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A Purified Adenovirus 289-Amino-Acid E1A Protein Activates RNA Polymerase III Transcription In Vitro and Alters Transcription Factor TFIIIC

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We have previously demonstrated that a purified bacterially synthesized E1A 289-amino-acid protein is capable of stimulating transcription from the promoters of genes transcribed by RNA polymerase II in vitro (R. Spangler, M. Bruner, B. Dalie, and M. L. Harter, Science 237:1044–1046, 1987). In this study, we show that this protein is also capable of transactivating in vitro the adenovirus virus-associated (VA1) RNA gene transcribed by RNA polymerase III. Pertinent to the transcription of this gene is the rate-limiting component, TFIIIC, which appears to be of two distinct forms in uninfected HeLa cells. The addition of an oligonucleotide containing a TFIIIC binding site to HeLa whole-cell extracts inhibits VA1 transcription by sequestering TFIIIC. However, the addition of purified E1A to extracts previously challenged with the TFIIIC oligonucleotide restores the level of VA1 transcription. When included in the same reaction, an E1A-specific monoclonal antibody reverses the restoration. Incubation of purified E1A with either HeLa cell nuclear or whole-cell extracts alters the DNA-binding properties of TFIIIC as detected by gel shift assays. This alteration does not occur if E1A-specific antibody and E1A protein are added simultaneously to the extract. In contrast, the addition of this antibody to extracts at a later time does not reverse the alteration observed in the TFIIIC binding activities. Never at any time did we note the formation of novel TFIIIC-promoter complexes after the addition of E1A to nuclear extracts. These results clearly establish that E1A mediates its effect on VA1 transcription through TFIIIC in a very rapid yet indirect manner. The results also establish that a bacterially produced E1A protein can directly participate in RNA polymerase II transcription without the requirement of cellular protein synthesis or other viral proteins.

The adenovirus 289-amino-acid E1A protein is well recognized for its ability to stimulate transcription from a number of viral and cellular promoters (reviewed in reference 7). The promoters responsive to this protein are not limited to genes transcribed by RNA polymerase II but also include those belonging to genes transcribed by RNA polymerase III (POLIII) (18, 19). In spite of what is known, it is still unclear exactly how the E1A protein is able to up-regulate transcription from the promoters of these two classes of genes. For instance, although E1A binds directly to DNA, as do many other transcriptional factors, it appears to do so only in a nonspecific fashion (4, 21). Furthermore, comparisons of wild-type and partially mutagenized promoters have not as yet identified an E1A inducible site apart from that which is required for full basal level transcription (20, 26, 33). Thus, it would seem that E1A does not act alone in stimulating transcription but instead acts indirectly, perhaps by affecting the levels of host-cell transcriptional factors (18, 20). However, it now appears unlikely that cellular protein synthesis is important to the E1A induction of class II genes, for several reasons. First, a purified bacterially synthesized E1A protein (2) and a peptide of E1A with transactivating activity (32) have recently proven to be quite capable of stimulating transcription from promoters in vitro (14, 37). Second, cellular factors such as ATF and MLTF, which appear to participate in E1A transactivation, remain unchanged in concentration during adenovirus infection (7, 25, 36). Taken together, these observations lead to the notion that certain cellular factors of class II genes may in themselves be directly affected by E1A. In fact, the prominent TATA-binding factor (i.e., TFIID) as well as the E4F, ATF, and E2F promoter factors all appear to be targets for E1A-mediated induction (15, 25, 27, 35), and both E2F and E4F appear to become modified by phosphorylation during adenovirus infection (1, 34). More recent studies have revealed a family of cellular ATF factors, one of which is thought to function by recruiting E1A to the promoter (29).

The only class III gene factor which appears to change during adenovirus infection is TFIIIC (18, 19, 40). Apart from POLIII, this factor, together with another factor termed TFIIIB, is the required component for in vivo transcription of the tRNA and adenovirus virus-associated (VA1) RNA genes (10, 19, 24). Both of these genes are unique in that they contain two highly conserved intragenic elements, termed the A and B blocks, which constitute internal transcriptional control regions (8, 10). TFIIIC, which has been defined as the rate-limiting factor of class III transcription, binds with high affinity to the internal B-block region (centered at about +60 relative to the start site) of both genes (8, 19, 24). However, it appears that TFIIIC is also capable of binding, albeit weakly, to the A-block region, but only under conditions that favor high protein concentrations (3, 39). The success of TFIIIB in becoming sequestered into a stable transcription complex on a mammalian class III gene seems to depend on its association (via protein-protein interaction) with TFIIIC (3, 8, 19) and not on its binding to upstream DNA.

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Transcription of the tRNA and VA1 RNA genes is dramatically increased in adenovirus-infected cells (11, 19, 38), and the E1A proteins appear to play a role in this process (18, 19, 29). Fractionation of these cells demonstrates an E1A-dependent stimulating activity in a fraction that contains TFIIIC, suggesting that this factor may be the target for E1A (18, 19, 29). Other experiments have recently shown that there are actually two forms of TFIIIC, one of which becomes altered by phosphorylation in response to adenovirus infection (18). Nevertheless, it is still not clear whether this alteration represents a direct involvement of the E1A protein. In the experiments that are reported here, we extend the analysis of class III transactivation by monitoring the activities of a purified bacterially synthesized E1A 289-amino-acid protein (2, 22) in extracts of HeLa cells programmed with the VA1 RNA gene. We show that E1A by itself is directly responsible for stimulating transcription from the VA1 promoter and does so without the requirement of cellular protein synthesis. We also show that E1A mediates its effect by adjusting the activities of TFIIIC in a manner that appears to be quite rapid and yet indirect.

MATERIALS AND METHODS

Cells and antibodies. HeLa cells in suspension culture were routinely maintained in S-MEM (GIBCO) containing 5.0% horse serum. Monoclonal antibodies specific for the E1A protein were obtained from the hybridoma cell lines M-2 and M-73 (kindly provided by E. Harlow, Cold Spring Harbor Laboratory [17]) and isolated by affinity chromatography on protein A-Sepharose as described by the manufacturer (Pharmacia). The Ab-1 monoclonal antibody specific for the adenovirus type 5 E1B 21-kDa protein was acquired from Oncogene Science, Inc.

Plasmids and oligonucleotides. The plasmid pVA* used for in vitro transcription contains the adenovirus type 2 *Hin*dIII fragment (nucleotides 6232 to 11559 [13]) inserted into the *Hin*dIII site of pBR322. The 129-bp *Xba*I-*Bst*EII fragment containing the VA1 gene for the gel retardation assays was derived from the pVA_1 plasmid, which has been described elsewhere (18) and which was kindly provided by W. K. Hoeffler, Genetech, Inc.

The double-stranded TFIIIC and mutant-TFIIIC (M-TFIIIC) oligonucleotides were formed by the hybridization (16) of two complementary 29-mers with the following sequences: TFIIIC, 5′-CCGGATCCGGGGTTCGAACCCCG GCCCAA-3′; M-TFIIIC, 5′-CCGGATCCAAAACTTACAT TTGCGCCCAA-3′. Another double-stranded TFIIIC oligonucleotide was formed by the hybridization of two complementary 51-mers; its sequence is 5′-AATTCATGGCGGAC GACCGGGGTTCGAACCCCGGATCCGGCCGTCCGT TAA. The sequences of oligonucleotides of 30 bp (AP3) and 48 bp (E2F), used as negative controls in the in vitro and gel retardation assays, are as follows: AP3, 5′-CCGGATCC AGTTAGGGTGTGGAAATATTAA-3′; E2F, 5′-CACTAT AGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGA AACTAGTCC-3′. Ligation of the M-TFIIIC oligonucleotide for competition in gel retardation assays was performed under standard conditions until a length of approximately 132 bp was obtained. All probes were gel purified before use.

Preparation of extracts. Whole-cell extracts were prepared from HeLa cells as previously described (37), but with modifications. Briefly, cells at a density of 4.5×10^5 cells per ml were harvested and washed twice (1,000 × *g*, 15 min) in cold phosphate-buffered saline. A volume of buffer A (10 mM Tris hydrochloride [pH 7.9], 1 mM EDTA, 5 mM dithiothreitol) equal to four times the packed cell volume was added, and the cells were allowed to swell for 20 min on ice. Cells were disrupted in a Kontes all-glass Dounce homogenizer by eight strokes with a B pestle. A volume of buffer B (50 mM Tris hydrochloride [pH 7.9], 10 mM $MgCl_2$, 2 mM dithiothreitol, 25% [wt/vol] sucrose, 50% [vol/vol] glycerol) equal to four times the packed cell volume was then added to the cell lysate. With continued stirring, a volume of saturated and neutralized ammonium sulfate equal to the packed cell volume was gradually added, and the lysate was stirred for an additional 30 min. The lysate was then centrifuged at 175,000 × *g* for 3 h. Afterwards, 0.35 g of solid ammonium sulfate per ml was added to the high-speed supernatant, and upon complete dissolution of the salt, 1 μl of 1 M NaOH per g of ammonium sulfate was added to the mixture. After 30 min of stirring, the mixture was centrifuged at 15,000 × *g* for 20 min. The pellet was retained and resuspended in a volume of buffer C (50 mM Tris hydrochloride [pH 7.9], 40 mM ammonium sulfate, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 15% glycerol) equal to 5% of the total volume of discarded supernatant. The resuspended pellet was dialyzed 8 to 12 h and then centrifuged (10,000 × *g*, 10 min) for the removal of insoluble material. The cleared whole-cell extract was aliquoted, rapidly frozen, and then stored at −80°C.

Nuclear extracts used in the gel retardation assays were prepared from HeLa cells by the method of Dignam et al. (6) with the modification of Hoeffler et al. (18) and dialyzed against buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol). Afterwards, the nuclear extract, with a protein concentration of about 6.0 to 7.0 mg/ml (determined by the Bio-Rad protein assay), was rapidly frozen and stored at −80°C.

In vitro transcription assays. In vitro synthesis was conducted in a 30-μl reaction volume containing 36 μg of whole-cell extract and (final concentrations) 10 mM Tris hydrochloride (pH 7.9), 45 mM KCl, and 5.4 mM $MgCl_2$. Transcription was initiated by the addition of the VA1 DNA template (50 ng) and a cocktail nucleoside triphosphate solution to a final concentration of 30 μM GTP, 5 μCi [α-^{32}P]GTP, 4 mM creatine phosphate, and 300 to 400 μM (each) ATP, CTP, and UTP. Transcription was allowed to proceed for 1 h at 30°C and was terminated by the addition of 200 μl of proteinase K buffer that contained 9.6 μg of proteinase K, 20 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 100 mM NaCl, and 1% sodium dodecyl sulfate. The RNA was extracted once with a mixture of phenol, chloroform, and isoamyl alcohol and then precipitated with ethanol. Afterwards, the RNA pellet was resuspended in 300 μl of deionized water containing 10 μg of glycogen as a carrier. After three successive ethanol precipitations, the pellet was resuspended in 30 μl of deionized formamide. One-third or one-fourth of the sample was then subjected to electrophoresis on a 6% polyacrylamide gel (acrylamide/bisacrylamide, 29:1) containing 8.3 M urea at a constant current of 55 mA. Gels were soaked in 10% glacial acetic acid, dried, and then exposed to X-ray film (Kodak X-Omat AR) at −70°C with an intensifying screen. Relative levels of transcript were quantitated by the densitometric scanning (Bio-Rad, model 620 densitometer) of appropriately exposed autoradiograms.

In some experiments, ^{32}P-5′-end-labeled fragments of 139 and 183 bp (derived from pUC19 with appropriate restriction endonucleases) were added to the reactions following the termination of transcription with proteinase K. This served

as a control to ensure that the quantity of RNA recovered from each of the reactions was the same.

Purification of the E1A protein. Plasmid pKHAO-T, encoding an authentic adenovirus type 2 E1A protein of 289 amino acids (2, 22), was propagated in a protease-deficient *Escherichia coli* strain, MC102. Growth conditions for the optimum expression of E1A in these cells, as well as its purification to near homogeneity from these cells, has been described elsewhere (2). Once obtained, the protein was stored in small aliquots at −80°C (not more than 2 months) and diluted immediately prior to the addition to the in vitro reactions.

Gel mobility shift. The binding reactions for gel mobility shift assays were essentially carried out as previously described (9, 18). Typically, the reactions (20 μl) contained 10 mM Tris hydrochloride (pH 7.5), 0.5 mM EDTA, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 50 mM KCl, 7 μg of either nuclear or whole-cell extract, 2.0 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia), and about 1 ng of 3′-end-labeled probe. The VA1 promoter-containing probe of 129 bp was prepared from pVA_1 by digestion with *Xba*1 and *Bst*EII and 3′-end labeling with the Klenow fragment of DNA polymerase I (sequence grade; Boehringer Mannheim). The binding reactions were incubated for 20 to 30 min at 30°C, and afterwards the samples were run on 4% nondenaturing polyacrylamide (acrylamide/bisacrylamide, 30:1) gels at 125 V for 3 to 4 h at room temperature in a TBE buffer system (6.25 mM Tris hydrochloride [pH 7.5], 6.25 mM boric acid, 0.25 mM EDTA). Gels were then dried and exposed to X-ray film (Kodak X-Omat AR). Prior to the loading of the samples, gels were preelectrophoresed for either 15 or 20 min.

The gel shift assays with purified E1A or with E1A and M-73 monoclonal antibody were carried out in an identical manner, except that nuclear or whole-cell extracts in binding buffer, with or without added E1A or E1A and antibody, were preincubated at 4°C for 30 min prior to the addition of poly(dI-dC)-poly(dI-dC) and radioactive probe.

RESULTS

A purified E1A protein stimulates VA1 transcription in vitro. We have previously shown that an E1A protein of 289 amino acids, synthesized and purified from *E. coli* (2, 22), is capable of transactivating transcription from promoters of class II genes in vitro (7, 37). To determine whether this function of E1A could be extended to class III genes, we examined the transcriptional response of the adenovirus VA1 RNA promoter in whole-cell extracts of uninfected HeLa cells supplemented with purified E1A. For these experiments, extracts were preincubated with and without E1A for 1 h at 4°C in the absence of VA1 DNA template and nucleoside triphosphates. Transcription was initiated with the simultaneous inclusion of these last two components, and the reactions were allowed to proceed for 60 min. The results of such an experiment are shown in Fig. 1A and B. In this case, transcription from the VA1 RNA promoter was increased dramatically (up to 4.5-fold; Fig. 1A, lane 3, and Fig. 1B) at optimal concentrations of E1A compared with that observed in extracts without E1A (lane 1). For reasons that we cannot explain, high amounts of E1A were not efficient in stimulating transcription from the VA1 RNA promoter. This result, however, was not entirely surprising, since we had observed earlier (37) a similar effect while exploring the activities of purified E1A on the adenovirus EIIaE promoter in vitro. Under these same assay conditions, transcription from the VA1 RNA promoter was severely inhibited by the addition of 200 μg of α-amanitin per ml (data not shown and reference 30), indicating that POLIII did indeed contribute to the transcription observed in the reactions. It should be noted that an E1A peptide of 49 amino acids, shown previously to be capable of transactivating class II genes in vitro (14), failed to stimulate the VA1 promoter when added to the same extract (data not shown). We also found this to be true regardless of whether the promoter being tested was of a class II or class III gene.

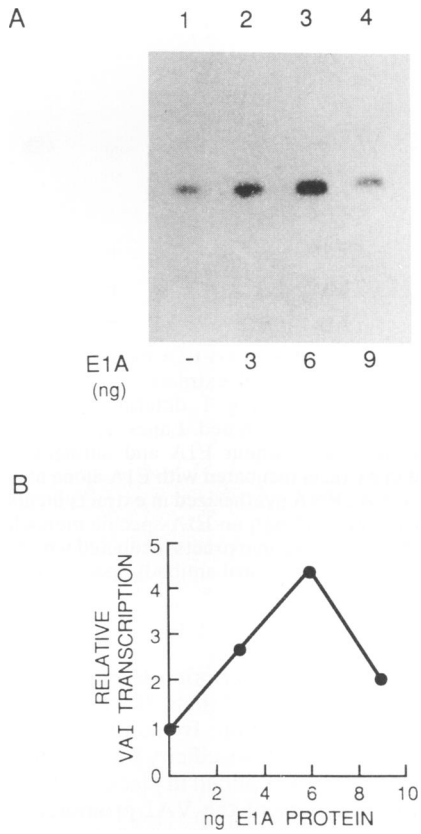

FIG. 1. Stimulation of transcription from the VA1 promoter by purified E1A in HeLa whole-cell extracts. The DNA template concentration for the analysis of the VA1 promoter was 50 ng. Extracts were incubated with or without E1A for 60 min at 4°C on a rotator before the addition of template DNA and nucleoside triphosphates. (A) VA1 RNA transcripts from in vitro transcription reactions. Lanes: 1, transcription from the promoter after the incubation of extract without the addition of E1A; 2 to 4, effect of incubating the extracts with increasing amounts of purified E1A (3, 6, and 9.0 ng) before adding template DNA. (B) Data obtained from the analysis of the autoradiogram in panel A, which is representative of four independent experiments. Autoradiographic exposures in the linear range of gels were scanned with a Bio-Rad densitometer, and the areas of peaks corresponding to the VA1 transcript were estimated. These values are expressed relative to the quantity of VA1 RNA synthesized from the template in the absence of E1A.

To determine whether E1A was involved in affecting the increased levels of transcription from the VA1 promoter, we examined its transactivating activity in the presence of an E1A-specific monoclonal antibody, M-2 (17). Prior to the start of transcription, whole-cell extracts with or without E1A or E1A with antibody were incubated under the same conditions as before. As shown in Fig. 2, the addition of E1A to extracts produced, in this case, a 2.5- to 3.0-fold increase

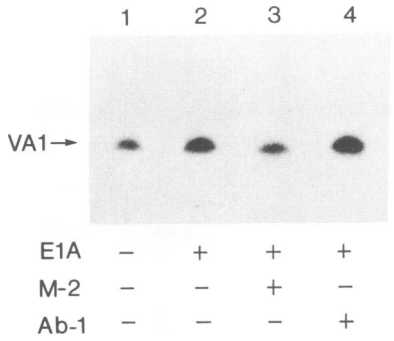

FIG. 2. Effect of antibody on E1A-mediated transcription of the VA1 promoter in whole-cell extracts. Assay conditions were as described in the legend to Fig. 1, differing only by the addition of appropriate antibody where noted. Lanes: 1, VA1 RNA synthesized in extracts incubated without E1A and antibody; 2, VA1 RNA synthesized in extracts incubated with E1A alone at a concentration of 5 ng/ml; 3, VA1 RNA synthesized in extracts incubated with both E1A (5 ng) and M-2 (12 ng), an E1A-specific monoclonal antibody; 4, VA1 RNA synthesized in extracts incubated with both E1A (5 ng) and Ab-1 (12 ng), a monoclonal antibody specific for the adenovirus E1B protein.

in the level of VA1 transcription (compare lanes 2 and 1), which was largely reversed (lane 3) when the M-2 antibody was included in the reaction. By contrast, when a monoclonal antibody (Ab-1) with specificity for the adenovirus type 5 E1B 21-kDa protein was added in place of M-2, the enhanced transcriptional activity of the VA1 promoter was sustained (lane 4). As was seen previously (37), no change in the efficiency of the VA1 transcription was observed when either of the antibodies was added to extracts without E1A, indicating that they themselves had no effect on the transcriptional events which were observed (data not shown). Thus, we conclude that our purified E1A is directly responsible for activating the VA1 RNA gene in vitro. In addition, neither cellular protein synthesis nor other viral proteins are required for this stimulation.

E1A restores VA1 transcription after depletion of active TFIIIC. It has been recently shown that TFIIIC can apparently assume two distinct forms, one of which becomes modified during adenovirus infection (18). Presumably, the form that is altered in the infected cell is initially without transcriptional activity and comparatively slow in its rate of association with the VA1 promoter (18). In light of these findings, we wanted to determine the effect of purified E1A on the transcriptional capacity of whole-cell extracts selectively depleted of active TFIIIC. One of the key components of this experiment is an oligonucleotide which contains the B-block region of the VA1 gene (8), a site previously identified as being the primary recognition site for TFIIIC binding (10, 18). We initiated experiments by titrating a fixed amount of whole-cell extract to determine the quantity of TFIIIC oligonucleotide required to block (almost completely) transcription from a subsequently added VA1 template. As shown in Fig. 3A, a 350-fold molar excess of TFIIIC oligonucleotide was sufficient to inhibit transcription from the VA1 promoter by about 4.0- to 5.0-fold (compare lanes 2 and 4). The specificity of this response was confirmed by the fact that a 350-fold molar excess of an AP3 control

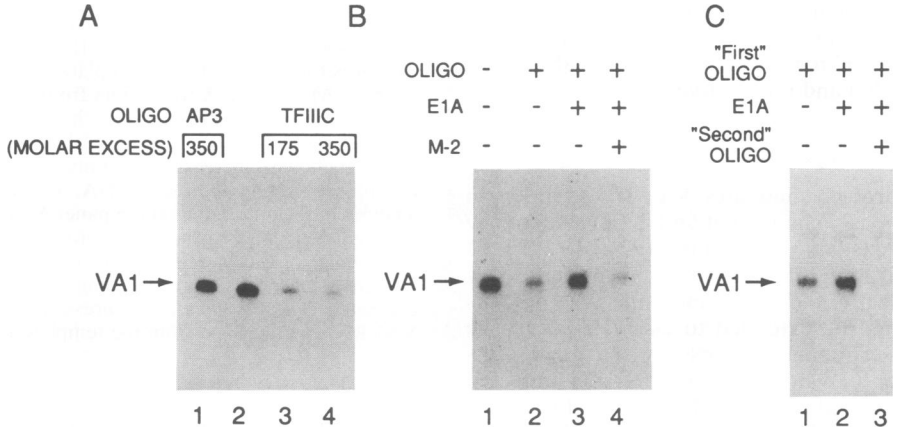

FIG. 3. Restoration of VA1 RNA synthesis by E1A in extracts challenged with TFIIIC oligonucleotide. (A) Transcription of the VA1 promoter in the presence of TFIIIC oligonucleotide. Transcription reactions containing whole-cell extracts were first incubated with (lanes 3 and 4) or without (lane 2) TFIIIC oligonucleotide for 1 h at 4°C prior to the addition of VA1 promoter (50 ng) and nucleoside triphosphates. The molar ratio of the TFIIIC oligonucleotide to the VA1 DNA template is 175 (lane 3) or 350 (lane 4). As a control, an identical transcriptional reaction (lane 1) containing an oligonucleotide of similar size (AP3) but with a random sequence (350-fold molar excess) was carried out in parallel. (B) Effect of E1A on transcription from the VA1 promoter after the addition of TFIIIC oligonucleotide. The conditions for VA1 transcription were essentially the same as described in the legend to panel A, except that the extracts with or without TFIIIC oligonucleotide were incubated an additional hour at 4°C in the absence or presence of added E1A or added E1A and M-2 antibody. Lanes: 1, RNA synthesis from the VA1 promoter after the incubation of extract without the addition of TFIIIC oligonucleotide, E1A, or E1A and M-2; 2, effect of incubating the extract with TFIIIC oligonucleotide (175-fold molar excess) before adding VA1 template; 3, effect of incubating the extract with TFIIIC oligonucleotide (175-fold molar excess) and then with E1A (10 ng) for 30 min before adding the VA1 template; 4, effect of incubating the extract with TFIIIC oligonucleotide (175-fold molar excess) and then with E1A (10 ng) and M-2 monoclonal antibody (13.2 ng) for 30 min prior to the addition of VA1 template. (C) Effect of E1A on VA1 transcription after a second addition of TFIIIC oligonucleotide. At time zero, three parallel reactions containing whole-cell extracts with TFIIIC oligonucleotide (lanes 1 to 3) were first incubated for 1 h at 4°C, and two of these were then furnished with 9 ng of E1A (lanes 2 to 3). All extracts were incubated an additional 30 min at 4°C. Afterwards, one of the extracts with E1A received a second round of TFIIIC oligonucleotide (lane 3). Transcription was initiated after 1 h at 4°C with the addition of VA1 template (50 ng) and nucleoside triphosphates. The molar ratios of TFIIIC oligonucleotide to VA1 template are 175 (lanes 1 and 2) and 535 (lane 3). The upper and lower bands serve as controls and reflect the accuracy of the amount of RNA recovered from each reaction.

oligonucleotide had little or no effect upon the transcription of VA1 (lane 1). These results stongly suggest that most of the transcriptionally active TFIIIC molecules have been sequestered on the TFIIIC oligonucleotide, thereby preempting VA1 transcription; the majority of those remaining unbound are presumably the TFIIIC molecules that are transcriptionally inactive and less stable in their association with the VA1 promoter (5, 18). If E1A does play a role in affecting inactive TFIIIC, then the addition of E1A to extracts previously challenged with TFIIIC oligonucleotide should rescue VA1 transcription, providing there is a sufficient amount of inactive TFIIIC for E1A to affect. That this indeed was the case is shown in Fig. 3B. The addition of E1A to extracts after TFIIIC oligonucleotide competition resulted in a restoration of VA1 transcription, with levels of synthesized RNA increasing about 2.5- to 3.0-fold (lane 3) compared with that observed in extracts challenged with TFIIIC oligonucleotide but containing no E1A (lane 2). However, when E1A and M-2 monoclonal antibody were incubated together in extracts also challenged with TFIIIC oligonucleotide, the transcriptional activity of the VA1 promoter was nearly the same as that observed in the same extract without E1A and antibody (Fig. 3B; compare lanes 2 and 4). Moreover, when extracts previously exposed to TFIIIC oligonucleotide and then to added E1A were challenged a second time with twice the amount of TFIIIC oligonucleotide (Fig. 3C), the activity of the VA1 promoter was dramatically reduced (lane 3). The results of this part of the experiment eliminate the possibility that E1A itself is acting directly at the VA1 promoter or that transactivation is occurring through a mechanism independent of TFIIIC. Taken together, these results indicate that, under these conditions, added E1A is able to directly rescue VA1 transcription and that this response may be predicated on the availability of inactive TFIIIC.

Purified E1A alters the interaction of TFIIIC with its recognition site. To determine whether the observed increase in transcriptional activity of extracts supplemented with E1A correlated with a change in the binding activity of TFIIIC, we performed gel shift analysis with the use of both nuclear and whole-cell extracts. The assays shown in Fig. 4 employed a labeled VA1 gene probe that extended from −30 to +99, as well as the inclusion of poly(dI-dC)-poly(dI-dC). Consistent with previous results (5, 18), the probe formed three complexes (A, B, and C) when it was incubated with nuclear proteins of HeLa cells (Fig. 4A, lane 1). The specificities of these three VA1 promoter complexes were confirmed when their formations became inhibited by the addition of unlabeled VA1 gene (lanes 2 and 3). By several criteria, however, we determined that only one of these complexes involved a TFIIIC interaction. The formation of complex B was prevented by competition with an unlabeled 51-bp oligonucleotide containing the TFIIIC sequence (lanes 6 and 7) but not with a nonspecific oligonucleotide of the same size (lanes 4 and 5). Furthermore, formation of complex B was not efficiently inhibited by the addition of an unlabeled ligated oligonucleotide (29 bp) which contained point mutations in the TFIIIC sequence and which was approximately the same length as the VA1 probe (Fig. 4B; compare lanes 1 and 2 to 4).

The formations of all three complexes were compared in reactions in which the VA1 probe was incubated in nuclear extracts with or without the addition of E1A (Fig. 5A). As expected, complexes A, B, and C were clearly visible when nuclear proteins with the probe and without E1A were analyzed (lane 3), and no complexes could be detected when

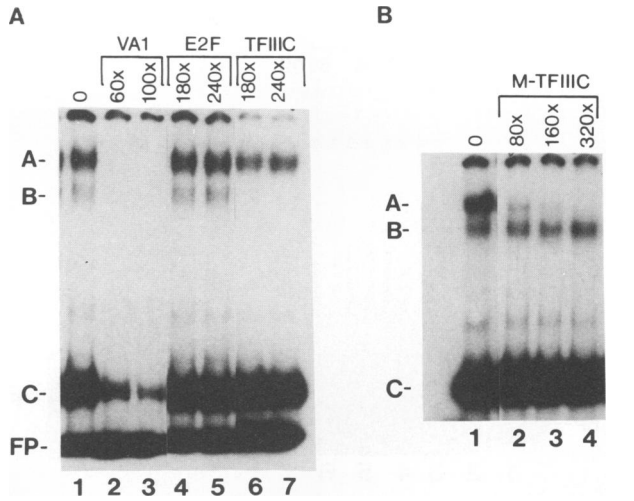

FIG. 4. Gel shift analysis for DNA-binding activity of TFIIIC. (A) The probe, a 129-bp end-labeled VA1 gene fragment containing the TFIIIC binding site, was incubated in a series of standard binding reactions containing nuclear extract (7 μg) and, where indicated, an unlabeled VA1 gene fragment (60- and 100-fold molar excess), a 51-bp oligonucleotide containing only the TFIIIC binding site (180- and 240-fold molar excess), or a nonspecific oligonucleotide of 48 bp (180- and 240-fold molar excess). After 30 min, the samples were directly loaded onto gels and electrophoresed. Migration of an upper (A) and a lower (B) TFIIIC-containing complex is indicated as well as an uncharacterized specific complex (C) which has been previously described (5, 18). (B) Reaction conditions are the same as in panel A, except that, where indicated, an unlabeled ligated 29-bp oligonucleotide of about 132 bp containing a mutated TFIIIC binding site (M-TFIIIC) was added as a competitor (see Materials and Methods).

E1A was incubated only with the probe (lane 2). By contrast, incubation of the VA1 gene probe with nuclear proteins containing incremental amounts of E1A resulted in a dramatic and highly reproducible decrease in the formation of the lower complex and in what appears to be, as judged by densitometry, a slight increase in the formation of the upper complex (lanes 4 to 6). Moreover, no new complexes in addition to the upper and lower complexes were ever observed under this condition. We also examined the effect E1A might have on the pattern of complexes induced by the probe when incubated in the same set of whole-cell extracts used for our in vitro transcription reactions shown herein. As expected, there was no significant difference in the pattern of complexes generated by the VA1 gene probe and, again, only complex B was efficiently competed for by the unlabeled TFIIIC oligonucleotide. Not surprisingly, upon the addition of E1A to the same extract, complex B was also substantially inhibited compared with what was observed in extracts containing no E1A (data not shown).

Figure 5B illustrates the effect that purified E1A has on the formation and stability of complex B in the presence of an E1A-specific monoclonal antibody. Compared with the control (lane 3), neither the A nor the B complex becomes significantly altered when E1A and antibody are added concomitantly to nuclear extract before the binding reaction (lane 1). In contrast, when the antibody used in this study was introduced into nuclear extracts after E1A had been added for a period of 15 min (lane 2), the formation of the B complex was reproducibly decreased after the addition of the probe. As anticipated, the addition of antibody or of E1A

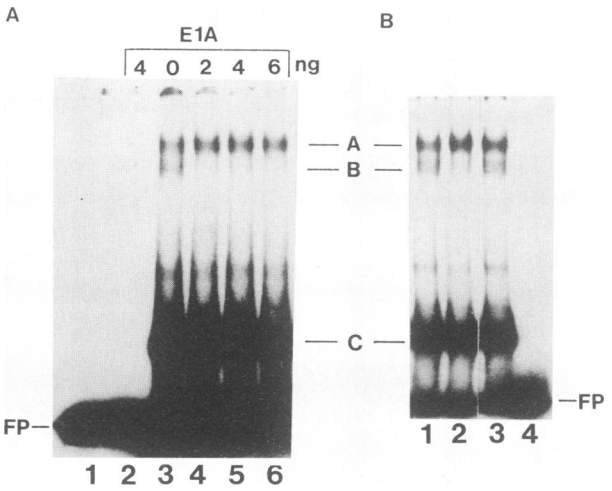

FIG. 5. Addition of E1A to nuclear extracts alters a TFIIIC-VA1 promoter DNA complex. (A) Effect of E1A in nuclear extracts. Binding reactions containing nuclear extract (7 μg) in buffer were incubated with or without E1A for 10 min at room temperature before the addition of the VA1 probe. Following a 30-min incubation at 30°C, the samples were electrophoresed as usual. Lanes: 3, formation of complexes without the addition of E1A; 4 to 6, effect on the formation of complexes after incubating nuclear extracts with increasing amounts of purified E1A (2.0, 4.0, and 6.0 ng); 1, free DNA probe (unbound); 2, free DNA probe without nuclear extract but with the addition of E1A. (B) The activities of E1A on the formation of the TFIIIC-VA1 complex are neutralized by monoclonal antibody. Prior to the addition of VA1 probe, binding reactions containing nuclear extract (7 μg) were first incubated for 30 min at 4°C without E1A (lane 3), with E1A added together with an E1A-specific monoclonal antibody (lane 1), and with the same antibody added 15 min after the addition of E1A (lane 2). Lane 4 represents VA1 probe without nuclear extract.

and antibody had no effect on the migration of free probe in the absence of nuclear proteins (data not shown). Taken together, these results clearly indicate that E1A itself is capable of altering, either directly or indirectly, the binding activity of the VA1 B-block factor, TFIIIC.

DISCUSSION

A purified E1A 289-amino-acid protein made in *E. coli* is capable of transactivating the adenovirus VA1 RNA gene in a mammalian cell-free system. When this protein was added to uninfected HeLa whole-cell extracts, the level of transcription observed from the VA1 promoter increased on an average about 4.5-fold (Fig. 1). The degree of transactivation occurring in these experiments depended primarily on the concentration of E1A molecules. That E1A indeed participates in enhancing transcription from the VA1 promoter was basically established in two ways. First, a purified 243-amino-acid E1A protein lacking the unique 46-amino-acid region was not at all efficient in activating transcription from the VA1 promoter when it was added to whole-cell extracts (17a). Second, an E1A-specific monoclonal antibody effectively reversed the stimulation of the VA1 promoter when it was added to extracts at the same time as E1A (Fig. 2). By contrast, when a monoclonal antibody specific for a protein other than E1A was added, the up-regulation of the VA1 promoter by E1A was sustained, thereby ruling out the possibility that the antibodies themselves were affecting the transcriptional machinery. These observations indicate that the E1A protein directly participates in stimulating transcription from the VA1 promoter. They also indicate that there is no requirement for the synthesis of other cellular factors or other viral proteins. Although these conclusions represent an important step in defining some of the mechanisms by which E1A transactivates class III genes, they are not unique, since similar conclusions were reached after studying the activities of purified E1A on class II genes in vitro (37). This scenario in itself may provide further testament to the mechanistic generality of transcriptional activation by E1A.

It can be inferred from our discussions given above that the E1A protein plays a direct role in enhancing transcription from the VA1 promoter. It has been suggested (40) that E1A transactivates the VA1 gene by effectively increasing the concentration of TFIIIC, the rate-limiting factor of VA1 transcription (24). However, the results presented herein show that this is not the case; instead, they argue for a more direct involvement between these two proteins. This is a reasonable notion in view of recent reports indicating that TFIIIC can be of two distinct forms, one of which appears to have greater transcriptional activity in vitro and a tendency to associate more rapidly (~1 min) with its cognate binding site (the B-block region) on the VA1 promoter. The other form of TFIIIC does not appear to be committed to transcriptional activity, and although the B-block element provides a binding site for it as well, maximal association usually occurs after 40 min (18). Our finding here that the levels of transcription from the VA1 promoter decrease appreciably in HeLa whole-cell extracts initially incubated with a 175- or 350-fold molar excess of B-block oligonucleotide and not with an equivalent amount of a nonspecific oligonucleotide (Fig. 3A) is somewhat consistent with these earlier reports. Conceivably, this change in VA1 transcription could be a result of the unavailability of active TFIIIC molecules depleted by virtue of its rapid association with the B-block oligonucleotide. If what remains is mostly TFIIIC molecules that are transcriptionally inactive (18), then this may be one reason why purified E1A is able to specifically restore the levels of VA1 transcription when added to whole-cell extracts initially incubated with the B-block oligonucleotide (Fig. 3B). In effect, the E1A protein could be stimulating VA1 transcription by operating through this particular form of TFIIIC. Not to be excluded, however, is the possibility that E1A may be affecting a set number of transcriptional complexes by making them more active in multiple rounds of transcription. This, nevertheless, defines a mechanism in contrast to that which has been recently reported (23), whereby E1A is instrumental in increasing the number of preinitiation complexes active in VA1 transcription. We also observed that restoration of VA1 transcription by E1A could be thwarted if the extract was challenged with a second round of B-block oligonucleotide prior to the addition of the DNA template (Fig. 3C). The implication of this last observation is twofold. First, E1A does not appear to be acting exclusively by itself at the VA1 promoter; second, it rules out the possibility of other mechanistic pathways being important to E1A's transactivating activities.

Exactly how does the E1A protein stimulate VA1 transcription through the TFIIIC factor? One possibility is that E1A either directly or indirectly alters TFIIIC through some form of modification such that it can no longer participate in the formation of a transcriptionally inactive complex. Consistent with this speculation is the recent finding that E1A expression in infected cells effects an increase in the con-

centration of complexes formed between the VA1 promoter and a transcriptionally active TFIIIC. Under these circumstances, very little, if any, of a complex consisting of inactive TFIIIC was detected, even though this particular complex had been shown to occur in uninfected cells (5, 18). The interconversion of these two complexes, whereby one increases in amount presumably at the expense of the other, is believed to be influenced by phosphorylation, and it has been suggested that E1A may be playing a role in this activity (18). Our next set of results, which reveals the effects of exogenous E1A on TFIIIC while interacting at the VA1 promoter, appears to support these observations. Most significant in this regard is the finding by gel shift analysis of a TFIIIC-specific complex (Fig. 4A and B) that becomes consistently reduced in quantity when purified E1A is preincubated with either HeLa nuclear or whole-cell extracts (Fig. 5A) (data not shown). This complex, whose formation is effectively blocked by E1A, is most likely equivalent to the one defined earlier (5, 18) which is generated by inactive TFIIIC. At this time, we have no clear evidence that E1A participates in the binding of the active form of TFIIIC to its site on the promoter or, for that matter, that a complex of this nature, with lower mobility, has been formed (5, 18). However, the possibility of it being produced and increased in abundance by the action of E1A cannot be eliminated, since under the present analysis its appearance would most likely be obscured by complex A (Fig. 4A, lane 1), whose composition does not include TFIIIC. In fact, the quantity of complex A does appear to be greater than that in extracts lacking E1A (Fig. 5A), although to a very small degree as judged by densitometry. To properly conclude, however, that this phenomenon is truly occurring, it will be necessary to examine the effect of purified E1A in fractionated extracts; experiments of this nature are now under way in our laboratory. The alternative, that E1A binds directly to TFIIIC in a manner reminiscent of the protein-protein interaction of herpes simplex virus VP16 and the host cell transcription factor OTF-1 (12), seems unlikely. For the most part, no novel TFIIIC-promoter complexes exhibiting reduced electrophoretic mobility were observed after E1A was preincubated in nuclear or whole-cell extracts (Fig. 5A and B and data not shown). This implies that E1A does not interact directly with the VA1 promoter through physical association with assembled transcriptional factors on the promoter, as has been previously proposed for the promoters of class II genes (28, 29, 31). Taken together, our observations can be best explained by assuming that E1A mediates a qualitative change in TFIIIC, which in turn affects its DNA-binding properties and, possibly, transcriptional activities.

It seems very likely that under the assay conditions used here, E1A is directly responsible for somehow altering the physical nature of TFIIIC, since its effect on the TFIIIC-containing complex can be readily neutralized when E1A-specific antibody is added to nuclear extracts together with E1A (Fig. 5B). Further, whatever mechanism E1A may be using to transcriptionally activate TFIIIC, it appears to be quite rapid and permanent, since the use of antibody appears to have no effect on E1A if it is added to nuclear extracts at a much later time.

Since at present our purified E1A has not been shown to have any kinase activity (17a), we believe that it serves more as a passive communicator in the pathway of POLIII transcriptional regulation. Whether this results in the activation of some type of cellular kinase or the inhibition of a protein phosphatase is a question amenable to experimental verification.

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