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An adenovirus E4 gene product trans-activates *E2* transcription and stimulates stable E2F binding through a direct association with E2F

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ABSTRACT **The adenovirus *E4* gene encodes a trans-activating function that can stimulate the *E2* promoter. *E2* promoter sequences required for *E4* trans-activation are identical to those required for *E1A* trans-activation, and these principally are the *E2* promoter binding factor (E2F) binding sites. Furthermore, full activation of E2F DNA binding activity requires both *E1A* and *E4* action. Analysis of a series of mutant E4 viruses identifies open reading frame (orf) 6/7 of the *E4* transcription unit as that required for activation of *E2F* binding activity. In addition, the assay of various E4 cDNAs demonstrates that the E4 orf 6/7 also is responsible for the trans-activation of *E2* transcription. Translation of the E4 orf 6/7 mRNA, but not a control mRNA, in a reticulocyte extract generates an activity that can stimulate cooperative binding of E2F *in vitro*, consistent with recent *in vivo* assays that demonstrate a role for the *E4* gene in E2F stable complex formation. This stimulation is due to a direct interaction of the E4 protein with E2F since an antibody that recognizes the E4 orf 6/7 polypeptide detects this E4 protein in the E2F–DNA complex. We conclude that the E4 orf 6/7 product interacts with the E2F factor altering binding to allow formation of a stable complex that results in a stimulation of transcription.**

Transcriptional control in eukaryotic cells is a complex process involving the interaction of a variety of DNA binding proteins with regulatory sequences, usually 5′ to the transcription initiation site, that then allow the subsequent initiation of transcription by RNA polymerase II (1). The trans-activation of the early genes of adenovirus dependent on the E1A gene product has proven to be an important system for the study of transcription control mechanisms (2). A set of five early transcription units are inefficiently transcribed in the absence of *E1A* function but are then stimulated upon expression of *E1A*. The promoter of the *E2* transcription unit has been mapped in detail and shown to include binding sites for three cellular transcription factors, including a TATA-specific transcription factor, the *E2* promoter binding factor (E2F) transcription factor, and the activating transcription factor (ATF) (3–6). All of these sites are important for transcription, including *E1A*-induced transcription (7–9). However, it is the E2F sites that appear to be the targets for regulation. Mutation of either E2F site generally has a more drastic effect on induced transcription than mutation of the ATF site (9). Of most importance is the fact that the E2F sites, when placed in a heterologous transcription unit, can confer *E1A* inducibility (10, 11). E2F DNA binding activity is increased significantly during an adenovirus infection (5), this increase parallels the activation of *E2* transcription (12), and purified E2F stimulates transcription *in vitro* from a promoter containing E2F binding sites (11).

All of these results point to the importance of E2F in the trans-activation of the *E2* transcription unit, dependent on *E1A* action. Recent experiments have demonstrated that expression of the viral *E4* gene is also necessary, in addition to *E1A*, for the activation of E2F DNA binding activity (13–15). Other experiments have demonstrated a role for *E4* in the activation of *E2* transcription and have shown that the *E4* gene in combination with *E1A* results in a greater stimulation than that achieved by either one alone (13). We have now further explored the roles of the E1A and E4 gene products in the trans-activation of *E2* and the activation of E2F. First, we have defined the *E2* promoter requirements for *E1A*-mediated and *E4*-mediated trans-activation. Second, we have determined which *E4* coding region, among the many possible (16), is responsible for the activation of E2F DNA binding activity and the trans-activation of *E2* transcription. Finally, we have analyzed the *E4*-mediated activation of E2F *in vitro* to determine the mechanism of activation.

METHODS

Cells and Virus. Monolayer cultures of HeLa cells (S3 line; American Type Culture Collection) or Vero cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Wild-type adenovirus type 5 (Ad5) was grown in HeLa cells, the dl312 mutant was grown in 293 cells, and the dl366 mutant was grown in the W162 cell line (17). Wild-type and mutant viruses were purified as described (18). The E4 mutant viruses were a gift from T. Shenk (19) and P. Hearing (20).

Preparation of Extracts. Extracts were prepared as described (12). For the gel retardation assays, the E2F preparation from Ad5-infected cells was purified through a phosphocellulose column (11). Mock E2F was partially purified by gradient elution from a heparin/agarose column (11).

E2F Assays. E2F binding assay procedures have been described (5). Exonuclease III assays used a 3′-end-labeled *E2* promoter fragment containing sequences from positions −21 to −98 (13). Gel-shift assays used a DNA probe containing *E2* promoter sequences between −28 and −85 derived from the ATF⁻ *E2* promoter clone described by Loeken and Brady (9). Where indicated, antisera were added to the gel-shift reaction mixtures after the normal 30-min binding reaction at room temperature. Antiserum was added (1 μl of a 1:5 dilution per 30-μl binding reaction mixture), and the tubes were incubated on ice for 60 min. The R3 and NR3 antisera were a gift from P. Hearing (21).

Transfections. Vero cells were transfected with calcium phosphate DNA precipitates. After 12 hr, precipitates were

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Abbreviations: Ad5, adenovirus type 5; CAT, chloramphenicol acetyltransferase; orf, open reading frame; ATF, activating transcription factor.

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removed and incubation was continued for an additional 36 hr. Extracts were prepared and assayed for chloramphenicol acetyltransferase (CAT) activity either by the organic extraction/TLC/autoradiography assay using [^{14}C]chloramphenicol (22) or by the LSC-fluor diffusion assay using [^{14}C]acetyl CoA (23).

Plasmids. pE2CAT is the previously described plasmid pEC113 (24) containing *E2* promoter sequences from positions −285 to +40 cloned upstream of the CAT gene in pCAT3M. The pE4 plasmid contains Ad5 sequences from 84.8 to 100 map units cloned in pBR322 (13). The pE1A plasmid has been described (24). The site-directed *E2* promoter mutants were a gift from M. R. Loeken and J. Brady (9).

Plasmids pil E4I, pil E4L, and pil E4D have been described (25). The E4 cDNA expression vectors were constructed as follows: pBC12/cytomegalovirus (CMV)/interleukin 2 [a gift from B. Cullen (26)] was digested with *Hin*dIII and *Bam*HI to remove all sequences between the CMV IE promoter and the 3′ end of the interleukin 2 cDNA. Polymerase chain reaction-generated E4 cDNA fragments with *Hin*dIII and *Bam*HI ends were then subcloned into this vector. The DNA fragments containing the E4 leader and coding sequences for E4 open reading frame (orf) 3, orf 6, or orf 6/7 were generated by using pil E4D, pil E4I, or pil E4L, respectively, as template (25) and oligonucleotides flanking the coding sequences as primers. The Perkin–Elmer/Cetus GeneAmp kit and DNA thermal cycler were used.

***In Vitro* Transcription/Translation.** The E4 orf 6/7 fragment from the CMV expression vector was subcloned into pGEM-1 (Promega Biotec) at the *Hin*dIII and *Bam*HI sites. The vector was linearized with *Eco*RI and used as template for SP6 RNA polymerase *in vitro* transcription reactions as described (27). This RNA was then translated in a nuclease-treated rabbit reticulocyte lysate (Promega Biotec).

RESULTS

***E2* Promoter Sequences Required for *E1A*- and *E4*-Mediated Trans-Activation.** Our previous experiments have demonstrated that the *E2* promoter could be trans-activated by the *E4* gene as well as by the *E1A* gene and that the two trans-activators together produced a greater stimulation of *E2* transcription, suggesting that the two regulatory gene products function differently (13). We could envision two possible explanations for this additive effect. First, the two trans-activators could alter the same transcription factor by different mechanisms. Second, given the fact that multiple transcription factors are involved in *E2* transcription, it is possible that the two trans-activators target different transcription factors. Although a previous study suggested that *E1A*-mediated trans-activation and *E4*-mediated trans-activation required the same *E2* promoter sequences (28), this work relied on linker scanning mutants that did not precisely define functional elements in the *E2* promoter as they are now known. To address this issue, we have made use of a series of *E2* promoters in which mutations have been precisely targeted to each of the known protein binding sites (9). As depicted in Fig. 1, mutations alter the ATF site, each of the E2F sites, and combinations of each. These plasmids were assayed by transfection into Vero cells along with pE1A or pE4. CAT activity was measured in extracts of the transfected cells and the results are shown in Fig. 1. Although mutation of the ATF site slightly impaired promoter activity, alteration of either one of the E2F sites was clearly the more significant effect. Most importantly, there was no significant difference observed in the promoter sequence requirement for allowing activation by *E1A* or *E4*. Thus, we find no evidence to suggest that the two trans-activators act through different transcription factors.

Identification of the E4 Product That Activates E2F. Previous experiments have demonstrated that the activation of E2F DNA binding activity depends on *E4* gene expression (13–15). These previous assays utilized the *E4* deletion mutant dl366, which eliminates all *E4* coding capacity, to demonstrate the *E4* requirement. However, as is the case for most of the early viral genes, the *E4* transcription unit is complex, encoding at least seven proteins by alternatively spliced mRNAs (16). We have now identified the E4 product that is responsible for the activation of E2F DNA binding activity, making use of a series of viral mutants (19, 20) that target each of the E4 orfs with the exception of orf 3/4 (Fig. 2*A*). HeLa cells were infected with each E4 mutant, and extracts were prepared and then assayed for E2F binding activity by an exonuclease III protection assay. As shown in Fig. 2*B*, each of the E4 mutants, with the exception of dl356 and dl366, induced E2F binding activity. The deficiency of dl356 or dl366 in the activation of E2F was not the consequence of a lack of infection by these viruses as demonstrated by coinfection with the mutant dl312, which lacks E1A function but provides E4 function. In each case, the two viruses complemented each other to allow efficient activation of E2F binding activity. We therefore conclude that dl356 is indeed deficient in E2F activation and that this then identifies the E4 orf 6/7 as that which is required for E2F activation. Since we did not have a virus that altered orf 3/4, we cannot exclude a role for this gene product based on these assays. However, additional experiments described below demonstrate that the orf 6/7 product is sufficient for E2F activation.

The E4 orf 6/7 Gene Product Trans-Activates the *E2* Promoter. If the *E4* activation of E2F binding activity is a critical aspect of the trans-activation of *E2* transcription, then we would expect that the E4 orf 6/7 product would be essential for trans-activation of *E2* transcription. This question has been addressed by utilizing several E4 cDNAs (25)

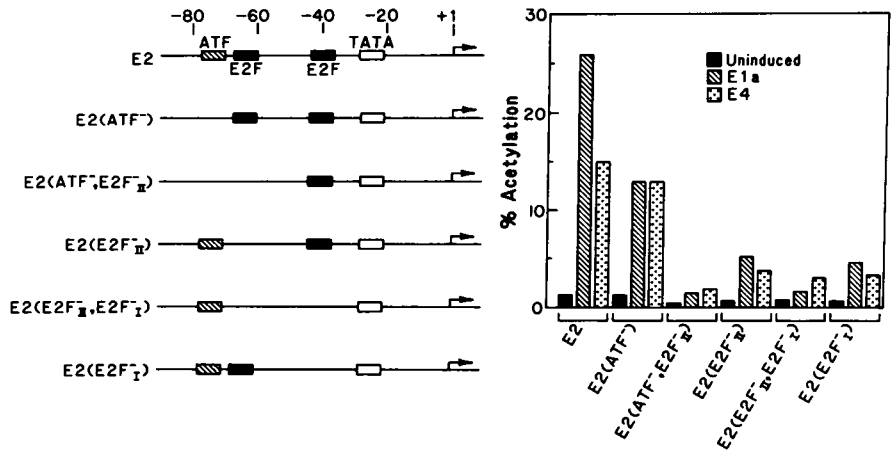

FIG. 1. E2 promoter requirements for *E1A*- and *E4*-mediated trans-activation. (*Left*) Schematic diagram of the *E2* promoter substitution mutants as described by Loeken and Brady (9). (*Right*) Results of trans-activation assays with the various *E2* promoter mutants. Vero cells were transfected with 10 μg of the *E2* promoter–CAT constructs either alone or together with pE1A (10 μg) or pE4 (10 μg). Cells were harvested for CAT assays as described. CAT activity was quantitated by scintillation counting of appropriate regions of the TLC plate.

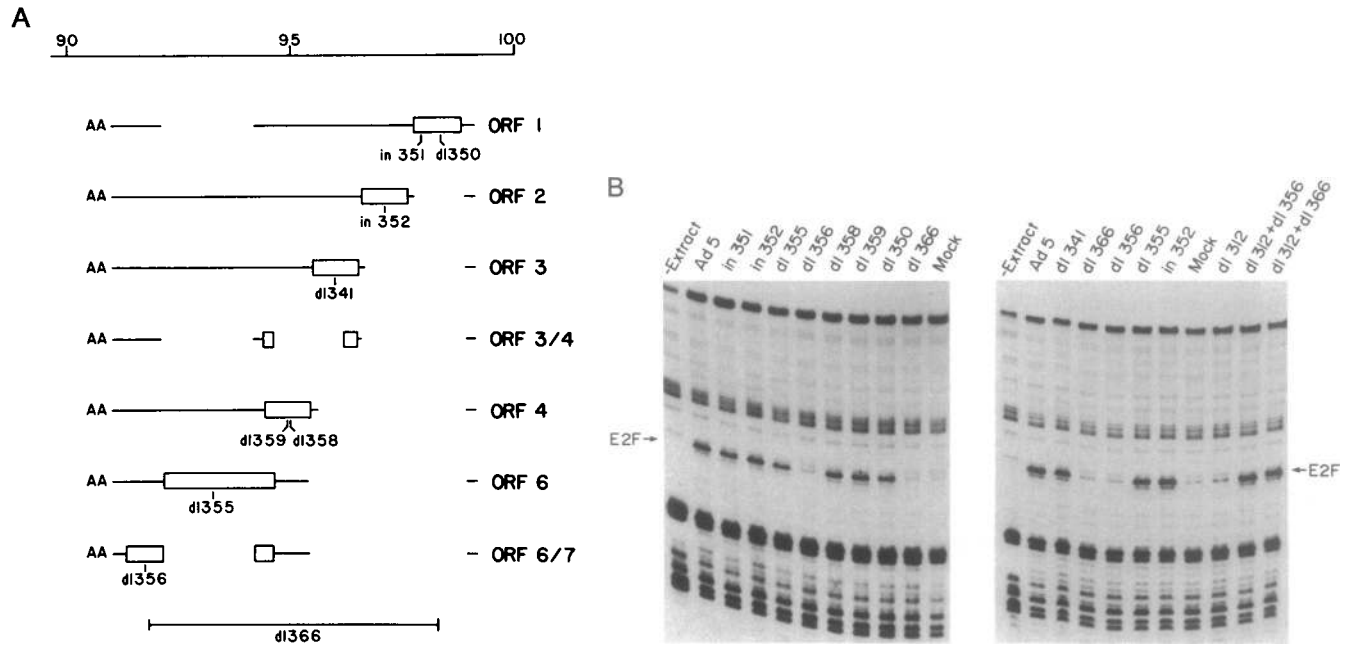

FIG. 2. Identification of the E4 protein that activates E2F. (*A*) Schematic diagram depicting the spliced E4 mRNA structures. The orfs are depicted as open boxes. The positions of the insertions and deletions in the E4 viral mutants are indicated. (*B*) Whole cell extracts were prepared from mock-infected HeLa cells or from HeLa cells infected with the indicated viruses. E2F binding activity was measured by an exonuclease III protection assay as described (5).

that have been cloned into an appropriate expression vector (Fig. 3*A*). The expression plasmids were constructed by amplifying the coding regions from the appropriate cDNAs by polymerase chain reaction using primers flanking the coding sequence. Importantly, this resulted in the elimination of splice sites that would allow production of orf 6/7 from orf 6 cDNA. Each was then used in cotransfection assays with E2 CAT to determine whether one possessed trans-activating function. As shown in Fig. 3*B* (*Left*), the addition of increasing amounts of the E4 orf 6/7 cDNA resulted in an increase in expression of E2 CAT, whereas a similar titration of the E4 orf 6 cDNA or an E4 *Sma* deletion equivalent to the dl366 deletion yielded no increase in E2 expression. Additional assays shown in Fig. 3*B* (*Right*) demonstrate again that the E4 orf 6/7 cDNA was functional as a trans-activator, whereas the E4 orf 3 cDNA and again the E4 orf 6 cDNA and the E4 *Sma* deletion were inactive. The trans-activation obtained with the E4 orf 6/7 cDNA was less than with the intact E4 transcription unit, but we suspect that this is a function of the difference in the makeup of the two plasmids. The clear result is that the E4 orf 6/7 product can function as a trans-activator of the *E2* promoter. This finding, together with the fact that the E4 orf 6/7 product is essential for the activation of E2F binding, clearly defines the role for this E4 product in the control of *E2* transcription in a viral infection.

The E4 orf 6/7 19-kDa Protein Stimulates Cooperative Binding of E2F. Our recent experiments have demonstrated that the activation of E2F is a two-step process involving an activation of the DNA binding capacity of the factor as well as an activation of stable complex formation dependent on adjacent E2F recognition sites (P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., unpublished work). These experiments further show that the first step is an E1A-dependent event, while the second step is E4 dependent, a result consistent with other studies of E4 action (14). We have now assayed for the ability of the E4 orf 6/7 protein to stimulate E2F binding *in vitro* and specifically to stimulate the formation of a stable complex. The E4 orf 6/7 mRNA was transcribed *in vitro* and then translated in a reticulocyte cell-free extract. As shown in Fig. 4, E2F from mock-infected cells forms a specific complex (lane 2) that migrates faster than that formed with E2F from adenovirus-infected cells (lane 1). Our previous experiments have shown that this difference results from the interaction of a single E2F molecule with the DNA (mock

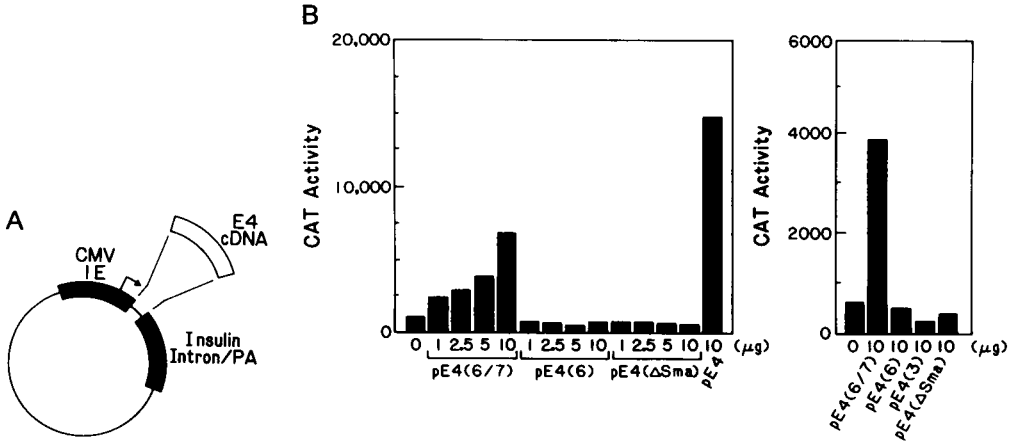

FIG. 3. Trans-activation of *E2* promoter by E4 cDNAs. (*A*) Structure of the E4 cDNA expression vectors. PA, poly(A). (*B*) Trans-activation of the *E2* promoter. Vero cells were transfected with 10 μg of pE2CAT either alone or together with the indicated amounts of pE4 or the various E4 cDNA plasmids. Cells were harvested for LSC-fluor diffusion CAT assays as described.

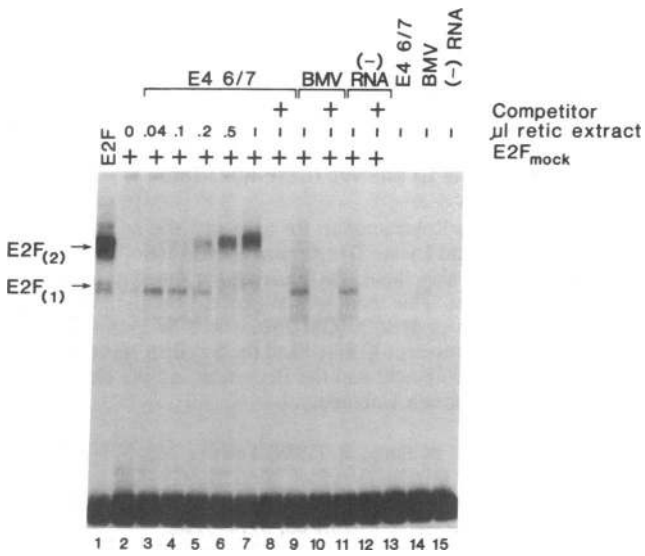

FIG. 4. *In vitro* translated orf E4 6/7 can stimulate E2F stable binding. E2F binding activity was measured by gel retardation using an ATF⁻ probe containing two E2F binding sites. E2F (lane 1) is a phosphocellulose fraction from infected cells. $E2F_{mock}$ is a heparin/agarose fraction from uninfected cells. $E2F_{mock}$ was incubated with the indicated amounts of reticulocyte (retic) lysates programmed with the E4 orf 6/7 mRNA (lanes 3–8), a control brome mosaic virus (BMV) RNA (lanes 9 and 10), or no RNA (lanes 11 and 12). The presence of competitor (+) indicates the addition of excess unlabeled competitor DNA containing the E2F recognition sequence. Lanes 13–15, DNA probe plus the reticulocyte lysates in the absence of E2F.

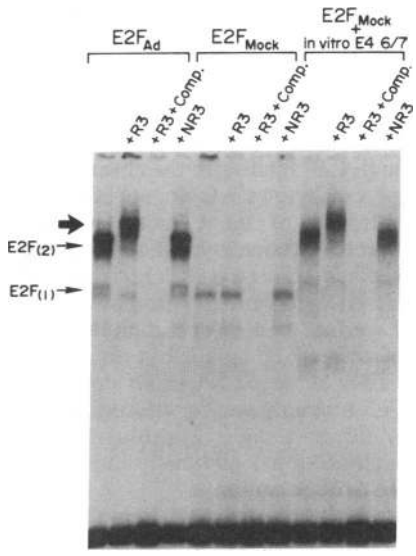

FIG. 5. The E4 orf 6/7 protein is a component of the E2F–DNA complex. Binding reactions were performed with the ATF⁻ probe containing two E2F binding sites along with $E2F_{Ad}$, $E2F_{Mock}$, or $E2F_{Mock}$ plus *in vitro* translated E4 orf 6/7 protein. After 30 min at room temperature, the reaction mixtures were incubated on ice either with preimmune serum (NR3) or with antiserum that recognized E4 orf 6/7 (R3); they were then assayed by gel retardation as described. + Comp., the presence of excess unlabeled competitor DNA containing the E2F recognition sequence.

E2F) or two E2F molecules with the DNA (Ad E2F) and that the larger complex predominates with Ad E2F due to a stabilized interaction between the two E2Fs. When increasing amounts of the E4 orf 6/7 programmed reticulocyte lysate was added to the preparation of E2F from mock-infected cells, the single factor complex was shifted to that typical of two factors interacting at adjacent sites (lanes 3–7). This difference in E2F binding, comparing mock E2F versus mock E2F plus E4 orf 6/7, is the same as that found in extracts of dl366 ($E4^-$) infected cells versus wild-type adenovirus-infected cells (P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., personal communication); that is, the E4 product promotes the formation of a stable E2F complex. The specificity of this complex was indicated by the competition of the complex with cold competitor DNA containing the E2F recognition sequence (lane 8), as well as by the fact that no complex forms with E4 orf 6/7 in the absence of mock E2F (lane 13). Furthermore, the addition of an equal amount of a lysate programmed with a control mRNA (BMV RNA) (lane 9) or no RNA (lane 11) failed to alter the mock E2F complex.

The results presented in Fig. 4 demonstrate that the E4 orf 6/7 protein can mediate a change in E2F binding to allow stable complex formation. One could imagine two alternative mechanisms for this effect. The orf 6/7 protein could function in a catalytic fashion to modify E2F or it could function stoichiometrically through an interaction with E2F. We have addressed this question by using an antiserum that recognizes an amino-terminal epitope common to the orf 6 and orf 6/7 proteins (21) to probe for the presence of the E4 orf 6/7 protein in the E2F complex. As shown in Fig. 5, the addition of the antiserum to an E2F–DNA complex formed with E2F from Ad5-infected cells altered the mobility of the complex to a slower-migrating form. That this result indicated the presence of the E4 orf 6/7 protein in the E2F–DNA complex was supported by a number of controls. First, formation of the altered complex was blocked by competition with excess cold DNA containing the E2F recognition sequence. Second, the control preimmune antiserum did not alter the mobility of the E2F–DNA complex. Third, the specific antiserum had no effect on the E2F–DNA complex formed with E2F from mock-infected HeLa cells. Finally, the $E2F_{(2)}$ complex formed as a result of mixing mock E2F with *in vitro* translated E4 orf 6/7 also was shifted with the antiserum in a manner identical to that seen with E2F from Ad5-infected cells. We therefore conclude that the product of the E4 orf 6/7 alters E2F binding to the DNA via a direct association with the E2F factor. A similar result has recently been obtained by Huang and Hearing (29) utilizing the same antibody approach.

DISCUSSION

From the data presented here as well as previous experiments, it appears that the activation of *E2* transcription depends critically on the activation of the E2F transcription factor. Certainly, interaction of the ATF transcription factor and a TATA factor with their cognate promoter elements is important for *E2* transcription but there is no evidence that either of these transcription factors plays a regulatory role in the activation of *E2* transcription. In contrast, activation of the E2F factor appears to be an important event for the stimulation of E2 transcription. Furthermore, the data presented in this paper demonstrate that the activation of *E2* transcription by either the addition of the 289-amino acid E1A protein or the E4 orf 6/7 gene product requires the E2F sites. Our recent experiments have shown that the activation of E2F is a two-step process involving an activation of both the DNA binding capacity of E2F as well as the ability of the protein to interact cooperatively with two adjacent sites on the promoter to form a stable complex (P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., personal communication). A two-step activation of E2F provides an explanation for the observations reported here and elsewhere that two early viral regulatory genes (*E1A* and *E4*) participate in E2F activation and *E2* trans-activation. Indeed, the activation of the DNA binding capacity of E2F appears to be a function of the action

of *E1A* and the activation of stabilized binding is a consequence of *E4* action (P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., personal communication). In this context, it is also clear how either trans-activator could stimulate *E2* transcription, whereas both together would be more efficient. *E1A* action alone would increase the level of active E2F in the cell and although E2F binding in the absence of modification by the E4 product is quite unstable, nevertheless an increase in the concentration of active factor would still lead to increased promoter occupancy. *E4* action alone, in the absence of *E1A*, could convert any preexisting active E2F to a form that could bind stably to the promoter. Clearly then, the extent of an *E4* effect would depend on the host cell; that is, the amount of active E2F existing in the cell. Thus, viewed in this way, *E1A* alone or *E4* alone could effect a transactivation of *E2* transcription by altering the E2F transcription factor in different ways. Expression of the two transactivators together would obviously then lead to a greater activation of *E2* transcription.

The results presented here now define the E4 orf 6/7 gene product, a 19-kDa polypeptide (21), as responsible for the activation of E2F. Most importantly, expression of the *E4* orf 6/7 is also capable of trans-activation of the *E2* promoter, whereas at least two other *E4* orfs are not functional in this assay. Thus, the ability of *E4* to enhance E2F binding is tightly linked to its ability to stimulate *E2* transcription. This conclusion is also supported by the fact that *E2* transcription in a virus infection is reduced 5- to 8-fold in the absence of *E4* (13). Clearly, *E1A* alone is sufficient to stimulate *E2* transcription but only to less than maximal levels. We also suspect that the contribution from E4 action may depend in part on the host cell environment, reflecting either an activity functionally equivalent to E4 or variation in the actual levels of active E2F.

The *E4*-mediated activation of E2F appears to involve a direct interaction between the 19-kDa E4 protein and the E2F–DNA complex resulting in the formation of a stable complex involving two adjacent E2F factors. In the absence of the E4 protein, E2F can bind to the DNA with specificity but it forms an unstable complex (ref. 14; P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., personal communication). Since previous experiments have shown that the stable complex is critically dependent on the spacing between the E2F sites as well as the orientation of the sites (ref. 14; P. Raychaudhuri, S. Bagchi, S.D.N., and J.R.N., personal communication), we assume that the *E4*-mediated stimulation involves protein–protein contacts. It does appear that the E4 protein can associate with E2F in the absence of DNA since highly purified E2F from infected cells does contain the E4 protein, as evidenced by the antibody shift experiment. In addition, the E4 antibody can deplete E2F from extracts (29). We find no evidence from the *in vitro* translation of *E4* orf 6/7 that the protein can bind to DNA independent of E2F. Thus, the most straightforward mechanism would involve the formation of a heteromeric complex of E2F with the E4 protein, which then interacts with the E2F recognition site. The presence of the E4 protein allows the adjacent complexes to interact so as to form a stable complex. The exact mechanism by which the association of the E4 protein promotes stable complex formation is unclear. One possibility is that the E4 protein itself possesses the ability to dimerize and then simply brings the adjacent E2F–E4 complexes together. Alternatively, the association of the E4 protein with E2F might alter E2F in such a way as to allow interaction between the two E2F molecules. Finally, we also do not know the details of the specificity of E4 action. This could be a viral-specific event such that E2F is the only target. Alternatively, other factors could also be targeted, allowing the formation of heteromeric stable complexes. The availability of an *in vitro* assay for the action of the E4 protein should allow experiments to answer these questions.

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