Adenovirus E1A Protein Activates Transcription of the E1A Gene Subsequent to Transcription Complex Formation

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Received 2 November 1990/Accepted 4 January 1991

The mechanism of transcriptional activation of the adenovirus E1A and E3 genes by E1A protein during infection was examined by using transcription-competition assays. Infection of HeLa cells with one virus led to inhibition of mRNA accumulation from a superinfecting virus. Synthesis of the E1A 289R protein by the first virus to infect reduced inhibition of transcription of the superinfecting virus, indicating that the E1A 289R protein was limiting for E1A-activated transcription. Infection with an E1A⁻ virus, followed 6 h later by superinfection with a wild-type virus, led to preferential transcriptional activation of the E1A gene of the first virus, suggesting that a host transcription component(s) stably associated with the E1A promoter in the absence of E1A protein and that this complex was the substrate for transcriptional activation by E1A protein. The limiting host transcription component(s) bound to the E1A promoter to form a complex with a half-life greater than 24 h in the absence of E1A 289R protein, as demonstrated in a challenge assay with a large excess of superinfecting virus. In the presence of the E1A 289R protein, the E1A gene of the superinfecting virus was gradually activated with a reduction in E1A mRNA accumulation from the first virus. The kinetics of the activation suggest that this was due to an indirect effect rather than to destabilization of stable transcription complexes by the 289R protein.

The adenovirus E1A gene encodes two related protein products translated from its two major, differentially spliced mRNAs: the 289R protein, encoded by the 13S mRNA, and the 243R protein, translated from the 12S mRNA, which lacks 46 amino acids present in the larger protein. The 289R protein can transcriptionally activate all of the early viral genes, as well as several host genes, and this activity requires its unique 46-amino-acid domain (reviewed in references 6 and 12). The 243R protein can induce transcription of certain host cell genes (24, 35), and in cooperation with cyclic AMP, it can efficiently induce both viral and cellular transcription units (10a). Since both E1A proteins can activate transcription and since the smaller protein lacks the activation domain present in the larger protein, it seems likely that the E1A proteins stimulate transcription through at least two distinct mechanisms (24).

The E1A 289R protein can influence existing RNA polymerase II transcription components to increase transcription, as demonstrated by the fact that its addition to nuclear extracts led to increased transcription *in vitro* (19, 31, 37). The E1A 289R protein binds DNA, although sequence specificity has not been demonstrated (8). Its unique 46-amino-acid region contains a Zn finger, and the Cys residues that make up this structure are critical for transcriptional activation (28). The C-terminal half of the 289R protein is responsible for sequence-independent binding to DNA (8) and has indirectly been shown to mediate interaction of E1A protein at the promoter (26).

The promoter elements through which E1A activates transcription have largely been shown to coincide with those

required for basal transcription (reviewed in references 6 and 13). E1A proteins appear to be capable of activating transcription through at least several different transcription factors (32). In particular, E1A protein appears to be capable of acting through TFIID (42) and ATF-2 (27) and to induce AP-1 activity (30).

Many explanations of the mechanism by which E1A and other viral transcriptional activator proteins stimulate transcription have been offered (reviewed in references 6 and 12). A proposal which has recently received wide support is that viral transcriptional activator proteins increase the rate of transcription by facilitating the binding of specific host transcription factors (2, 5, 14, 25) in competition with histones (1, 41) to form stable transcription complexes. It has also been proposed that E1A protein activates transcription through interaction with the stable transcription complex after it has been formed (33).

Formation of the preinitiation complex (10) or stable transcription complex has been studied in detail for the adenovirus major late promoter (39). The initial steps involve stable binding to upstream control elements by the TATA box binding factor (TFIID) and the upstream stimulatory factor. Following binding of specific factors to form a committed complex, general transcription factors and RNA polymerase II bind. Finally, an energy-dependent rearrangement in the complex occurs and transcription is initiated in the presence of nucleoside triphosphates (39).

In this study, we investigated the role of stable complexes in transcriptional activation of the adenovirus E1A and E3 genes by E1A protein. Examination of the requirement for a limiting host transcription component through the use of competition assays *in vivo* suggested that the limiting host component(s), the nature of which was not ascertained, bound rapidly and stably to the E1A promoter in the absence of E1A protein and that the complex formed was then transcriptionally activated by E1A protein.

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FIG. 1. The role of E1A gene products in inhibition of E1A mRNA accumulation. (A) The E1 region and mutant viruses are presented schematically. The antisense probe transcript, which extends from base pair 1838 to the left end of the viral chromosome, is indicated above. The regions of the wild-type E1A and E1B mRNAs which overlap the probe are indicated in the middle, with the nucleotide numbers of the first and last bases remaining within the regions of the 12S and 13S mRNAs presented. The deleted regions of the mutant viruses are indicated by closed boxes below, with the nucleotides adjacent to the deleted regions indicated. (B) As indicated in the flow diagram at the top, HeLa cells were infected with competitor viruses (noted above the lanes) or mock infected (lanes labeled with minus signs). At 6 h later, the cells were superinfected with *d/310* or mock superinfected (lanes labeled 343 alone). After an additional 6 h, the cells were harvested and

MATERIALS AND METHODS

Cells and viruses. HeLa suspension cells were grown in medium supplemented with 5% calf serum or 10% horse serum. HeLa suspension cells at a concentration of 5×10^6 /ml were infected for 30 min at a multiplicity of 25 PFU per cell, except where noted otherwise, and then diluted 10-fold with serum-containing medium. Cells were maintained in circulating water baths at 37°C.

Viruses were grown and titered by using 293 cells, a human embryonic kidney cell line transformed by and expressing the E1-encoding region of adenovirus type 5 (18). The viruses used were wild-type *w/300* (22); *d/309*, which is phenotypically wild type (23); *d/310*, which is phenotypically wild type (23) but has an in-frame deletion of 27 bp within the portion of the E1A gene which encodes the 3' exon (21); *d/312*, an E1A⁻ virus with a large deletion of transcription control and coding sequences (23); *d/343*, an E1A⁻ virus with a 2-bp deletion near the 5' end of the E1A-coding region (21); *d/347*, which has an E1A 12S mRNA cDNA in place of the E1A-coding region (40); and *d/348*, which has an E1A 13S mRNA cDNA in place of the E1A-coding region (40). All of the viruses used except *w/300* have the same deletion within the gene for E3. This deletion does not affect growth of the virus in 293 or HeLa cells (22).

RNA isolation and analysis. Cytoplasmic RNA was isolated by lysing cells in hypotonic buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 3 mM MgCl₂) containing 0.5% Nonidet P-40, removing nuclei by centrifugation, and deproteinizing by digestion with 250 µg of proteinase K per ml at 37°C for 15 min, followed by phenol extraction and ethanol precipitation. Nuclear RNA was isolated by washing the nuclei in hypotonic buffer containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate, pelleting them by centrifugation, suspending the nuclear pellet in 7 M guanidinium thiocyanate, and pelleting the RNA through 5.7 M CsCl (9). RNA was analyzed by RNase protection (29) with 10 µg of RNA and labeled probes in sufficient excess to saturate binding. E1A and E1B mRNAs were analyzed by using a probe which overlaps all of E1A and the first 139 bases of the E1B mRNAs. The E3 probe was transcribed from a clone containing the adenovirus sequence from the *Eco*RI site at 83.5 map units to the *Alu*NI site at 81.8 map units. Concentrations of specific mRNAs were determined by densitometric scanning of preflashed autoradiographs.

RESULTS

Inhibition of E1A mRNA accumulation from a superinfecting virus. The ability of wild-type and E1A mutant adenoviruses to inhibit E1A mRNA accumulation from a superinfecting wild-type virus was examined. The E1A promoter was chosen for study because (i) it responds to the E1A 289R protein by increasing the rate at which it directs transcription (e.g., reference 21) and (ii) a phenotypically wild-type virus,

cytoplasmic and nuclear RNAs were isolated. Relative concentrations of E1A and E1B mRNAs were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis and are indicated. *d/343* contains a 2-bp deletion approximately 120 bases from the E1A mRNA cap site. During RNase digestion, the deleted region forms a structure which is cleaved with variable efficiency. Thus, both the 13S and 12S 5' exons from *d/343* yield smaller digestion products; the 13S product runs just behind the wild-type 12S exon species, and the 12S product runs in front of the common 3' exon protection fragment.

FIG. 2. DNA from the superinfecting virus reaches the nucleus efficiently. DNAs from HeLa cells which were mock infected (lane identified with a minus sign) or infected with *dl312* or *dl347*, superinfected 6 h later with *dl310*, and harvested 6 h later were purified, digested with *Hind*III, resolved by agarose gel electrophoresis, denatured, transferred to nitrocellulose, and probed with the left-terminal adenovirus 2,805-bp *Hind*III fragment. The fragments are identified.

dl310, encodes E1A mRNAs that can be distinguished from E1A mRNAs of other adenoviruses in coinfecting cells (21). The region that encodes the 3' exon of the *dl310* E1A mRNAs contains a 27-bp deletion (Fig. 1A). This leads to a discontinuity between a wild-type probe RNA and *dl310*-encoded E1A mRNAs. RNase cleaves at the site of the discontinuity during RNase protection assays to produce a shortened band corresponding to the mutated 3' exon (Fig. 1B, lanes 1 and 8).

Cells were initially infected with a competitor virus, which was either wild type or mutated in the E1A gene (Fig. 1A) and then superinfected 6 h later with *dl310*, the phenotypically wild-type reporter virus. After an additional 6 h, the cells were harvested and the RNA was analyzed. When the wild-type virus, *wt300*, was the first to infect, it caused an approximately threefold reduction in E1A mRNA accumulation from the superinfecting virus, *dl310* (Fig. 1B, lane 1 versus lane 2). When *dl348*, a mutant virus that expresses only the E1A 13S mRNA (Fig. 1A), was the first to infect, it inhibited *dl310* E1A mRNA accumulation to the same extent as the wild-type virus (Fig. 1B, lane 1 versus lane 6). The viruses that express only the E1A 12S mRNA (*dl347*; Fig. 1A) or no E1A function (*dl312* and *dl343*; Fig. 1A) inhibited *dl310* E1A mRNA accumulation to a greater degree, approximately eightfold (Fig. 1B, lane 1 versus lanes 3 to 5). The inhibition observed was reproducible, ranging between 5- and 10-fold with *dl343* as the competitor (data not shown), as long as the temperature was maintained at 37°C (unpublished data). Thus, inhibition of E1A mRNA accumulation from a superinfecting reporter virus was greater when the first virus did not express the E1A 289R transcriptional activator protein.

E1A mRNA accumulation from E1A⁻ virus *dl343* was low when cells were infected with *dl343* alone (Fig. 1B, lane 7) but was significantly increased by superinfection with *dl310*, which provided wild-type E1A protein (Fig. 1B, lane 4). Thus, the presence of the *dl343* chromosome in the nuclei for 6 h before synthesis of wild-type E1A protein did not prevent it from being transcriptionally activated by E1A protein. Further, the *dl310* E1A protein preferentially activated the E1A gene from the previously introduced *dl343* chromosome

rather than that from the superinfecting *dl310* chromosome (compare the *dl343*-specific E1A mRNA 3' exon [Fig. 1B, lanes 4 and 7] and the *dl310*-specific E1A mRNA 3' exon [Fig. 1B, lanes 1 and 4]).

The ratio of nuclear to cytoplasmic *dl310* E1A mRNAs was not significantly altered in any of the infections (Fig. 1B), suggesting that the inhibition of E1A mRNA accumulation was unlikely to be the result of a reduction in the rate of nucleocytoplasmic transport. Southern analysis of DNA isolated from the nuclei of superinfected cells demonstrated that DNA of the superinfecting virus, *dl310*, reached the nucleus of preinfected cells at the same concentration as in mock-preinfected cells (Fig. 2 and data not shown). These findings are consistent with the occurrence of inhibition of mRNA accumulation from the superinfecting virus at the level of transcription.

The effect of increasing the multiplicity of infection of *dl343* on mRNA accumulation from superinfected *dl310* was analyzed. Inhibition was efficient at a low multiplicity of infection and was increased with increasing multiplicities of infection (Fig. 3, lanes 2 to 4 versus lane 1). This suggests that a component required for efficient transcription was bound by the first viral DNA to enter the nucleus and that the half-life of the complex formed with this factor(s) was long relative to the time of these assays.

FIG. 3. Dependence of competition on multiplicity of infection (moi). HeLa cells were infected with *dl343* at various multiplicities or mock infected (lane labeled 310 alone). At 30 min later, the cells were superinfected with *dl310*. At 4.5 h after infection with *dl310*, the cells were harvested and cytoplasmic RNA was isolated. Relative concentrations of E1A mRNA were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis, and their identities are indicated.

FIG. 4. Binding of the limiting transcription component by the first virus to infect. HeLa cells were infected with *dl310* alone, superinfected at various times with *dl343* (lanes labeled 310 first), or infected with *dl343* first, followed by superinfection with *dl310* at various times (lanes labeled 343 first). At 4.5 h after infection with *dl310*, the cells were harvested and cytoplasmic RNA was isolated. Relative concentrations of E1A mRNA were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis, and their identities are indicated.

Dependence of inhibition on the order of infection. To examine the time dependence of infection on inhibition of E1A mRNA accumulation from a superinfecting virus, cells were infected with *dl310* for up to 30 min before superinfection with *dl343* and the cells were harvested 4.5 h after *dl310* infection. Prior infection with *dl310* for 10 min was sufficient to virtually abolish the inhibition of *dl310* E1A mRNA accumulation caused by *dl343* (Fig. 4, lane 1 versus lane 6). Infection with *dl343* for as little as 10 min led to a nearly maximal level of inhibition of E1A mRNA accumulation from superinfecting *dl310* (Fig. 4, lane 1 versus lane 4). Thus, it appears that the first viral DNA to reach the nucleus efficiently and stably bound the limiting component(s) required for efficient transcription and that the wild-type and E1A⁻ viruses both bound the limiting component(s) rapidly.

Inhibition of an E1A⁻ virus by an E1A⁻ virus. E1A 289R⁻ viruses efficiently inhibited E1A mRNA accumulation from a superinfecting wild-type virus. To determine whether the same component was competed for in E1A-independent and E1A-dependent transcription of the gene for E1A, inhibition of an E1A⁻ virus (*dl343*) by an E1A⁻ virus (*dl312*, which produces no E1A mRNAs) was compared with inhibition of an E1A⁺ virus (*dl309*) by an E1A⁻ virus (*dl312*) (Fig. 5). Accumulation of *dl309* E1A mRNA was reduced approximately fivefold by coinfection with *dl312* (Fig. 5, lane 1 versus lane 2), while accumulation of *dl343* E1A mRNA was reduced less than twofold by coinfection with *dl312* (Fig. 5, lane 3 versus lane 4). Inhibition caused by infection with *dl312* and superinfection with either *dl309* or *dl343* was also examined (data not shown). Inhibition of *dl309* E1A mRNA accumulation was consistently greater than inhibition of *dl343* E1A mRNA accumulation. The slight inhibition of *dl343* E1A mRNA accumulation suggested that in the absence of the E1A 289R protein, a host transcription component(s) was limiting and the two viral chromosomes com-

FIG. 5. Inhibition of an E1A⁻ virus by an E1A⁻ virus. HeLa cells were either singly infected with *dl309* or *dl343* or coinfecting with *dl312* and either *dl309* or *dl343*. At 4.5 h after infection, the cells were harvested and cytoplasmic RNA was isolated. Relative concentrations of E1A mRNA were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis, and their identities are indicated.

peted for it. The greater inhibition of *dl309* E1A mRNA accumulation relative to *dl343* E1A mRNA accumulation suggested that the major part of the inhibition was specific to E1A-activated transcription and that the E1A 289R protein or a cellular factor which responds to the E1A protein was the limiting component.

Stability of the complex formed on the E1A promoter. Inhibition of E1A mRNA accumulation from a superinfecting virus suggested that the limiting host transcription component(s) bound stably to the E1A promoter. To examine the half-life of the complex formed on the E1A promoter, a challenge assay was used. Cells were infected with E1A⁻ virus *dl343* and superinfected 30 min later with a large excess of the E1A 12S cDNA virus *dl347* to provide a sink for transcription components released from the *dl343* E1A promoter. Accumulation of *dl343* E1A mRNA in cells superinfected with *dl347* was only slightly reduced relative to cells infected with *dl343* alone through 24 h after *dl343* infection (Fig. 6A). *dl347* E1A mRNA accumulation increased slowly (compare the concentration of the 12S 5' exon in Fig. 6A), indicating that relatively little binding of the limiting host transcription component(s) to the *dl347* E1A promoter occurred. Similar results were obtained when *dl343* E1A mRNA accumulation was measured in the presence or absence of a large excess of superinfecting *dl312* (data not shown). Thus, transcription complexes formed on the E1A promoter appeared to be very stable, with a half life of greater than 24 h in the absence of the E1A 289R protein.

The challenge assay was also performed by using infection with E1A⁺ virus *dl310* and superinfection with a large excess of E1A⁺ virus *dl309* (Fig. 6B). Accumulation of *dl310* E1A

FIG. 6. Stability of complexes formed with the E1A promoter. (A) As indicated in the flow diagram at the top, HeLa cells were infected with *dl343* at a multiplicity (moi) of 25 PFU per cell. At 30 min later, the cells were either mock superinfected or superinfected with *dl347* at a multiplicity of 200 PFU per cell. At 2 h after infection with *dl343*, DNA replication was blocked by addition of 10 mM hydroxyurea. At various times after *dl343* infection, the cells were harvested and cytoplasmic RNA was isolated. Relative concentrations of E1A mRNA in cells infected with *dl343* alone (lanes labeled with minus signs) or cells superinfected with *dl347* (lanes labeled 347) were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis and are indicated. (B) As indicated in the flow diagram at the top, HeLa cells were infected with *dl310* at a multiplicity of 25 PFU per cell. At 30 min later, the cells were either mock superinfected or superinfected with *dl309* at a multiplicity of 200 PFU per cell. At 2 h after infection with *dl310*, DNA replication was blocked by addition of 10 mM hydroxyurea. At various times after *dl310* infection, the cells were harvested and cytoplasmic RNA was isolated. Relative concentrations of E1A mRNA in cells infected with *dl310* alone (lanes labeled with minus signs) or cells superinfected with *dl309* (lanes labeled 309) were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis, and their identities are indicated.

mRNA was unaffected at 4 h but was reduced approximately threefold by 8 h in the presence of superinfecting *dl309*. Gradual activation of the E1A gene of *dl309* was observed. Infection with *dl310*, followed by superinfection with a large excess of *dl343*, or infection with *dl343*, followed by superinfection with a large excess of *dl310*, also led to gradual activation of the superinfecting virus with a reduction in E1A mRNA accumulation from the first virus (data not shown). Synthesis of the E1A 289R protein led to a shift to transcription of the more numerous superinfecting virus E1A-encoding genes over time. This suggests the possibility that the E1A 289R protein destabilized transcription complexes on the E1A promoter. Alternatively, it is possible that the concentration of the limiting host transcription component(s) was increased in the presence of the 289R protein. Forma-

tion of transcription complexes on the superinfecting viral DNA could then have led to titration of 289R protein and/or host transcription components which did not remain stably associated with transcription complexes formed on the first viral chromosome, leading to reduced transcription from the preinfecting virus. Thus, these data do not appear to permit unequivocal determination of the half-life of transcription complexes on the E1A promoter in the presence of E1A 289R protein, although it is clear that they are stable for greater than 3.5 h.

Inhibition of E3 mRNA accumulation. To determine whether competition involves early-region promoters other than that which controls the gene for E1A, viruses with distinguishable E3 transcripts were used to infect and superinfect cells. Analysis of the RNA from such cells demon-

FIG. 7. Inhibition of E3 mRNA accumulation. HeLa cells were either mock infected (lane labeled with a minus sign) or infected with the virus listed above and then superinfected 30 min later by the virus listed below. Cells were harvested 4.5 h after infection with *w*300, cytoplasmic RNA was isolated, and relative E3 mRNA concentrations were determined by RNase protection. The protected fragments were resolved by denaturing polyacrylamide gel electrophoresis, and their identities are indicated.

strated that infection with either an E1A⁻ or an E1A⁺ virus led to inhibition of E3 mRNA accumulation from a superinfecting virus (Fig. 7). The inhibition was greater when an E1A⁻ virus was used in the initial infection (compare the levels of *w*300 E3 from lanes 1 and 5 in which *d*312 and *d*310, respectively, were infected first). Thus, stable binding of a limiting component(s) also appeared to be involved in activation of the gene for E3 by E1A protein.

DISCUSSION

Transcriptional activation of viral genes by E1A protein was assayed in the presence of competitor viruses. These analyses suggested that transcriptional activation of the gene for E1A immediately after infection occurred through interaction, either directly or indirectly, of E1A protein with transcription complexes already formed (see below).

Most of the assays reported here involved analysis of total cytoplasmic RNA. The differences observed most likely reflect differences in transcription rather than in a posttranscriptional step for several reasons. (i) The relative concentrations of specific RNAs varied little between the cytoplasm and the nucleus (Fig. 1), arguing against a block in mRNA transport. (ii) Inhibition of E1A mRNA accumulation was dependent on the time of preinfection of the competitor over a very short period (Fig. 4), arguing against a change in mRNA stability. (iii) The E1A 289R protein, which activates transcription of the genes for E1A and E3, helped to alleviate the inhibition of a superinfecting virus (Fig. 1 and 7).

At least some of the viral DNA reached the nucleus and bound the limiting transcription component(s) very quickly after infection, as indicated by the short initial period of infection required to cause a nearly maximal level of inhibition or to prevent inhibition (Fig. 4). Thus, although the measurement of transcription component binding is indirect, this assay appears to measure very early steps required for transcriptional activation by E1A protein.

The inhibition of transcriptional activation (Fig. 1, 3 to 5, and 7) appears primarily to involve competition between viral promoters for direct or indirect interaction with the

E1A 289R protein. Down regulation of E1A transcription by E1A proteins (36, 38) did not appear to play a significant role, since the level of inhibition caused by viruses which make no E1A protein was equal to that caused by the virus which expresses only the 243R protein (Fig. 1). Competition between wild-type and mutated E1A proteins for sites of interaction, as was suggested to explain the inhibition caused by the mutant *hr5* E1A protein in transfection and infection assays (16), also does not explain these data, since competition was observed in the complete absence of E1A protein from the competing virus (Fig. 1, 5, and 7).

Implications for the mechanism of transcriptional activation. Transcriptional activation of the E3 promoter by E1A protein in *Xenopus* oocytes is dependent on E1A protein-independent binding of a factor to the ATF site (33). This indicates that E1A protein can act after transcription complex formation to activate transcription. Our data suggest that during infection of HeLa cells, E1A protein also activates transcription from the E1A promoter after transcription components are stably bound.

The first step in transcription of the gene for E1A appears to involve rapid binding of host cell transcription components to the promoter to form stable complexes (the molecular makeup of which is not clear from these data) in the absence of E1A protein. The rapidity is demonstrated by the brief (10 min or less) initial infection required to cause or alleviate inhibition (Fig. 4). The stability is demonstrated by the long half-life of the complex (Fig. 6) and the time dependence of competition on superinfection (Fig. 4). The independence from E1A protein is demonstrated by the ability of E1A⁻ viruses to inhibit mRNA accumulation from a superinfected wild-type virus (Fig. 1, 3 to 5, and 7).

The E1A 289R protein appeared to activate transcription through recognition of the transcription complexes (26; Fig. 1). The 289R protein was limiting and was competed for in these assays, as expected on the basis of the linear dependence of transcription of early-region genes other than that for E1A on the 289R protein concentration (7). During transcription-competition assays with an E1A⁺ competitor virus, transcriptional activation of the competitor virus E1A promoter led to synthesis of additional E1A protein, and inhibition of superinfecting virus transcription was reduced. If the first virus did not synthesize the 289R protein, interaction of 289R protein synthesized by the wild-type superinfecting virus with the first viral genome did not lead to additional 289R protein synthesis and inhibition of superinfecting virus transcription was accentuated (Fig. 1 and 7).

Transcriptional activation and stable complex formation. It has been suggested that for a number of viral transcriptional activator proteins, activation is accomplished through an increased rate of formation of stable transcription complexes (2, 5, 14, 25). We cannot rule out such a role for E1A protein. However, our data provide evidence for a different site of E1A protein action in activation of the gene for E1A during the early phase of infection. The gene for E1A of E1A⁻ virus *d*343 was transcriptionally activated by *d*310 E1A protein 6 h after *d*343 infection (Fig. 1), long after transcription of the *d*343 gene for E1A had begun and long after stable binding of a host transcription component(s) had occurred (Fig. 4 and 6). Further, the *d*343 gene for E1A appeared to be preferentially activated relative to the superinfecting *d*310 gene for E1A, suggesting that the steps occurring after infection made the *d*343 E1A promoter a better substrate for E1A protein activity. Therefore, our results suggest (i) that the limiting host transcription component(s) bound stably to the E1A promoter in the absence of E1A protein, (ii) that the

rate-limiting step in transcriptional activation occurred subsequent to stable binding of the limiting host transcription component(s), and (iii) that E1A protein recognized transcription complexes already formed in activating transcription from the E1A promoter.

For a variety of reasons, it is reasonable that, immediately after infection, E1A protein should act on stable complexes already formed rather than in their formation. De novo synthesis of E1A protein requires that transcription factors bind before the protein is synthesized. Thus, for E1A protein to affect stable complex formation on the E1A promoter immediately after infection, it would have to be brought to the nucleus by the virion. This does not appear to happen. The *d1343* genome did not appear to have been activated prior to superinfection with *d1310* (Fig. 1), since activation appears to be stable (3; unpublished data). Further, levels of E1A mRNA accumulation in HeLa cells infected with *d1343* grown in either 293 cells (which could provide E1A protein in the virion) or HeLa cells (which cannot) were the same (unpublished data), indicating that E1A-dependent transcriptional activation did not occur in either case.

It would be possible for E1A protein to affect formation of stable transcription complexes if the complexes were first destabilized by E1A protein after infection. The shift in transcription from the E1A-encoding gene of the first virus to that of the superinfected virus in the presence of the 289R protein (Fig. 6B) could be indicative of such a function. E1A protein has been shown to destabilize complexes containing transcription factor E2F and other host proteins (4). However, this effect was accomplished by both the 289R and 243R proteins (4), while the shift to transcription of the superinfecting virus was dependent on the presence of the 289R protein (Fig. 6 and data not shown). Further, the time required for a reduction in E1A mRNA accumulation from the first virus in the presence of the 289R protein was between 3.5 and 7.5 h, which was greater than the time necessary for transcriptional activation of the E1A-encoding gene to be apparent and, thus, too long to represent the rate-limiting step in transcriptional activation.

The fact that the E1A-encoding gene is the first viral gene to be expressed after infection (17) means that E1A protein could, in theory, be synthesized before transcription factors bind to other viral promoters. Thus, it is possible that other viral promoters are activated through a mechanism different from that of the E1A promoter. However, *d1312*, which has a deletion that removes part of the E1A promoter, including the TATA box, and other E1A 289R⁻ viruses competed to the same level. This suggests that the host component(s) that is limiting for E1A-encoding gene transcription is shared with other viral promoters and that the other promoters stably bind this component(s) in the absence of E1A protein. The fact that transcriptional activation of the E3 promoter (33) and host promoters directly activated by E1A protein appears to occur through recognition of stable complexes further suggests that the E1A promoter is not unique with respect to transcriptional activation by E1A protein.

Chromatin domains and transcriptional activation. The adenovirus chromosome appears to act as a single domain with respect to transcription during the early phase of infection (34). Transcriptional activation of viral genes by E1A protein is dependent on proper function of the covalently attached terminal protein in a manner consistent with a role for nuclear matrix association of the DNA (34). This suggests that transcriptional activation involves recognition of domains of chromatin. Transcriptional activation of a gene could increase the likelihood of transcription of nearby

genes, or the domain-dependent state of the promoters could lead to recognition by the transcriptional activator protein. Support for these possibilities comes from studies of transcriptional activation of the β -globin promoter by herpes simplex virus infected-cell protein 4 (ICP4; Vmw175). The β -globin promoter in its normal chromosomal location is not activated by ICP4. However, ICP4 activated the β -globin promoter after it was used to transform cells stably, although only when transcription was relatively high in the absence of ICP4 (11). While increased transcription has been observed in vitro in the presence of E1A protein and other viral transcriptional activators, the apparent dependence on chromatin domain function for transcriptional activation in vivo raises the possibility that the mechanism of transcriptional activation of the E1A promoter by E1A protein during infection is not recreated in soluble in vitro systems.

Certain promoters are not transcriptionally activated by E1A protein during infection, but they direct increased transcription when present on DNA transiently transfected into cells in which E1A protein is expressed (15, 20). E1A protein may facilitate the binding of transcription factors to naked DNA in competition with histones during transient transfection as pseudorabies virus immediate-early protein does in vitro (41). Such an activity could be reflective of a role for E1A protein in activating newly replicated viral promoters during the late phase of infection. Alternatively, naked DNA transiently transfected into cells could assume a structure which resembles that of domains of chromatin (either viral or host) which are activated by E1A protein during infection. The possibility that E1A stimulation of transcription in vitro is related to that which occurs during transient transfection of DNA leads to the prediction that host promoters unaffected during infection, as well as those activated by E1A protein, can direct increased transcription in vitro in the presence of E1A protein. If this proves correct, it will provide further evidence that the rate-limiting step differs between transcriptions in vitro and in vivo during infection and, therefore, that the mechanism of transcriptional activation by E1A protein during infection is not entirely reproduced in soluble in vitro transcription systems.

ACKNOWLEDGMENTS

We thank D. Engel, P. Beard, R. Everett, and A. Berk for helpful discussions and C. Doerig, C. Wilcox, S. Baim, M. Marton, M. Roberts, and J. Flint for critical reading of the manuscript.

This project was supported by a Public Health Service grant from the National Cancer Institute (CA 41086) to T. Shenk; by grant BRSG-05357 awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health; and by a research grant from the University of Colorado Cancer Center to J. Schaack. J. Schaack was a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research during the initial phase of this work. E. Vakalopoulou was a postdoctoral fellow of the Boehringer Ingelheim Fonds and the Howard Hughes Medical Institute, and T. Shenk is an American Cancer Society Professor.

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