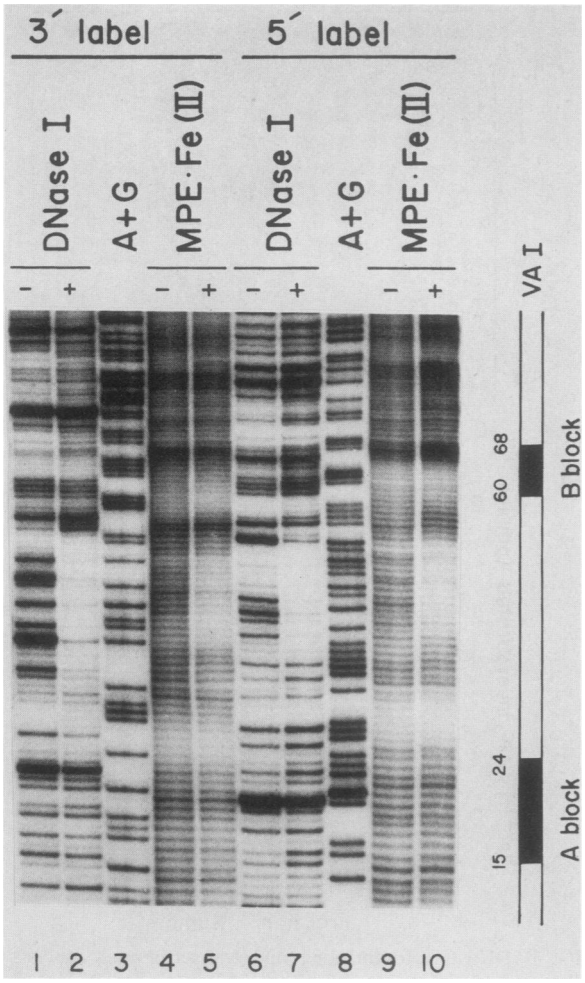

FIG. 2. High-resolution footprint analysis of Pr1 on the VAI gene. A 672-bp DNA fragment containing the VA genes was singly end labeled on either the 3′ (transcribed) or 5′ (nontranscribed) strand and subjected to limited DNase I or MPE digestion in the absence (−) or presence (+) of 4 μl of phosphocellulose gradient fraction 12 (Fig. 1). Maxam-Gilbert chemical sequencing reactions specific for purines (A+G) were used as markers. Shown is an autoradiogram of the cleavage products surrounding Pr1 (left), flanked by a scale representation of the corresponding section of the VAI gene (right). Numbers within this representation refer to nucleotide distances downstream from the transcriptional start site; the A- and B-block consensus elements within the internal promoter are indicated.

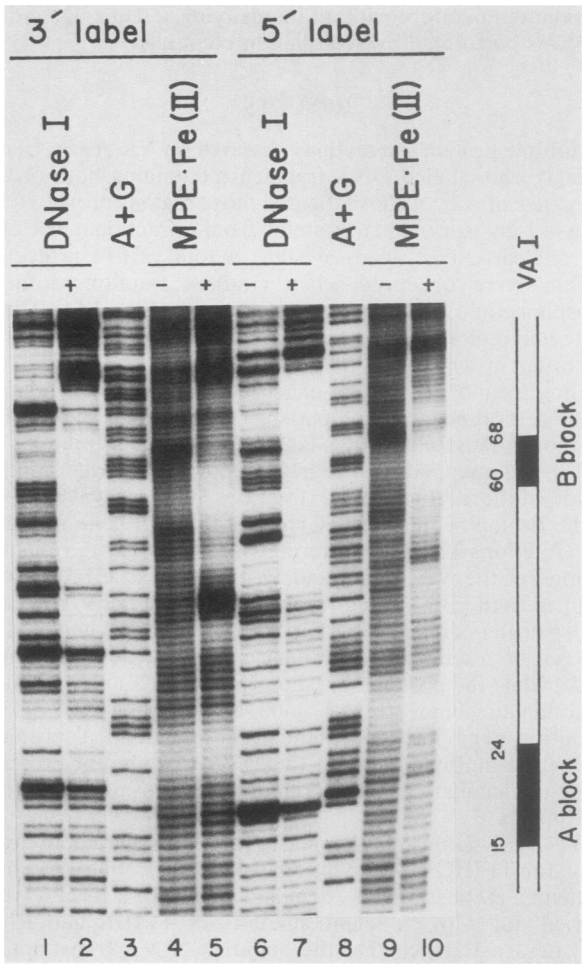

FIG. 3. High-resolution footprint analysis of Pr2 on the VAI gene. Conditions were identical to those stated in the legend to Fig. 2, with the exception that 2.5 μl of phosphocellulose gradient fraction 22 was used instead of fraction 12 in the + lanes.

volved in the various protected regions, making comparison with known consensus sequences possible.

Figure 2 shows an analysis of Pr1 in the VAI gene. DNase I protection extended from nucleotides +37 to +54 on the nontranscribed stand (lane 7) and from +35 to +53 on the transcribed strand (lane 2). The MPE-protected sites were subsets of the DNase I-protected sites; a region from +41 to +53 was protected on the nontranscribed strand (lane 10), and a region from +40 to +52 was protected on the transcribed strand (lane 5). A DNase I-hypersensitive cleavage was observed 3′ to the protected region on the transcribed strand. Sequence analysis (see Fig. 6) showed that Pr1 was located within the interblock region of the VAI gene, lying closer to the B-block consensus element. No protection of a similar nature was observed for the VAII gene or for any other class III gene investigated (*Xenopus laevis* $tRNA_i^{Met}$, *X. borealis* 5S; data not shown). Comparison with the consensus sequences of several eucaryotic DNA binding proteins yielded no positive matches.

Figure 3 shows an analysis of Pr2 in the VAI gene. A region of complete DNase I protection extended from +45 to +84 on the nontranscribed strand (lane 7) and from +42 to +80 on the transcribed strand (lane 2). A region of partial protection was found to extend from Pr2 to the labeled end of the DNA fragment. DNase I-hypersensitive cleavages were located primarily 3′ to the protected region. MPE cleavage protection was found between +53 and +75 on the nontranscribed strand (lane 10) and between +52 and +72 on the transcribed strand (lane 5), with the strongest protection located between +60 and +71 (nontranscribed) and +57 and +68 (transcribed). (For sequence information, see Fig. 6.) The region of greatest MPE protection in Pr2 coincided with the B-block consensus element of VAI. It should also be noted that no specific protection from either DNase I or MPE was observed in the region which correlates with the corresponding A-block consensus element. Interestingly, a region of enhanced MPE cleavages was observed on each strand approximately 16 bp upstream of the center of Pr2. This pattern of enhanced cleavages mimics that produced by

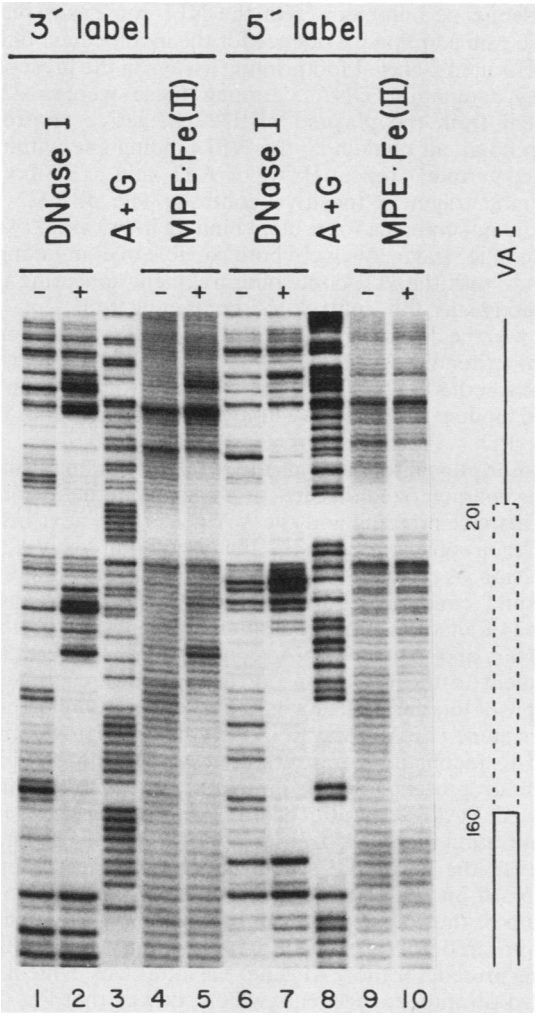

FIG. 4. High-resolution footprint analysis of Pr3 and Pr4 on the termini of the VAI gene. A 523-bp DNA fragment containing the 3′ half of the VAI gene and all of VAII was singly end labeled on either the 3′ (transcribed) or 5′ (nontranscribed) strand and subjected to limited DNase I or MPE digestion in the absence (−) or presence (+) of 4 μl of phosphocellulose gradient fraction 12. Purine (A+G)-specific Maxam-Gilbert sequencing reactions were used as markers. Shown is an autoradiogram (left) illustrating Pr3 (below) and Pr4 (above). The scale representation of the corresponding DNA section includes the primary termination site (solid line, +160) for VAI and the readthrough termination site (dotted line, +201) for the VA200 transcript.

a fixed EDTA-chelated iron located near the DNA phosphodiester backbone which generates a diffusible, nonspecific DNA cleaving species (i.e., the hydroxyl radical) (36). Thus, the observed cleavages suggest that the intercalation site between base pairs 47 and 48 is a preferred MPE binding site and may be indicative of a protein-induced conformational change in the DNA (such as kinking) which promotes the stability of the MPE-DNA interaction (21).

Figure 4 shows an analysis of Pr3 (near +160) and Pr4 (near +201) in the intergenic region between VAI and VAII. DNase I protection was evident from +157 to +178 on the nontranscribed strand (lane 7) and from +154 to +176 on the transcribed strand (lane 2) for Pr3. Similarly, DNase I protection was evident from +197 to +217 on the nontranscribed strand (lane 7) and from +194 to +215 on the transcribed strand (lane 2) for Pr4. A repeated pattern of DNase I-hypersensitive sites was seen 3′ to each protected region. MPE-protected regions covered from +159 to +174 on the nontranscribed strand (lane 10) and from +159 to +172 on the transcribed strand (lane 5) for Pr3. The corresponding regions on Pr4 extended from +199 to +211 on the nontranscribed strand (lane 10) and from +199 to +210 on the transcribed strand (lane 5). Similar hypersensitive cleavages were observed in both MPE and DNase I reactions. This coincidence seemed inconceivable, given the different mechanisms by which these DNA cleaving reactions effect increased rates of DNA strand scission at specific sites. In fact, these enhanced cleavages were observed even when no DNase I or MPE was present, indicating that they were due to endogenous nucleases (data not shown). No further evidence exists that the same proteins are involved in both cleavage protection and cleavage enhancement. (For sequences corresponding to Pr3 and Pr4, see Fig. 6.) From these studies it could be determined that Pr3 and Pr4 are located immediately 3′ to the two polymerase III transcription terminators of VAI (+160 and +201). Comparison against known consensus sequences for DNA binding proteins gave a good correlation between Pr3 (8 of 10 nucleotides) and Pr4 (7 of 10 nucleotides) and the TGGCA binding protein, which appears to be the same as cellular NFI (23, 28), a protein involved in initiation of adenovirus replication. The close proximity (41 nucleotides, center to center) of Pr3 and Pr4 and their separation by an integral number of DNA helical turns may contribute to stabilization of presumptive NFI binding to these less-than-ideal sequences and to their periodic cleavage sensitivity, which is not characteristic of other NFI binding sites (29, 30).

Figure 5 shows an analysis of Pr5 in the VAII gene. DNase I protection was observed from +45 to +76 and +80 to +82 on the nontranscribed strand (lane 7) and from +42 to +71 and +75 to +80 on the transcribed strand (lane 2). DNase I-hypersensitive cleavages were observed on each strand both within the 3′ half of Pr5 and immediately adjacent to the 3′ border. Regions protected from MPE cleavage were found between +58 and +73 on the nontranscribed strand (lane 10) and between +55 and +69 on the transcribed strand (lane 5). No enhanced MPE cleavages were noted. Sequence analysis (Fig. 6) showed that Pr5 correlated well with the B-block consensus element of the VAII internal promoter. As with Pr2, no protected regions were evident in the vicinity of the A-block consensus element. Thus, Pr2 and Pr5 are most likely results of the same B-block binding protein(s) interacting with the VAI and VAII genes, respectively.

At least three proteins interact with the VA genes. The above-described phosphocellulose gradient fraction analysis showed five different DNase I-protected regions on the VA genes. These could be subdivided into two groupings (Pr1, Pr3, and Pr4 on the one hand and Pr2 and Pr5 on the other) on the basis of similar chromatographic behavior. High-resolution footprinting indicated sequence homologies between Pr3 and Pr4 (NFI binding site) and between Pr2 and Pr5 (B-block consensus element). Pr1 exhibited no sequence homology with Pr3 and Pr4, although all three were generated with the same chromatographic fractions. To determine the minimum number of proteins involved in forming the observed protected regions, we performed further chromatography.

Phosphocellulose gradient fractions 12 to 16, previously shown to contain those proteins responsible for Pr1, Pr3, and Pr4 (Fig. 1), were subjected to chromatography over a DEAE-Sephacel column as described in Materials and Meth-

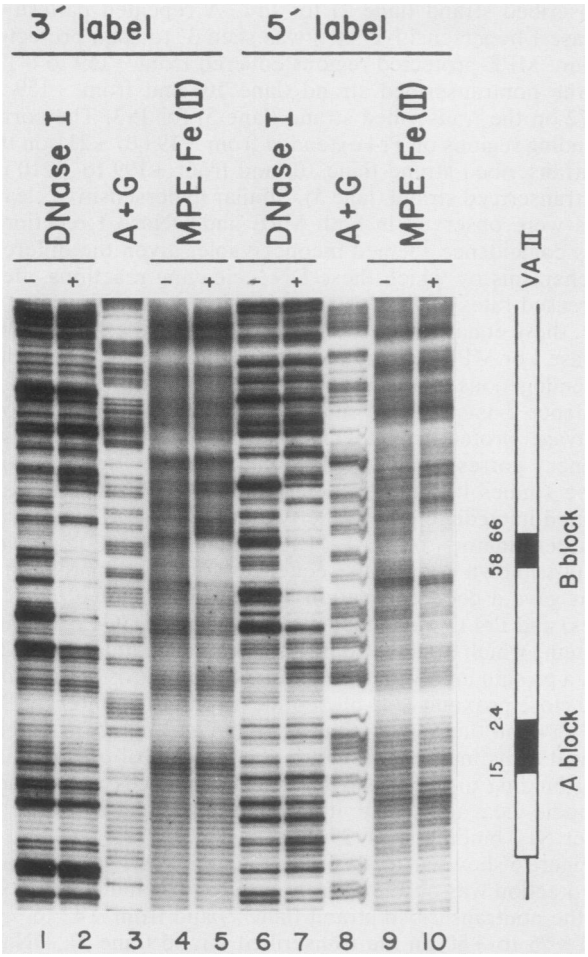

FIG. 5. High-resolution footprint analysis of Pr5 on the VAII gene. A 523-bp DNA fragment was singly end labeled on either the 3′ (transcribed) or 5′ (nontranscribed) strand and subjected to limited DNase I or MPE digestion in the absence (−) or presence (+) of 2.5 μl of phosphocellulose fraction 22. Purine (A+G)-specific chemical sequencing reactions were used as markers. Shown is an autoradiogram (left) illustrating Pr5, flanked by a scale representation of the corresponding VAII gene section (right). Numbers within this representation refer to nucleotide distances downstream from the VAII gene start site; A block and B block refer to consensus elements within the internal promoter.

ods. Gradient fractions eluted from this column were analyzed by DNase I footprinting (Fig. 7). Pr3 and Pr4 were both observed in reactions containing the flowthrough fraction, whereas Pr1 was observed only with fractions which eluted late in the gradient. When phosphocellulose fractions containing the protein responsible for generating Pr2 and Pr5 were subjected to DEAE-Sephacel chromatography under similar conditions, no separation of proteins responsible for the respective footprints was achieved; however, the appearance of Pr5 was usually more sensitive to alterations in protein concentration (data not shown; Fig. 1A). From this and the aforementioned sequence information, it was concluded that the five protected regions observed on the VA genes may be classified into three groups (Pr1, Pr2 and Pr5, and Pr3 and Pr4), each containing a site(s) for the binding of a distinct protein.

Evidence for NFI binding to Pr3 and Pr4. Pr3 and Pr4 were shown through high-resolution footprinting to possess a good sequence homology with the NFI consensus binding site. To gain additional evidence for the involvement of NFI, we performed DNase I footprinting assays in the presence of various competitor DNAs. Among these were a 227-bp fragment from the plasmid pBR322 (negative control), a 450-bp fragment containing the NFI binding site within the inverted terminal repeat (ITR) of Ad2, and a 673-bp VA-containing fragment (positive control). The pBR322 fragment did not compete for protein binding in Pr3 and Pr4 (Fig. 8). The other fragments were both capable of eliminating Pr3 and Pr4, with the ITR-containing fragment appearing twice as effective as the control VA-containing fragment. Also noted was the disappearance of Pr4 before Pr3 as the amount of competitor DNA was increased. These data support the conclusion that both Pr3 and Pr4 bind NFI (or a protein with related binding specificity), with their relative affinities being ITR > Pr3 > Pr4.

Transcriptional influence of the VA gene binding proteins. Having characterized the sites of physical interaction among three distinct proteins and the VA genes, we next investigated their possible roles in the regulation of VA gene expression. A reconstituted in vitro transcription assay with a plasmid containing both VA genes was chosen as the method of analysis. Reaction conditions (ionic strength and promoter and carrier DNA concentrations) were made equivalent to those used in our footprinting reactions. An example of this assay is shown in Fig. 9. The complementary factors alone (TFIIIB and RNA polymerase III) were insufficient to reconstitute transcription. Addition of a TFIIIC-containing protein fraction initiated transcription, with an observed VAI-VAII ratio of 10:1. Increasing the TFIIIC concentration increased transcription from both VA genes. However, the VAI-VAII ratio dropped to a level of approximately 4:1 and exhibited an inverse linear relationship with the sum of the transcripts from both genes. This phenomenon appeared to reflect the greater affinity of the B-box binding protein for the VAI gene; the latter was evident from detailed titration experiments which showed that Pr2 (VAI) appeared at lower protein concentrations than did Pr5 (VAII) (data not shown; Fig. 1A). Thus, whereas the protein responsible for Pr2 and Pr5 need not be identical to TFIIIC (Fig. 1; Discussion), it remains plausible that the ratio of VAI-VAII transcription reflects the binding equilibrium of a DNA binding transcription factor to competing substrates of differing affinities.

Addition of VBP, the protein responsible for Pr1, affected both the absolute levels and relative distributions of the two VA RNA products. The increased transcriptional level is due in part to a contamination of VBP-containing fractions with TFIIIC activity. Such could be expected given the similar chromatographic behavior of the two proteins on DEAE-Sephacel. Interestingly, the ratio of VAI to VAII transcription was reduced from 10:1 in the presence of TFIIIC alone to 2:1 in the presence of VBP (Fig. 9). Given the total amount of transcription present, the VAI-VAII ratio is approximately one-third that expected in a TFIIIC-alone reaction. This depression of the anticipated VAI-VAII ratio remained unchanged even after addition of exogenous TFIIIC. Thus, it appears that VBP, in quantities sufficient to generate Pr1 (Fig. 7), was capable of perturbing the relative transcriptional efficiencies of VAI and VAII, perhaps through competition between TFIIIC and VBP for interfering binding sites on VAI (Fig. 6).

In contrast, the putative NFI-containing fraction (NFI is the protein responsible for Pr3 and Pr4) did not appear to affect either the amounts of transcription from the VA genes

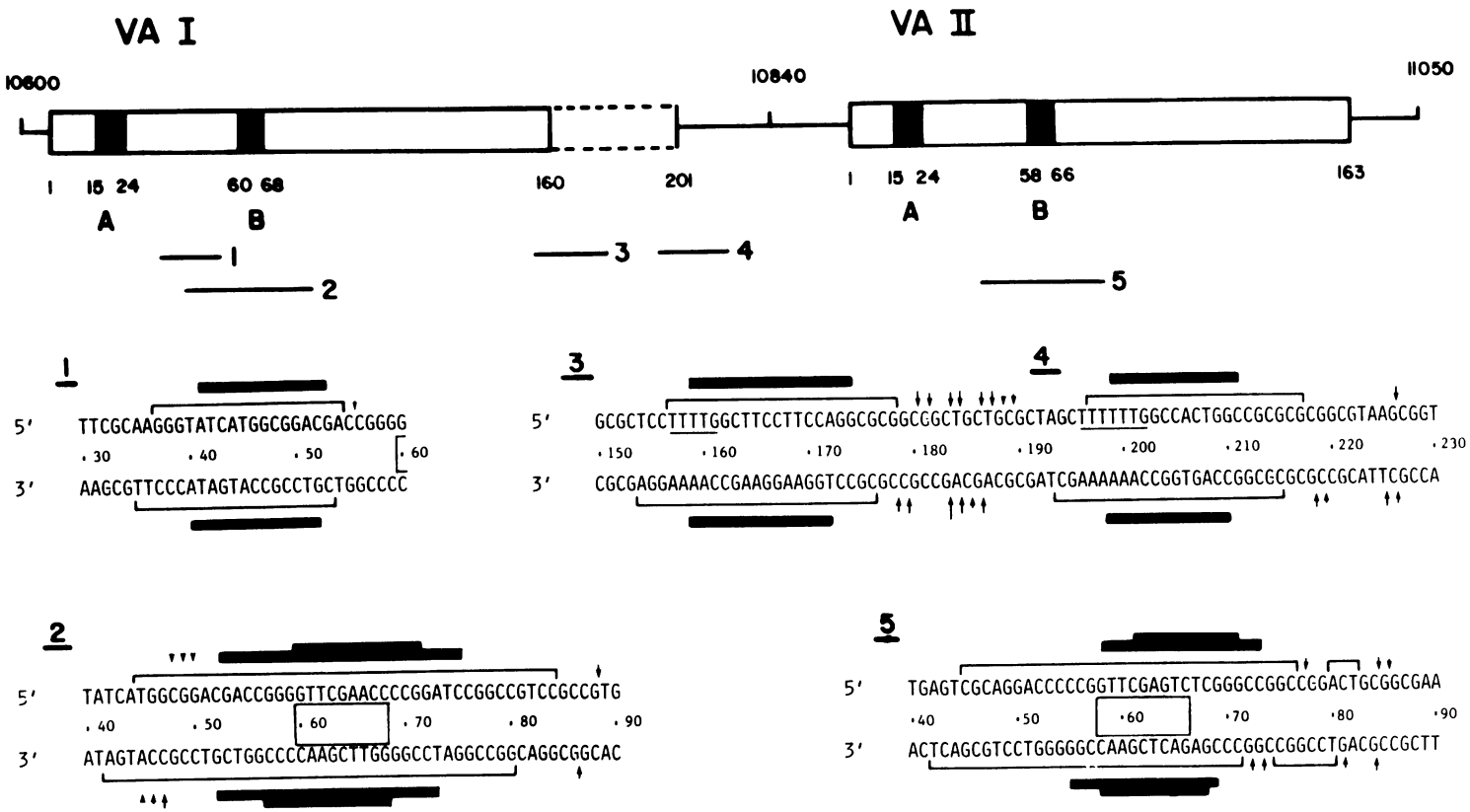

FIG. 6. Sequence information for Pr1 to Pr5. (A) Schematic representation of the VA gene cluster together with the locations of the protected regions. VAI and VAII are represented by open boxes; the VA200 transcript is represented by a dashed box extending beyond the VAI terminator. Closed boxes indicate the A- and B-block consensus elements within the VA gene internal promoter. Numbers above these genes refer to distances (in nucleotides) from the leftward end of the Ad2 genome. Numbers below refer to distances (in nucleotides) downstream from the closest VA gene transcription start site. Numbered lines beneath these genes refer to the locations and sites of the protected regions. (B) Nucleotide sequences of Pr1 to Pr5. The 5′ and 3′ end-labeled strands refer to the nontranscribed and transcribed strands, respectively. MPE protections are indicated by black bars; DNase I protections are indicated by fine brackets. Hypersensitive cleavages are indicated by arrows whose lengths indicate the degrees of hypersensitivity observed. Between sequences, numbers indicate the distances (in nucleotides) from the VA gene transcription start site, boxes delineate the B-block consensus elements, and underlined sequences refer to the RNA polymerase III transcription terminators.

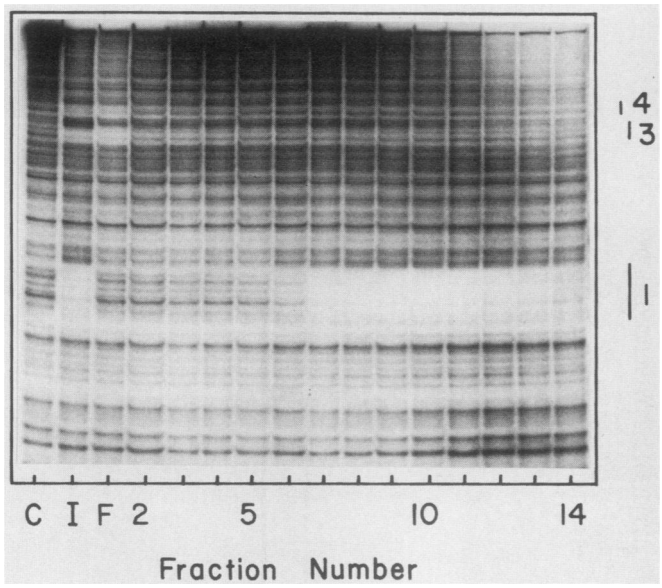

FIG. 7. Chromatographic separation of the proteins involved in Pr1, Pr3, and Pr4. Those fractions containing proteins interacting with Pr1, Pr3, and Pr4 (phosphocellulose gradient fractions 12 to 14 [Fig. 1A]) were submitted to additional chromatography on DEAE-Sephacel as described in Materials and Methods. Footprinting reactions were performed as described in the legend to Fig. 1, except for the addition of 6.0 to 3.5 μl of the various protein fractions to maintain a constant KCl concentration. Abbreviations: C, Control DNase I reaction; I, input fraction; F, flowthrough fraction. Numbered bars on the right indicate the locations of the protected regions.

or the ratio of their products greatly (Fig. 9). A concentration of NFI sufficient to generate Pr3 and Pr4 was relatively free of TFIIIC activity, as demonstrated by its poor ability to reconstitute transcription. Addition of a TFIIIC-containing fraction provided a linear increase in transcription with a slope equivalent to that observed in a titration of TFIIIC alone. This indicated that neither inhibitory nor stimulatory transcriptional activity was present in the NFI fraction. It should also be noted that, although NFI binds adjacent to both transcription terminators downstream from the VAI start site, the lengths of the major (156 nucleotide) and minor (200 nucleotide) VAI RNAs were not detectably changed when NFI was present. Thus, as assayed by our in vitro assay, NFI exhibited no significant effect on VA gene transcription.

DISCUSSION

Our observation of multiple DNase I cleavage protections (footprints) on the VAI and VAII genes of Ad2 led us to investigate these DNA binding sites and the proteins responsible for them. Identification of the DNA binding sites was obtained by DNase I and MPE footprinting assays, the latter method proving more useful in this respect. Sequence analysis allowed classification of the five original protected regions into three groups: one located immediately upstream of the B-block concensus element in VAI (Pr1), two corresponding to the B-block concensus elements of VAI and VAII (Pr2 and Pr5, respectively), and two located immediately downstream of the transcription terminators (+160 and +200) of VAI (Pr3 and Pr4, respectively). The proteins responsible for these protected regions were further characterized by their chromatographic behaviors on both cationic and anionic exchange columns and by footprinting competition experiments with DNA fragments containing known protein binding sites. Through these methods we were able to identify two of the proteins tentatively. One, interacting at Pr2 and Pr5, is most likely related to the transcription initiation factor TFIIIC; another, interacting at Pr3 and Pr4, it similar to the DNA replication protein NFI. Before this investigation, the only factor implicated in binding to sequences within the VA genes was TFIIIC. With the present discovery of two other proteins interacting with these genes, the possibility arises that various interactions between these proteins could affect the transcriptional process and thereby control the differential regulation of the two VA genes during the course of adenovirus infection.

MPE footprinting was of significantly greater benefit than DNase I footprinting in our characterization of the various VA gene binding proteins. Possessing only a moderate binding propensity for G+C sequences (inherent in the methidium intercalator moiety), MPE-mediated DNA cleavage exhibited less specificity than that observed with DNase I (41). Given the more uniform cleavage pattern of MPE, footprints may be analyzed with higher resolution, thereby allowing a more precise definition of protein binding sites on DNA (Fig. 3, lanes 2 and 5). Since MPE has a significantly lower molecular weight than DNase I (0.7 versus 31 kilodaltons, respectively), it provides less possibility for steric interference between itself and a protein bound to the DNA. This resulted in smaller protected regions obtained with MPE and thus increased the accuracy with which

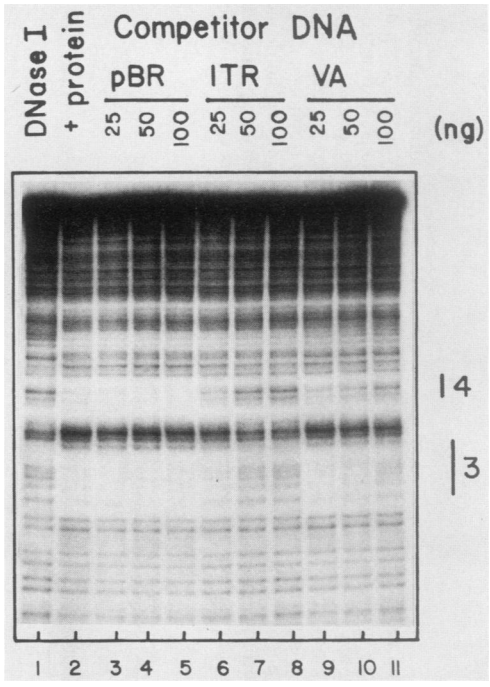

FIG. 8. Footprint competition of Pr3 and Pr4 by NFI binding sites. Reactions included the 5′ end-labeled 523-bp DNA fragment and, except in the case of the DNase I control (lane 1), 7.0 μl of a DEAE-Sephacel flowthrough fraction. For each reaction, carrier DNA was composed of the listed amount of competitor DNA, with the remainder being plasmid pUC12. Abbreviations: *pBR*, Fragment from pBR322; *ITR*, fragment upstream of the E1a promoter in Ad2, including the NFI binding site in the inverted terminal repeat; *VA*, fragment containing both VA genes.

protein binding sites were located (Fig. 6). The binding constant of MPE to DNA is manyfold greater than that of DNase I (12, 17). MPE was therefore better able to compete against weak protein-DNA interactions, thereby identifying only those regions which exhibited the strongest interactions and which more closely matched the regions identified through mutational analysis (Fig. 6, Pr2 and Pr5) (35). Considering the improved precision, accuracy, and discriminating potential of footprinting with MPE, protected regions determined by this method may provide enough information for the design of oligonucleotides suitable for affinity purification or point mutational analysis.

TFIIIC was initially defined as an activity which was present in the 0.5 M KCl eluate of a phosphocellulose column and allowed reconstitution of polymerase III-mediated specific transcription in vitro (37). Subsequent studies demonstrated that this fraction contained a protein(s) which interacts with sequences within the internal promoters of the VAI and tRNA genes (8, 15, 22). Template competition experiments demonstrated the involvement of TFIIIC in the formation of stable preinitiation complexes on all class III genes (8, 22). Thus, the transcriptional activity of TFIIIC was apparently correlated to its initial binding to the gene promoter (VA and tRNA), which was followed by its interaction with the other transcription factor, TFIIIB, and RNA polymerase III.

Recent studies investigating the interactions between TFIIIC, the VAI promoter, and the other transcription factors have yielded conflicting results when analyzed by functional assays (in vitro transcription) versus physical methods (DNase I footprinting). Thus, Carey et al. (8) demonstrated that presumptive TFIIIC-VAI gene complexes were resistant to high (1 M KCl) salt concentrations when assayed in transcription reactions but labile in the presence of moderate (0.2 M KCl) ionic strengths when assayed by DNase I footprinting. Similarly, functionally active preinitiation complexes could be isolated after addition of TFIIIB and RNA polymerase III, although addition of these other proteins failed to alter the basic TFIIIC footprint. To account for their observations, Carey et al. advanced a unique model advocating two different modes of TFIIIC-DNA interaction: (i) a highly localized site-speciifc mode which was observable by DNase I footprinting and (ii) a delocalized though gene-dependent mode capable of interaction with TFIIIB and remaining functional in transcription. However, given that the TFIIIC fraction used was not highly purified, there is an alternative and perhaps more plausible explanation. Thus, there might simply be two populations of B-block binding proteins in HeLa cell nuclear extracts: (i) a predominant form which is readily detectable by footprinting and which need not be capable of further interactions and (ii) a minor form capable of both site-specific DNA binding and subsequent interactions with TFIIIB and RNA polymerase III to effect transcription. Detection of the minor form is made possible by the extreme sensitivity inherent in transcription assays; whereas these assays necessitate that only a small fraction of the total templates be active, footprinting reactions require complete saturation of all potential sites. Thus, Carey et al. might simply have not detected a minor fraction of functionally active TFIIIC-VA complexes in their footprinting assays. It should be possible to confirm the existence of the minor form by physical methods, given either an assay of appropriate sensitivity or a means for its selective enrichment. Our results demonstrating lack of complete coincidence between B-block protection and TFIIIC activity in gradient fractions from a phosphocellulose column not only concur with this possibility but also lend support to the feasibility of chromatographically separating the two species. Further investigations on this point are currently under way in our laboratory. It should be stressed that the existence of both forms of B-block binding proteins need not necessarily reflect an experimental artifact (e.g., limited proteolysis or other covalent modification during extract preparation creating an inactive form of TFIIIC) but may rather be indicative of a means for class III gene regulation in vivo (e.g., by interconversion of active and inactive forms of TFIIIC).

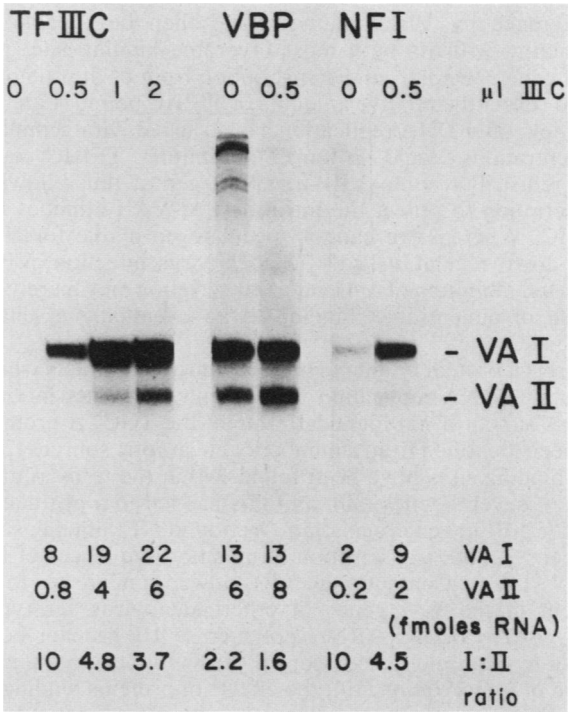

FIG. 9. Effect of TFIIIC, VBP, and NFI on in vitro transcription of the two VA genes. Shown is an autoradiogram of transcripts from the VA genes of Ad2, with quantitation tabulated below. All reactions contained an excess of TFIIIB and RNA polymerase III. Other proteins present included TFIIIC (phosphocellulose gradient fraction 22, quantities listed above the lanes), VBP (DEAE-Sephacel gradient fraction 8, 3 μl), and NFI (DEAE-Sephacel flowthrough, 5 μl) where indicated. Quantitation of the VAI and VAII transcripts is given for each species as femtomoles of RNA synthesized.

The novel VAI gene binding protein described here is a cellular protein which binds specifically to a region of the VA gene immediately upstream of the B-block consensus element. Although mutations in this region greatly affect the translational enhancing function of the resulting VAI RNA (2), deletion, insertion, substitution, and linker scanning mutants have shown no indication that this region is essential for transcription in vitro (3, 7). However, we have found that inclusion of VBP in in vitro transcription assays caused depression of VAI transcription with a concomitant increase in VAII. Under these conditions, the resulting VAI-VAII product ratio mirrors that observed early in adenovirus infection (38). This observation, coupled with our demonstration of overlapping binding sites between VBP and TFIIIC, lends support to a model in which VBP and TFIIIC compete for occupancy on a limited quantity of VAI genes. Thus, TFIIIC not bound to the VAI gene as a result of

interference by VBP binding would then be capable of interacting with its next most favorable binding site, the VAII gene promoter, and transcription from each promoter would reflect the relative amounts of TFIIIC bound. Later in infection, after DNA replication has occurred, viral template concentrations would no longer be limiting. TFIIIC could then redistribute onto VBP-free VAI genes, thus allowing transcription to reflect the intrinsic VAI-VAII affinities for TFIIIC. Whereas we cannot speculate on a role for VAI gene down regulation early in adenovirus infection, VBP-mediated inhibition of VAI gene transcription may merely be a case of adventitious binding to an eventually essential sequence.

NFI is a protein found in HeLa cell nuclear extracts which stimulates DNA replication from adenovirus ITRs in vitro (28). A similar if not identical protein, the TGCCA protein, has been identified from a number of eucaryotic sources (23). NFI binding sites have been found within the transcription units of several genes (30), and this has led to a postulated role for NFI in gene regulation. We found NFI binding sites adjacent to both transcription termination sequences of the Ad2 VAI gene. Computer analysis showed similar sites to be present in the VAI genes of other adenovirus serotypes (Ad5, Ad7). Whereas RNA polymerase III has not been shown to require any accessory proteins for accurate termination of transcription (10), the effects of proteins binding to class III gene terminator regions on either termination efficiency or transcription reinitiation rates are as yet unknown. Within the limitations of our in vitro assay, a crude preparation of NFI had apparently no effect on transcription from either of the VA genes. Therefore, regardless of the implications engendered by conserved NFI binding sites, we presently cannot predict what role, if any, NFI might play in either the efficiency of VAI transcription or the differential regulation of VA genes in vivo.

ACKNOWLEDGMENTS

We thank Paul Martin for providing partially purified TFIIIB and Peter B. Dervan for the gift of MPE. We are especially grateful to Michele Sawadogo for many helpful discussions.

This research was supported by Public Health Service grants CA 42567 and CA 34223 from the National Institutes of Health to R.G.R. and with general support from the Pew Trusts. M.W.V. was supported by a postdoctoral fellowship (GM 09949) from the National Institutes of Health.

LITERATURE CITED

1. **Akusjarvi, G., M. B. Mathews, P. Andersson, B. Vennstrom, and U. Pettersson.** 1980. Structure of genes for virus associated RNA_I and RNA_{II} of adenovirus type 2. Proc. Natl. Acad. Sci. USA **77**:2424–2428.
2. **Bhat, R. A., P. H. Domer, and B. Thimmappaya.** 1985. Structural requirements of adenovirus VAI RNA for its translation enhancement function. Mol. Cell. Biol. **5**:187–196.
3. **Bhat, R. A., B. Metz, and B. Thimmappaya.** 1983. Organization of the noncontiguous promoter components of adenovirus VAI RNA gene is strikingly similar to that of eucaryotic tRNA genes. Mol. Cell. Biol. **3**:1996–2005.
4. **Bhat, R. A., and B. Thimmappaya.** 1984. Adenovirus mutants with DNA sequence pertubations in the intragenic promoter of VAI RNA allow the enhanced transcription of VAII RNA gene in HeLa cells. Nucleic Acids Res. **12**:7377–7388.
5. **Bogenhagen, D. F., and D. D. Brown.** 1981. Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell **24**:261–270.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the priciple of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Cannon, R. E., G.-J. Wu, and J. F. Railey.** 1986. Functions of and interactions between the A and B blocks in adenovirus type 2-specific VARNA1 gene. Proc. Natl. Acad. Sci. USA **83**:1285–1289.
8. **Carey, M. F., S. P. Gerrard, and N. R. Cozzarelli.** 1986. Analysis of RNA polymerase III transcription complexes by gel filtration. J. Biol. Chem. **261**:4309–4317.
9. **Celma, M. L., J. Pan, and S. M. Weissman.** 1977. Studies of low molecular weight RNA from cells infected with adenovirus 2. I. The sequence at the 3′ end of VA-RNAI. J. Biol. Chem. **252**: 9032–9043.
10. **Cozzarelli, N. R., S. P. Gerrard, M. Schlissel, D. D. Brown, and D. F. Bogenhagen.** 1983. Purified RNA polymerase III accurately and efficiently terminates transcription of 5S RNA genes. Cell **34**:829–835.
11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
12. **Drew, H. R.** 1984. Structural specificities of five commonly used DNA nucleases. J. Mol. Biol. **176**:535–557.
13. **Fowlkes, D. M., and T. Shenk.** 1980. Transcriptional control regions of the adenovirus VAI RNA gene. Cell **22**:405–413.
14. **Francoeur, A. M., and M. B. Mathews.** 1982. Interaction between VA RNA and the lupus antigen La: formation of a ribonucleoprotein particle in vitro. Proc. Natl. Acad. Sci. USA **79**:6772–6776.
15. **Fuhrman, S. A., D. R. Engelke, and E. P. Gueiduschek.** 1984. HeLa cell RNA polymerase III transcription factors. J. Biol. Chem. **259**:1934–1943.
16. **Galas, D. J., and A. Schmitz.** 1978. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
17. **Hertzberg, R. P., and P. B. Dervan.** 1984. Cleavage of DNA with methidiumpropyl-EDTA · Fe(II): reaction conditions and product analyses. Biochemistry **23**:3934–3945.
18. **Hofstetter, H., A. Kressman, and M. Birnstiel.** 1981. A split promoter for a eucaryotic tRNA gene. Cell **24**:573–585.
19. **Jaehning, J. A., C. C. Stewart, and R. G. Roeder.** 1975. DNA-dependent RNA polymerase levels during the response of human peripheral lymphocytes to phytohemagglutinin. Cell **4**: 51–57.
20. **Kasher, M. S., D. Pintel, and D. C. Ward.** 1986. Rapid enrichment of HeLa transcription factors IIIB and IIIC by using affinity chromatography based on avidin-biotin interactions. Mol. Cell. Biol. **6**:3117–3127.
21. **Kean, J. M., S. A. White, and D. E. Draper.** 1985. Detection of high-affinity intercalator sites in a ribosomal RNA fragment by the affinity cleavage intercalator methidiumpropyl-EDTA · Fe(II). Biochemistry **24**:5062–5070.
22. **Lassar, A. B., P. L. Martin, and R. G. Roeder.** 1983. Transcription of class III genes: formation of preinitiation complexes. Science **222**:740–748.
23. **Leegwater, P. A. J., P. C. van der Vliet, R. A. W. Rupp, J. Nowock, and A. E. Sippel.** 1986. Functional homology between the sequence-specific DNA-binding proteins nuclear factor I from HeLa cells and the TGGCA protein from chicken liver. EMBO J. **5**:381–386.
24. **Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz.** 1981. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science **211**: 400–402.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Maxam, R. A. M., and W. Gilbert.** 1980. Sequencing end labelled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–559.
27. **Monstein, B., and L. Philipson.** 1981. The conformation of adenovirus VAI-RNA in solution. Nucleic Acids Res. **9**:4239–4250.
28. **Nagata, K., R. A. Guggenheimer, T. Enomoto, J. H. Lichy, and**

J. Hurwitz. 1982. Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA **79**:6438–6442.

29. **Nagata, K., R. A. Guggenheimer, and J. Hurwitz.** 1983. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA **80**:6177–6181.
30. **Nowock, J., U. Borgmeyer, A. W. Puschel, R. A. W. Rupp, and A. E. Sippel.** 1985. The TGGCA protein binds to the MMTV-LTR, the adenovirus origin of replication, and the BK virus enhancer. Nucleic Acids Res. **13**:2045–2061.
31. **O'Malley, R. P., T. M. Mariano, J. Siekierka, and M. B. Mathews.** 1986. A mechanism for the control of protein synthesis by adenovirus VA RNA_I. Cell **44**:391–400.
32. **Pettersson, U., and L. Philipson.** 1975. Location of sequences on the adenovirus genome coding for the 5.5S RNA. Cell **6**:1–4.
33. **Price, R., and S. Penman.** 1972. A distinct RNA polymerase activity, synthesizing 5.5S, 5S and 4S RNA in nuclei from adenovirus 2-infected HeLa cells. J. Mol. Biol. **70**:435–450.
34. **Reich, P. R., B. Forget, J. Rose, and S. M. Weissman.** 1966. RNA of low molecular weight in KB cells infected with Ad2. J. Mol. Biol. **17**:428–438.
35. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
36. **Schultz, P. G., and P. B. Dervan.** 1983. Sequence-specific double-strand cleavage of DNA by penta-*N*-methylpyrrole-carboxamide-EDTA · Fe(II). Proc. Natl. Acad. Sci. USA **80**: 6834–6837.
37. **Segall, J., T. Matsui, and R. G. Roeder.** 1980. Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. J. Biol. Chem. **255**:11986–11991.
38. **Sonderlund, H., U. Pettersson, B. Vennstrom, L. Philipson, and M. B. Mathews.** 1976. A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. Cell **7**:585–593.
39. **Thimmappaya, B., N, Jones, and T. Shenk.** 1979. RNA mutation which alters initiation of transcription by RNA polymerase III on the Ad5 chromosome. Cell **18**:947–954.
40. **Thimmappaya, B., C. Weinberger, R. J. Schneider, and T. Shenk.** 1982. Adenovirus VAI RNA is required for efficient translation of viral mRNA at late times after infection. Cell **31**:543–551.
41. **Van Dyke, M. W., and P. B. Dervan.** 1983. Methidiumpropyl-EDTA · Fe(II) and DNase I footprinting report different small molecule binding site sizes on DNA. Nucleic Acids Res. **11**:5555–5567.
42. **Weil, P. A., J. Segal, B. Harris, S. Y. Ng, and R. G. Roeder.** 1979. Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. J. Biol. Chem. **254**:6163–6173.
43. **Weinmann, R., H. J. Raskas, and R. G. Roeder.** 1974. Role of DNA dependent RNA polymerase II and III in transcription of the adenovirus genome late in productive infection. Proc. Natl. Acad. Sci. USA **71**:3426–3430.