Activation of Adenovirus Promoters by the Adenovirus E1A Protein in Cell-Free Extracts

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The primary product of the adenovirus E1A gene is a protein that is sufficient for controlling host-cell proliferation and immortalizing primary rodent cells. The mechanism by which the protein induces these cellular effects is poorly understood, but might be linked to its ability to regulate RNA transcription from a number of viral and cellular genes. The mechanism of E1A's transcriptional-activation (trans-activation) was studied here by monitoring the protein's effect on specific adenovirus promoters in two types of transcriptional systems in vitro. One of these systems consisted of extracts from transformed cells constitutively expressing E1A, and the other consisted of extracts of HeLa cells supplemented with a plasmid-encoded E1A protein purified from *Escherichia coli*. The results show that the E1A protein specifically stimulates transcription from adenovirus promoters; thus, the induction of cellular transcription factors is not necessary to explain the stimulation of transcription by E1A.

THE ADENOVIRUS (Ad) E1A PROtein of 289 amino acids has the ability to either activate or repress RNA transcription from promoters of both viral and nonviral origin (*1*). Because of this paradoxical behavior, the protein is viewed as an important factor in the study of transcriptional control. The first indication of E1A's regulatory nature came from the analysis of E1A mutants. These studies revealed that E1A was required for efficient expression of the other adenoviral genes (*2, 3*). Later experiments established that the control of this expression was at the level of transcription and the E1A specifically operated in trans on the various viral promoters (*1*). Since these studies, a number of nonviral genes (β-globin and preproinsulin) on transfected plasmids have also been shown to respond to E1A (*4, 5*). Although the transcription of these genes is stimulated by E1A, curiously, their endogenous counterparts are not activated (*5, 6*). In fact, other experiments have suggested that transcription of most endogenous genes is not induced by E1A (*7*); however, it does appear that E1A can stimulate transcription from promoters of at least two endogenous cellular genes, namely, β-tubulin and *hsp70* (*1*). It has also been found that E1A can effectively repress the activity of certain transcriptional enhancers (cis-acting regulatory sequences). The enhancers negatively regulated by E1A include not only those active in a wide variety of cells (SV40 and polyoma virus enhancers) (*8*), but also cell-specific enhancers, such as the mouse immunoglobulin heavy and light chain enhancers, active only in lymphoid β cells (*9*). By contrast, the same enhancer of the mouse immunoglobulin heavy chain gene is activated by E1A in nonlymphoid cells (*10*). This rather surprising result once again confirms the diversity of E1A's trans-acting ability.

How the E1A protein is able to accomplish these diverse forms of transcriptional control is not clear, but several lines of evidence suggest that there may be other cellular factors involved in E1A's regulatory pathway. First, it has been reported that E1A by itself is not a DNA-binding transcription factor (*11*). Instead, there are strong indications that E1A associates indirectly with DNA, presumably mediated by another factor (or factors) (*12*). Second, E1A does not seem to require (at least in most cases) a unique set of sequences to affect transcriptional regulation (*1*). Finally, transcriptional factors of cellular origin that have binding affinities to upstream sequences of the Ad E1lAE (*13*) and to major late (ML) (*14*) have now been detected. An important question from all these studies is whether the activity of E1A either physically modifies or increases the actual concentration of cellular transcription factors as has been previously proposed (*13*). Therefore, in the present study of gene activation by E1A, we added purified E1A (Fig. 1) expressed in *Escherichia coli* by our pKHAO-T plasmid (*15*) to extracts of HeLa cells programmed with either the Ad E1lAE or ML promoters. We found addition of E1A is sufficient to up-regulate transcription from both of these promoters, and extracts derived from human 293 cells (which constitutively produce the E1A protein) (*1*) can also effectively stimulate transcription from the same Ad promoters. Although it remains possible that E1A also leads to an increase in the supply of cellular transcription factors in vivo, our results indicate that it is not necessary to invoke this mechanism to explain a significant fraction of the stimulation of transcription by E1A.

Previous studies indicated that transcription from both the Ad E1lAE and ML promoters can be activated in human 293 cells (*16, 17*). It was of interest then to determine whether extracts of these cells could also stimulate the same Ad promoters in vitro. First we contructed Ad promoter–containing recombinants (*18*) that would be easily assayed in an in vitro transcription system (*19*). The first of these (pE1lD) contains the E1lAE upstream promoter elements; the second (pMLR) is a derivative of pH3B*R (*20*) and contains the boundary of the ML promoter sequences required for optimal transcription in vitro (*20, 21*). Transcription of these linearized recombinants was next analyzed in vitro by run-off transcription with whole-cell extracts prepared from either HeLa (human cells containing no adenoviral sequences) or 293 cells. The ML promoter, which generated a run-off transcript of length 3500 nucleotides, was slightly more active (≈2.0-fold) in extracts of 293 cells (Fig. 2A, lane 2) when compared to HeLa cell extracts (Fig. 2A, lane 1). Furthermore, the ratio of activity of this promoter, derived from the levels of transcription occurring in both extracts, did not

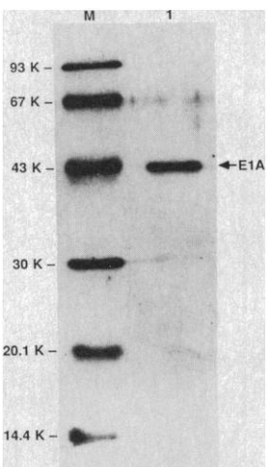

Fig. 1. Analysis of the E1A protein after purification from extracts of *E. coli*. Proteins were resolved on an SDS-polyacrylamide gel and visualized by staining with Coomassie brilliant blue R-250. Lane 1 contains 5 μg of purified E1A protein. Lane M shows the position and molecular size (in kilodaltons) of markers (Pharmacia). A modified version of the expression plasmid pKHAO, previously shown to encode an authentic adenovirus-2 E1A protein of 289 amino acids (*15*), was propagated in a protease-deficient *E. coli* strain, SG4140. Growth conditions for optimum expression of the E1A protein in these bacterial cells as well as a detailed description of its purification to near homogeneity will be given elsewhere (*33*).

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vary significantly when a range of ML-promoter concentrations was used (*22*). Although both extracts were fully capable of supporting RNA synthesis from the ML promoters, a small additional stimulatory activity was present in the 293 extracts, possibly because of the endogenous E1A protein.

In agreement with others (*23*), we found that the E11AE promoter at the optimum concentration (*24*) was poorly recognized in the HeLa cell extracts, and the promoter consistently yielded a low level of run-off transcripts (Fig. 2B, lane 1) that were barely visible. However, when the E11AE promoter was added to 293 cell extracts, the level of run-off transcripts was about 6.0-fold higher (Fig. 2B, lane 2) than those observed with the HeLa cell extract, under the same optimized conditions. Since transcription from the E11AE promoter in Ad-infected cells appears to be dependent at least in part on the action of the E1A protein, we incubated the extracts of 293 cells with a monoclonal antibody to E1A (M-2) (*25*) to determine whether the endogenous E1A was in fact responsible for the up-regulation of this promoter. As shown in Fig. 2B, lane 4, the M-2 antibody at its optimal inhibitory concentration did perturb to some extent the transcriptional capacity of the 293 cell extracts, and the level of run-off transcripts derived from the E11AE promoter was about 3.0-fold lower when compared to that of the same extract without antibody (lane 2). In contrast, there appeared to be no change in the level of activity of the E11AE promoter in HeLa cell extracts after incubation with the same antibody (lane 3); therefore, it can be concluded that the M-2 antibody has no apparent effect on the action of other transcriptional factors. Collectively, these results suggest that the endogenous E1A protein does stimulate transcription from the E11AE promoter, although a requirement for other transcriptional factors cannot be excluded.

Our comparisons of RNA synthesis in extracts of HeLa and 293 cells suggest that the E1A protein plays an essential role in the overall activity of the E11AE promoter, although a much lesser one for the ML promoter. Therefore, to provide more direct evidence for E1A's effect on these promoters, we again used an optimized in vitro transcriptional system (*24*), but in this case the HeLa cell extracts were incubated with a molar excess of our purified E1A protein (Fig. 1) prior to the addition of template DNA. The basal level of transcription from the E11AE promoter (Fig. 3A, lanes 2 to 5) increased dramatically (up to 7.0-fold) with increasing amounts of E1A compared to that observed in control extracts without E1A (lane 1). Elevated levels of transcription from the E11AE promoter began to plateau (compare lanes 4 and 5) beyond a 28-fold excess of E1A, which suggests that the assay system had reached a point of saturation. These results indicate that the purified E1A protein is capable of functioning in an in vitro transcription system and is responsible for the up-regulation in transcription observed from the E11AE promoter.

In the second part of the transcriptional analysis (Fig. 3B), stimulation of the ML promoter in HeLa cell extracts after incubation with the E1A protein (lane 2) was also apparent when compared to control extracts without E1A (lane 1), but, as observed previously for the 293 cell extracts (Fig. 2A), the increase in activity of this promoter was again only 1.5- to 2.0-fold. Moreover, stimulation was maximal even at the lowest E1A concentration tested in the HeLa cell extracts (*22*) and thus accentuates the importance of other transcriptional factors that have been recently identified for the activity of the ML promoter in vitro (*14*).

The results in Fig. 2B indicated that M-2 antibody to E1A added to extracts of 293 cells can partly inhibit the stimulatory activity of E1A on the E11AE promoter (lane 4). Since this inhibitory effect on transcription is probably a consequence of the antibody's specificity for 293 cells, endogenous E1A, we used this antibody to prove more rigorously the role of purified E1A in the stimulation of the E11AE promoter. As shown above, when HeLa cell extracts were supplemented with incremental amounts of purified E1A, the transcriptional activity of the E11AE promoter (Fig. 4A, lanes 2 and 3) was significantly higher (in this case up to 4.0-fold) when compared with the activity of the control extract without addition of E1A (lane 1). However, when a reaction mixture in parallel, again containing HeLa cell extract, was incubated with an equivalent amount of E1A and M-2 monoclonal antibody, the transcriptional activity of the promoter was sharply reduced (lane 4) and almost comparable with that observed in the extract containing no E1A (lane 1). As before (Fig. 2B), incubation of HeLa cell extract with M-2 antibody alone did not affect the observed transcriptional activity of the E11AE promoter (*22*). Only one-third of the amount of M-2 antibody was needed to eliminate E1A-stimulated RNA synthesis from the E11AE promoter compared to that which was required when the promoter was in the 293 cell extract (Fig. 2, lane 4). The M-2 antibody itself might possess an uncharacteristic activity and not be truly specific in neutralizing the effect of E1A; to test this, we performed an additional experiment similar to that described above, but in this case, a monoclonal antibody with specificity for the Ad5 E1B 21-kD protein was used. As shown in Fig. 4B, the increased level (7.0-fold) of transcripts derived from the E11AE promoter in HeLa cell extracts incubated with E1A alone (lane 2) or in the presence of monoclonal antibody to E1B (lane 3) was similar to the level of transcripts of the control extract without addition of E1A (lane 1).

Our results show that the Ad E1A protein stimulates RNA transcription from the E11AE promoter and that the induction of cellular factors, if necessary, is not the only mechanism stimulating activation by E1A.

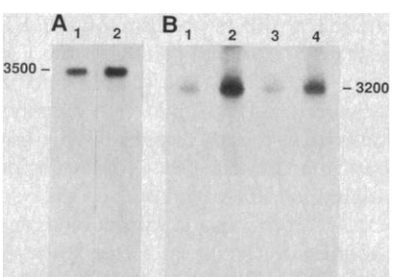

Fig. 2. Stimulation of transcription from the ML and E11AE promoters in extracts of 293 cells. The in vitro transcription assay and the preparation of whole-cell extracts have been described (*34*). Reaction conditions were optimized (*24*) for synthesis of ML- and E11AE-specific RNA in HeLa cell extracts. The transcription mixtures (25 μl) contained: 90 μg of whole-cell extract (as determined by the Bio-Rad protein assay); 0.4 and 0.6 μg of template DNA digested to completion with either Pst I (ML promoter) or Xho I (E11AE promoter), respectively; and other components as described (*34*). All the reaction mixtures were incubated for 60 minutes at 4°C on a rotator before the addition of template DNA and nucleoside triphosphates. After addition of template, the in vitro synthesis was continued for 60 minutes at 30°C and then terminated by the addition of 300 μl of proteinase K buffer that contained 0.15 mg per milliliter of proteinase K, 20 m*M* tris HCl (*p*H 8.0), 10 m*M* EDTA, 100 m*M* NaCl, and 1% SDS. Afterwards, the RNA was extracted once with a mixture of phenol, chloroform, and isoamyl alcohol, and twice with chloroform and isoamyl alcohol. The RNA was precipitated with ethanol, glyoxalated, and fractionated (one-fourth of the sample) through 1.4% agarose gels as described (*34*). The gel was dried, and then exposed to x-ray film for 15 to 96 hours. Relative levels of transcript were quantitated by densitometric scanning of appropriately exposed autoradiograms. M-2 monoclonal antibodies in mouse ascites fluid were isolated by affinity chromatography on protein A–Sepharose as described by the manufacturer (Pharmacia). (**A**) Run-off RNA synthesized from the ML promoter in extracts of HeLa (lane 1) and 293 (lane 2) cells. (**B**) Run-off RNA synthesized from the E11AE promoter in extracts of HeLa (lanes 1 and 3) and 293 (lanes 2 and 4) cells. The whole-cell extracts in lanes 3 and 4 were incubated with 30 ng of E1A antibody (M-2) before addition of DNA template.

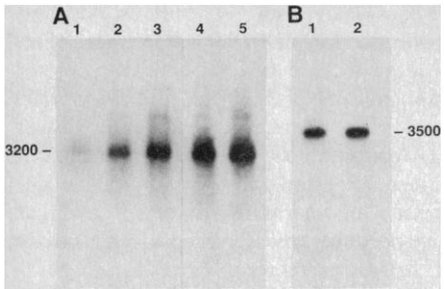

Fig. 3. Stimulation of transcription from the E11AE and ML promoter by purified E1A in HeLa cell extracts. Preparation of whole-cell extracts and assay conditions were as described in Fig. 2. DNA template concentrations for the analysis of the E11Ae and ML promoters were 0.6 and 0.4 μg, respectively. HeLa cell extracts at concentrations of 90 and 135 μg of protein (Bio-Rad protein assay) were incubated with or without E1A for 60 minutes at 4°C before the addition of DNA from ML and E11AE promoter, respectively. (**A**) In vitro transcription run-off analysis of the E11AE promoter: lane 1, transcription from the promoter after incubation of HeLa cell extracts without addition of E1A; lanes 2 to 5, effect of incubating the HeLa cell extracts with increasing amounts of purified E1A (3.3, 5.0, 9.0, and 12.5 ng) before adding template DNA. (**B**) In vitro transcription run-off analysis of the ML promoters: incubation of HeLa cell extracts without E1A (lane 1) and with addition of 3.3 ng of E1A (lane 2) before the introduction of template DNA.

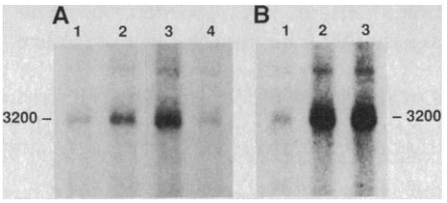

Fig. 4. Inhibition by antibody of the E1A-stimulating activity of the E11AE promoter in HeLa cell extracts. Preparation of whole-cell extracts and assay conditions were as described in Figs. 2 and 3. HeLa cell extract and DNA template concentrations for the analysis of the E11AE promoter were 135 and 6.0 μg, respectively. The Ab-1 monoclonal antibody specific for the Ad5 E1B 21-kD protein was acquired from Oncogene Science, Inc. (**A**) Effect of monoclonal antibody to E1A (M-2) on transcription: lane 1, RNA synthesized in extracts incubated without addition of E1A and M-2; lanes 2 and 3, RNA synthesized in extracts incubated with E1A alone at concentrations of 1.5 and 4.5 ng, respectively; lane 4, RNA synthesized in extracts incubated with both E1A (4.5 ng) and M-2 monoclonal antibody (4.5 ng). (**B**) Effect of monoclonal antibody to E1B (Ab-1) on transcription: lane 1, RNA synthesized in extracts incubated without E1A and Ab-1; lane 2, RNA synthesized in extracts incubated only with E1A (8.0 ng); lane 3, RNA synthesized in extracts incubated with both E1A (8.0 ng) and Ab-1 (62.5 ng).

We propose the E1A functions in a similar fashion to the immediate early protein of pseudorabies virus, which appears to stimulate transcription by altering the actions of a preexisting cellular factor (or factors), either directly or indirectly (*26*). Recent studies of the E11AE and ML promoters have led to the identification of putative regulatory factors of cellular origin. For example, a protein that specifically binds to a control sequence (positions −66 to −82) upstream from the cap site of the E11AE promoter has been detected at comparable levels in nuclear extracts from uninfected or adenovirus-infected HeLa cells (*27*). Other reports have also suggested that a protein factor (or factors) present in much reduced amounts in uninfected HeLa cells binds to upstream sequences between −33 and −74 of E11AE (*13*); it is not clear, however, whether this factor is similar to or different from the one mentioned above. There are at least two sequence-specific cellular factors associated with the ML promoter, one of which interacts with an upstream promoter element at −63 to −52 and the other which establishes contact with the TATA box region (*14, 28*). Of significance is that the upstream sequences identified by interaction with the cellular factors specific for either E11AE or ML are the only sequences required for E1A-mediated induction, both in vivo and in vitro (*1, 17, 27*).

It has been recently proposed that E1A might act by increasing the concentration of a limited number of transcription factors (*13, 29*). Our work, however, is consistent with the idea that E1A may operate by further activating preexisting cellular factors that bind to key transcriptional elements within certain promoters, either by associating with such factors (protein-protein contact) or by chemically modifying them. Alternatively, the mechanism of the E1A protein may be similar to that of the yeast activator protein GCN4. This protein has recently been shown to have two functional regions that are both responsible for transcriptional activation. One of these regions is required for DNA binding, and the other appears to act as a ligand for the binding of yet another transcription factor (*30*). Although previous studies have failed to show DNA-binding activity for E1A (*31*), our earlier studies with purified E1A were not inconsistent with the possibility that E1A did bind to DNA (*12*). In fact, we have recently demonstrated that our genetically engineered E1A protein purified from *E. coli* does bind directly to DNA (*32*). Certainly, further analysis on the interaction between the E1A protein and purified cellular components as they relate to regulatory sequences of DNA will be required to provide additional insight into the mechanism of E1A trans-activation.

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18. The recombinant plasmid pE11D was constructed by cloning the Sma I-A fragment of Ad (extending from 56.8 map units to 76.0 map units on the Ad genome) into the Pst I site of plasmid pBR322. pE11D was prepared for in vitro transcription analysis by digestion with Xho I, which cleaves the Ad sequence at coordinate 66.5 map units. This results in a correctly initiated run-off transcript (as judged by S1 nuclease mapping) of ≈3200 nucleotides. The recombinant pMLR is derived from pH3B*R (*20*) and contains a deletion that removes the Ad-associated VAI and VAII genes but leaves the ML promoter of Ad intact. This template, when linearized at the pBR322 Pst I site, yields a correctly initiated run-off transcript of ≈3500 nucleotides in the cell-free system.
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