Genetic Analysis of the Adenovirus E4 6/7 *trans* Activator: Interaction with E2F and Induction of a Stable DNA-Protein Complex Are Critical for Activity

SUZANNE D. NEILL AND JOSEPH R. NEVINS*

Section of Genetics, Department of Microbiology and Immunology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

Received 6 May 1991/Accepted 8 July 1991

The adenovirus early E4 transcription unit encodes a 19-kDa polypeptide that *trans* activates transcription of the early E2 gene and is dependent on the binding sites for the E2F transcription factor. Biochemical assays have shown that the E4 protein, a product of the 6/7 open reading frame, interacts with the E2F transcription factor and alters its DNA binding characteristics, resulting in the formation of a very stable DNA-protein complex. We have generated a series of E4 mutants to determine the requirements for the interaction with E2F and the induction of a stable E2F complex on the E2 promoter in relation to the *trans* activation of E2 transcription. We find that the *trans*-activation function of E4 is dependent on the ability of the protein to interact with E2F and that full *trans* activation is dependent on the induction of the E2F stable complex. Interestingly, several mutants distinguish these events, since they retain the ability to interact with E2F but have lost the capacity to induce the stable complex. Since these mutants can still *trans* activate, albeit at reduced levels, these results suggest that the E4 protein contributes to *trans* activation that is independent of stable complex formation.

A variety of experiments have demonstrated the importance of protein-protein interactions in the control of transcription initiation. In many cases, the DNA binding capacity or the specific sequence recognition of a factor is dependent on the formation of a homodimer of the protein. In other cases, the functional DNA binding molecule is a heterodimer of two different polypeptides. Additionally, the capacity of a factor to activate transcription may be influenced by interactions with other proteins, including those that are postulated to bridge the promoter-binding factors, the so-called coactivators (24).

It is also true that viruses encode proteins that interact with cellular transcription factors to alter their function. For example, the herpesvirus VP16 protein has been shown to interact with the ubiquitous OTF-1 octamer binding factor (8, 18, 22, 23, 27). By so doing, the viral protein alters the DNA binding characteristics of OTF-1, allowing it to specifically recognize the TAATGARAT motif found in the promoters of the herpesvirus immediate-early genes. Moreover, the VP16 protein also contributes *trans*-activating potential to OTF-1.

In addition, there is strong evidence for an interaction between an early adenovirus gene product and a cellular transcription factor. The 6/7 open reading frame (ORF) of the adenovirus E4 transcription unit encodes a 19-kDa protein that is synthesized during the early phase of the lytic viral infection (6). Recent experiments have shown that in a lytic infection the E4 gene function is required for full activation of E2 transcription (1, 26). Moreover, transfection experiments have shown that the E4 gene, and more specifically an E4 6/7 cDNA, can function independently of other viral gene products to *trans* activate the E2 promoter (9, 20, 26). Biochemical analyses have shown that the E2F tran-

scription factor, a critical component for E2 transcription (15, 29), binds cooperatively to the adjacent sites in the E2 promoter (10, 11, 25), dependent on E4 6/7 function (13, 17, 20, 25). Moreover, the 19-kDa E4 protein has been found to physically interact with E2F and is a component of the E2F-promoter complex (13, 17, 20, 25). Although the E2F-DNA complex is quite unstable, the E2F-E4-DNA complex that forms on the adjacent E2F binding sites of the wild-type E2 promoter is very stable. Through an analysis of a series of mutations introduced into the E4 6/7 sequence, we sought to define the E4 requirements for the alteration of E2F DNA binding and in so doing to elucidate the role of these E4-mediated effects in the *trans* activation of the E2 promoter.

MATERIALS AND METHODS

Construction of molecular clones. Targeted nucleotide substitutions were introduced into the full-length cDNA copy of the E4 6/7 gene encoded by the previously described expression vector pE4 (6/7) (20) by using a bacteriophage M13-based oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.). The DNA sequence of the introduced mutations was confirmed by dideoxynucleotide sequencing (2). Each of the mutations introduced a unique *Bgl*II restriction site into the E4 6/7 gene sequence, resulting in the replacement of the boxed residues indicated in Fig. 1B with the amino acids arginine and serine. The *Bgl*II sites were all inserted in the same translational frame, permitting construction of the various N-terminal, C-terminal, and internal deletion mutants of E4 6/7 (Fig. 2). All of the mutant E4 6/7 cDNAs were subcloned into pGEM-1 (Promega Biotec) for use in in vitro transcription reactions and into the cytomegalovirus immediate-early promoter-based expression vector pBC12/CMV (5) as previously described for the wild-type E4 6/7 cDNA (20) for use in transfection assays. The secreted

* Corresponding author.

FIG. 1. Products of the E4 transcription unit. (A) Schematic depiction of the spliced E4 mRNA structures. Open boxes depict the ORFs. (B) Location of site-directed mutations introduced into the E4 ORF 6/7 gene. The E4 ORF 6/7 gene encodes a 150-amino-acid protein with the predicted sequence as shown. Missense mutations affecting two adjacent codons, as indicated by the boxed residues, were introduced by oligonucleotide-directed mutagenesis. Each mutation introduced a unique *Bgl*II restriction site into the ORF 6/7 gene sequence, resulting in the substitution of the indicated amino acids with arginine and serine. The mutations were named according to their location within E4 ORF 6/7, with S1 corresponding to the mutation closest to the amino terminus and S15 corresponding to the mutation closest to the carboxy terminus.

alkaline phosphatase expression vector (pBC12/CMV/SEAP), a gift from B. Cullen, was constructed from previously described plasmids (5). pE2CAT is the previously described plasmid pEC113 (14) containing E2 promoter sequences from positions -285 to +40 cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in pCAT3M.

In vitro transcription-translation. The mutant E4 ORF 6/7 cDNAs were subcloned into pGEM-1 (Promega Biotec). The vectors were linearized with *Bam*HI and used as templates for SP6 RNA polymerase in in vitro transcription reactions as described previously (20). The RNAs were translated in

nuclease-treated rabbit reticulocyte lysates (Promega Biotec) in the presence of [35 S]methionine. The relative levels of the 35 S-labeled proteins produced in vitro were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography and scanning densitometry.

Gel retardation assays. Gel retardation assay procedures were described previously (15). The DNA probes (Fig. 3A) contain E2 promoter sequences between positions -28 and -85 derived from the E2 promoter substitution mutants described by Loeken and Brady (16). Both probes are devoid of the ATF binding site. The single site probe contains only

FIG. 2. Schematic representation of the E4 mutants. Arrows depict the locations of the site-directed mutations (S1 through S15). The introduction of a unique *Bgl*II restriction site at each location facilitated the construction of N-terminal, C-terminal, and internal deletion mutants. Deletion mutants are named for the extent of the deletion, e.g., Δ 1-7 has a deletion between the introduced S1 and S7 mutations.

FIG. 3. Assays for E4 interaction with E2F and induction of altered E2F binding. (A) Schematic representation of DNA probes and the putative DNA-protein complexes that form. The probes are derived from the E2 promoter substitution mutants described by Loeken and Brady (16) and contain E2 promoter sequences between positions -28 and -85. Both probes lack the ATF site of the E2 promoter. The single site probe also lacks the distal E2F site. (B) Gel retardation assays for the formation of the E2F-E4-DNA complex and the stability of E2F-DNA interactions as a function of E4. Binding reactions utilized the indicated probe along with partially purified E2F from mock-infected cells with or without in vitro-translated wild-type E4 6/7 protein. After incubation at room temperature for 30 min, the time zero aliquot was removed. Then a 400-fold excess of the cold two-site probe was added to the remainder of the binding reactions. Aliquots were removed at the indicated times (minutes) and loaded directly onto a running gel. Thus, the sample from the zero time point has been in the gel 60 min longer than has the sample from the 60-min time point. (C) The E4 ORF 6/7 protein is a component of the E2F DNA complex. Binding reactions contained the indicated probe along with either mock E2F plus in vitro-translated wild-type E4 6/7 protein (a) or partially purified E2F from adenovirus type 5-infected cells (b and c). 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 in the text. +Competitor indicates the addition of excess unlabeled competitor DNA containing the E2F recognition sequence.

the proximal E2F binding site and lacks the distal E2F site and the ATF site. Mock E2F was partially purified from a HeLa cell whole-cell extract by gradient elution from a heparin-agarose column (29). E2F_{Ad}, the E2F preparation from adenovirus type 5-infected HeLa cells, was purified through a Mono Q column (29). Where indicated, antisera

were added to the gel shift reaction mixture (1 μ l of a 1:5 dilution per 30 μ l of binding reaction) after the normal 30-min binding reaction at room temperature, and the tubes were incubated on ice for an additional 60 min. The R3 and NR3 antisera were a gift from P. Hearing (6).

Cell culture and transfections. Vero cells, maintained in

Dulbecco modified Eagle medium with 10% fetal calf serum, were transfected with calcium phosphate DNA precipitates. The levels of plasmid DNAs used are indicated in the legend of Fig. 6. After 16 h, the precipitates were removed and incubation was continued for an additional 48 h. Extracts were prepared and assayed for CAT activity by the LSC-fluor diffusion assay with ^{14}C -labeled acetyl coenzyme A (21). The secreted alkaline phosphatase (SEAP) levels in the supernatant were determined as previously described (3). Within a given transfection experiment, the supernatant SEAP levels were normalized by setting the lowest value equal to 1. Protein levels for the CAT extracts were normalized in the same manner. CAT activities were corrected for the variability in SEAP and protein levels by dividing the value for CAT activity by the normalized values for SEAP and protein.

RESULTS

Generation of E4 mutants. The E4 6/7 open reading frame encodes a 150-amino-acid protein in which the first 58 amino acids derive from the ORF 6 sequence and the C terminal 92 amino acids derive from ORF 7 (Fig. 1). Mutations were introduced throughout the entire E4 6/7 sequence at the positions indicated in Fig. 1B. Through the introduction of a unique *Bgl*III restriction site into the E4 6/7 gene, each mutation replaced two adjacent codons with the codons for arginine and serine. Since each site-specific mutation created a unique restriction site, it was possible to generate a series of N-terminal and C-terminal deletions as well as various internal deletions (Fig. 2).

In vitro assays for E2F-E4 interactions and alteration of E2F DNA binding. Our assay for measuring the interaction of the E4 protein with E2F relies on the in vitro system described previously (20). The E2F factor used for these assays is a partially purified preparation derived from HeLa cell extracts. The E4 protein is synthesized in a reticulocyte lysate programmed by an SP6 transcript from the E4 6/7 cDNA. Two DNA probes have been used for the E2F binding assays (Fig. 3A). A probe containing a single E2F binding site (one site probe) allows E2F binding but does not allow stable complex formation. The interaction of the E4 protein with E2F on a one-site probe can be observed as the formation of a somewhat slower migrating complex that is also unstable. This property is easily observed in an off-rate measurement (25) (Fig. 3B). Direct evidence that this slower-migrating complex is due to the interaction of the E4 protein with E2F is provided by an antiserum that recognizes the E4 19-kDa protein. The addition of the E4-specific antiserum eliminates the E2F/E4 complex and generates a more slowly migrating complex, whereas a preimmune serum had no effect (Fig. 3C).

When a two-site E2F probe is employed, the interaction of E4 with E2F allows cooperative binding and the formation of a stable complex, as demonstrated by the off-rate measurement (Fig. 3B). Once again, the addition of the E4-specific antiserum shifts this complex to a more slowly migrating form (Fig. 3C), a result seen in previous experiments in which the complex was formed with in vitro-produced E4 (20), confirming the presence of the E4 protein in the complex. Although we sometimes observe the E2F-E4 complex migrating as a doublet [(E2F-E4)₂], as in this particular assay, the lower, faster-migrating band is variable from assay to assay and may represent partial breakdown products of E2F or of the complex. Thus, through the use of a probe that contains a single E2F binding site and a probe that

contains two E2F binding sites, we can distinguish between the ability of the E4 6/7 protein to interact with E2F and its ability to stimulate cooperative binding and stable complex formation.

Separate requirements for E2F interaction and stable complex formation. The various E4 6/7 mutant proteins, synthesized in vitro in reticulocyte lysates, were assayed for their ability to interact with E2F and for their ability to stimulate formation of the stable, two-site complex. We began with an analysis of the N-terminal and C-terminal truncations. Deletion of the first 64 amino acids at the N terminus of the protein (Δ 1-7 mutant), which deletes all of ORF 6, had no effect on either the interaction with E2F (formation of the E2F-E4 complex) or the induction of the stable complex [the (E2F-E4)₂ complex] (Fig. 4A). Clearly, the amino acids derived from ORF 6 are not essential for this activity of E4.

In sharp contrast to the N-terminal mutants, each of the C-terminal deletion mutants was deficient in the induction of the stable E2F complex (Fig. 4A). Moreover, there was no indication that any of these mutants could even interact with E2F, as evidenced by the inability to form the E2F-E4 complex. Clearly, the C terminus of the E4 protein (and thus the ORF 7 sequences) is essential for the interaction of the protein with E2F.

Assays of the entire series of point mutants indicated that most of the alterations had no measurable effect on the formation of the stable E2F complex (Fig. 4B). Exceptions were the C-terminal S13 and S14 mutants. Both were impaired, although not completely deficient, in their ability to form the stable, two-site complex. Moreover, although S13 retained the ability to interact with E2F, as indicated by the formation of the E2F-E4 complex, the S14 mutant did not show evidence of interaction with E2F on a single site.

Further assays of substitution and internal deletion mutants, employing probes containing two E2F sites (Fig. 5A) or one E2F site (Fig. 5B), extended these results and demonstrated that, indeed, the region between residue 70 and the C terminus of the protein was important for the interaction with E2F and the formation of the stable two-site complex. Assays with the one-site probe, to measure formation of the E2F-E4 complex, defined two separate regions in the C terminus that are essential for E2F interaction. One region is defined by mutations surrounding S14 and includes the deletion mutants Δ 13-14 and Δ 14-15 as well as the S14 mutant. The second region is defined by the deletion mutations surrounding S10 and includes Δ 9-10 and Δ 10-11. All other mutants, including a Δ 8-9 mutant (data not shown), were found to interact with E2F in a manner equivalent to that of the wild type.

As noted above, the S14 mutant did not form the E2F-E4 complex, but it did promote stable complex formation, albeit at a much reduced level compared with that of the wild type. The small amount of two-site complex formed with the S14 mutant was stable in an off-rate assay (data not shown), and the addition of the E4 6/7-specific antiserum eliminated this complex and generated a more slowly migrating complex (Fig. 5C). Thus, although the level of stable complex formed with the S14 mutant is reduced, the complex is authentic and does contain the E4 protein. Further evidence that this low level of stable complex formation was significant was provided by a comparison with the Δ 10-11, Δ 13-14, and Δ 14-15 deletions (Fig. 5D). These mutants also do not interact with E2F, but, in contrast to S14, they were not able to form the stable complex. Finally, similar to the S14 mutant, the Δ 9-10 deletion mutant failed to interact with E2F on the single-site probe but did form the stable two-site complex. In contrast

FIG. 4. The C-terminal ORF 7 sequence is essential for E2F interaction. (A) The E4 6/7 truncation mutants were assayed for E2F interaction and stable complex formation by gel retardation. Binding reactions utilize the two-site probe along with partially purified E2F from mock-infected cells plus 1 μ l of the indicated in vitro-translated E4 mutants. (B) The E4 6/7 point mutations were assayed as described for panel A.

to the S14 mutant, however, the Δ 9-10 mutant did so at nearly wild-type efficiency. These results suggest that the impaired ability of these mutant proteins to interact with E2F is stabilized in the two-site complex.

The Δ 11-12 and Δ 12-13 deletion mutants were similar to the S13 mutant in that they were able to form the E2F-E4 complex with wild-type efficiency. But whereas S13 did promote stable complex formation, albeit at reduced levels (Fig. 5A), Δ 11-12 and Δ 12-13 were completely negative in this regard (Fig. 5D). Thus, the Δ 11-12 and Δ 12-13 mutations appear to define a function for E4 in promoting stable complex formation that is distinct from its ability to interact with E2F.

From these results we conclude that, although the N

terminus of the E4 6/7 product is completely dispensable for activity relating to interaction with E2F, the C terminus is absolutely essential. In general, the mutations appear to define two distinct phenotypes: the ability to interact with E2F and the capacity to form the stable (E2F-E4)₂ complex. The region essential for stable complex formation appears to lie in between the two regions required for E2F interaction.

trans activation by E4 is dependent on E2F interaction. The product of the E4 6/7 cDNA has been shown to *trans* activate the E2 promoter in cotransfection assays (20). The availability of E4 mutants that alter the interaction with E2F and stable complex formation now allows a definition of the role of these biochemical events in the *trans*-activation process. Vero cells were cotransfected with each of the

FIG. 5. Definition of E4 6/7 sequences required for E2F interaction and stable binding. (A) Gel retardation assays of internal deletion mutants and site-directed mutants on the two-site probe. Binding reactions contained the indicated probe together with mock E2F and 1 μ l of each of the in vitro-translated E4 mutants as shown. (B) Gel retardation assays of internal deletion mutants and site-directed mutants on the one-site probe. (C) E4 is a component of the stable two-site complex formed by the S14 mutant. Binding reactions contained the two-site probe, mock E2F, and 2 μ l of the in vitro-translated S14 mutant. Incubations with antisera were as described in the legend to Fig. 3C. (D) The S14 mutant can form the two-site complex. Binding reactions contained the two-site probe, mock E2F, and 2 μ l of the indicated in vitro-translated mutants.

FIG. 6. The C-terminal ORF 7 sequence is essential for *trans* activation of the E2 promoter. Vero cells were transfected with 5 μ g of pE2CAT either alone with increasing amounts (4-fold increments starting with 0.63 μ g) of the expression vector encoding the indicated E4 mutants; i.e., 16-fold means co-transfection of 10 μ g of the indicated plasmid construction. A SEAP gene expression vector, pBC12/CMV/SEAP (1 μ g), was cotransfected as an internal control for transfection efficiency. pGEM4 carrier DNA was included to maintain total plasmid DNA at 25 μ g/ml in the calcium phosphate precipitates. Cells were harvested for LSC-fluor diffusion CAT assays as described previously (21). The units of CAT activity are counts per minute of [14 C]acetylchloramphenicol produced per minute; the scale is in thousands. The values presented here are corrected for the variability observed in supernatant SEAP levels and protein levels in the cell extracts as described in Materials and Methods.

mutant E4 6/7 expression vectors together with a reporter plasmid containing the E2 promoter linked to the bacterial CAT gene. In addition, a plasmid containing the SEAP reporter gene linked to the cytomegalovirus immediate-early promoter was included as an internal control for transfection efficiency. Western analysis of extracts from COS cells cotransfected with the mutant E4 6/7 expression vectors revealed that each of the mutant E4 proteins is stably expressed at a level comparable to that of the wild type (data not shown).

Assays of the N-terminal deletion mutants indicated that this portion of the protein was not essential for *trans*-activation function, since deletion of the N-terminal 64 amino acids (Δ 1-7) had little, if any, effect on activity (Fig. 6A). Moreover, the individual point mutations spanning this region (S1 through S8) exhibited wild-type activity (data not shown).

In contrast, each of the C-terminal truncations was inactive in *trans* activation of the E2 promoter (Fig. 6B). Thus, the C terminus of the E4 6/7 product, including the extreme terminal sequence as defined by the Δ 14-15 mutant, is absolutely essential for both E2F interaction as well as for *trans* activation of the E2 promoter. Analyses of the various point mutants and internal deletions within the C-terminal region are shown in Fig. 7. The S12, S13, and S14 mutants were impaired in *trans*-activation function but did retain approximately 50% of the wild-type activity. The same result was also true for the internal deletion mutants Δ 11-12 and Δ 12-13. Each of these mutants produced proteins that could interact with E2F. In contrast, the Δ 13-14 and Δ 14-15 deletions, which encoded proteins that failed to interact with E2F, were completely inactive in *trans* activation of the E2 promoter. This was also true for the Δ 10-11 mutant, which again failed to interact with E2F. Finally, the Δ 9-10 and the S10 mutants were functional in *trans* activation but exhibited a reproducible sharp decline in activity at high input levels. Apparently, the mutant proteins interfere with transcription at high concentrations.

The clear result from these assays is that an E4 protein that cannot interact with E2F, as exemplified by the Δ 10-11, Δ 13-14, and Δ 14-15 mutants, is also inactive for *trans* activation, whereas E4 proteins that are wild type with respect

to E2F binding and stable complex formation are also wild type for *trans* activation. Moreover, mutants that can still interact with E2F but do not allow stable complex formation (Δ 11-12 and Δ 12-13) are reduced in *trans*-activation function. Thus the capacity to form the stable E2F complex is tightly linked to *trans* activation. Nevertheless, it is also true that E4 *trans*-activation function is not obligatorily dependent on the formation of the stable complex, indicating that another property of E4 may contribute to *trans* activation.

DISCUSSION

The cellular E2F transcription factor is a critical component of the functional transcription complex that drives adenovirus E2 transcription and is a target for *trans* activation by the E1A gene product (15, 29). At least a part of the E1A activation event is direct and appears to involve an increase in the level of active E2F (free E2F that can bind to DNA) (25). E1A also has an indirect effect, since a series of experiments has shown that full E2 transcription requires the expression of the early E4 gene, which itself is E1A inducible (1, 26). Finally, recent experiments have shown that E1A can also release E2F from cellular interactions that prevent the association with the E4 protein (1a). It would thus appear that in a lytic infection the activation of E2 transcription is a result of the combined effects of E1A and E4 on the E2F factor. Various studies have shown that the E4 protein converts the E2F factor to a form that binds to the E2 promoter in a cooperative manner and is dependent on the arrangement of the adjacent E2F recognition elements in the promoter (10, 11, 25). Moreover, the E4 protein has been shown to directly interact with E2F and is found in the stable DNA-protein complex (13, 17, 20). The experiments we present here demonstrate that the interaction of E4 with E2F is indeed critical for transcription activation by E4. Without exception, mutants that failed to interact with E2F in any demonstrable fashion also failed to *trans* activate (Fig. 8), strongly suggesting that this interaction is critical for function.

The phenotypes of the E4 6/7 mutants clearly demonstrate that the N terminus, including all sequences derived from ORF 6, is completely dispensable for the interaction with

FIG. 7. Definition of E4 6/7 sequences required for *trans* activation. Vero cells were transfected with pECAT either alone or together with expression vectors encoding the indicated E4 mutants as described in the legend to Fig. 6.

E2F and stable complex formation and for *trans* activation of the E2 promoter (Fig. 8). It would appear that ORF 6 is required solely for the purpose of expression, providing the ATG for translation initiation. However, the C terminus of the E4 6/7 protein is absolutely essential for wild-type function. The C terminus can be subdivided into two regions required for interaction with E2F, which flank a third region required for stable two-site complex formation (Fig. 9). This conclusion is particularly evident from the analysis of mutants such as $\Delta 11-12$ and $\Delta 12-13$, which demonstrate that interaction with E2F can be separated from the formation of the stable complex.

In two cases, the S14 mutant and the $\Delta 9-10$ mutant, there was no apparent formation of the one-site E2F-E4 complex, whereas there was formation of the stable complex. In the case of the S14 mutant, the level of stable complex formed was markedly reduced compared with that of the wild type,

but for the $\Delta 9-10$ mutant, the level was only slightly reduced compared with that of the wild type. The fact that the stable complex is generated, together with the direct evidence of the presence of E4 provided by the E4 antisera, demonstrates that these mutant E4 proteins do associate with E2F. We can only speculate as to the reason for the lack of one-site complex formation with these mutants, but it surely must reflect a reduced capacity to interact with E2F while retaining the capacity for stable complex formation. This indirect indication that the events of E2F interaction and stable complex formation are distinct functions is strongly supported by the identification of mutants, such as $\Delta 11-12$ and $\Delta 12-13$, that form the E2F-E4 complex but do not form the stable complex. We therefore suggest that distinct regions of the E4 6/7 protein are required for interaction with E2F and for stimulation of cooperative binding.

A computer-assisted analysis of the E4 ORF 7 sequence

FIG. 8. Summary of phenotypes of the E4 mutants. E2F interaction and E2F stable complex formation phenotypes are based on the ability to form the E2F-E4 complex and the (E2F-E4)₂ complex, respectively, as determined by gel retardation assays (Fig. 4, 5, and 6). *trans*-activation phenotypes were determined as described in the legends to Fig. 6 and 7. E2F interaction: +, comparable to that of the wild type; -, not detectable. E2F stable complex: ++, comparable to that of the wild type; +, reduced levels of authentic complex formed; -, not detectable. *trans* activation: ++, comparable to that of the wild type; +, 40 to 60% of the wild-type level; -, ≤5% of the wild-type value. ND, not done. ^a, The S10 and the Δ9-10 mutants *trans* activated like the wild type at low plasmid concentrations but exhibited a reproducible sharp decline in activity at high plasmid concentrations.

suggests that a helix-loop-helix domain may be important for the ability of E4 to promote dimerization of E2F (Fig. 9). The Δ12-13 mutant deletes helix 2, whereas the Δ11-12 mutant deletes the loop and a portion of helix 1. These mutants abolish formation of the stable two-site complex but do not impair the interaction of E4 with E2F. Sequences removed by the Δ10-11 deletion, which includes the remainder of helix 1, are required for interaction with E2F. Thus, helix 1 may contribute to the interaction with E2F in addition to allowing formation of the stable two-site complex. There are, of course, numerous examples of the involvement of helix-loop-helix domains in the formation of protein dimers, both homodimers and heterodimers. Since the formation of the

stable two-site complex on the E2 promoter clearly involves the dimerization of E2F and clearly requires the E4 protein, we suggest that the putative helix-loop-helix domain of the E4 protein may be critical for this event. Sequences in E4 that flank the helix-loop-helix domain appear to be involved in the interaction of E4 with the E2F molecule.

In general, the *trans*-activation phenotypes of the E4 6/7 mutants correlate well with the E2F interaction phenotypes (Fig. 8). Mutants that are able to form the stable, two-site complex *trans* activate as well as the wild type. Mutants that interact with E2F but fail to form the cooperatively binding complex *trans* activate 50% as well as the wild type. Finally, mutants that fail to interact with E2F also fail to *trans*

FIG. 9. Schematic depiction of the E4 protein and regions of functional importance. A putative helix-loop-helix domain, predicted by a computer analysis by the method of Garnier et al. (7), is schematically depicted with respect to E4 ORF 7 sequence. Shown at the bottom are the regions of the protein that are essential for full wild-type function (E2F interaction, stable complex formation, *trans* activation) as well as the separate regions involved in E2F interaction and induction of the stable complex.

activate the E2 promoter. The fact that a significant level of *trans* activation by the E4 6/7 product is achieved in the absence of stable E2F complex formation suggests that *trans* activation by E4 may involve two events. Clearly, the formation of the stable E2F complex on the E2 promoter is one important component of the *trans* activation. We can only speculate on the potential role of a second event that is dependent on an interaction with E2F but does not involve stable complex formation. In the most straightforward scenario the E4 protein would be similar to the herpesvirus VP16 protein, providing a transcriptional activation domain to E2F. In specific terms, this might involve a facilitation of interactions with other components of the transcription complex. If this is the case, it certainly cannot be an absolute requirement since a variety of studies suggest that E2F, independent of E4, can activate transcription.

Finally, these analyses that reveal a molecular mechanism for the action of a viral regulatory protein in stimulating transcription also provide insight into the evolution of viral strategies for enhancing gene expression. Although a variety of cellular promoters have now been identified that utilize the E2F factor (12, 19, 28), it is only on the E2 promoter that the E2F-E4 complex can bind cooperatively to form the stable complex. It appears to be the particular arrangement of E2F sites in the E2 promoter that is critical for the formation of the stable complex (11, 25). In no other instance are the E2F sites arranged in this fashion; the E2F-E4 complex can bind to sites in the *c-myc* promoter, but the spatial arrangement does not allow the stable complex to form. Therefore, the net effect of E4 action, the formation of a stable E2F complex on the E2 promoter, appears to be limited to the E2 promoter (12, 19). In short, the E2F factor is converted into an E2 promoter-specific factor as a result of the association with the E4 protein. Since we have found no evidence to indicate an E4 interaction with other cellular transcription factors, this process may represent the evolution of a viral function to recruit a cellular transcription factor for the specific purpose of enhancing E2 transcription.

ACKNOWLEDGMENTS

We thank Pat Hearing for the gift of E4-specific antiserum, Richard Randall and Beth Jones for the synthesis of oligonucleotides, and Laszlo Jakoi for supplying the purified E2F.

This work was supported by Public Health Service grant GM 26765 from the National Institutes of Health.

REFERENCES

- Babiss, L. E. 1989. The cellular transcription factor E2F requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression. *J. Virol.* **63**:2709-2717.
- Bagchi, S., P. Raychaudhuri, and J. R. Nevins. 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A *trans*-activation. *Cell* **62**:659-669.
- Bankier, A. T., and B. G. Barrell. 1983. Shotgun DNA sequencing. *Tech. Life Sci.* **85**:1-33.
- Berger, J., J. Hauber, R. Hauber, R. Geiger, and B. Cullen. 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66**:1-10.
- Blake, M. C., and J. C. Azizkhan. 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. *Mol. Cell. Biol.* **9**:4994-5002.
- Cullen, B. R. 1986. *trans*-Activation of human immunodeficiency virus occurs via a bimodal mechanism. *Cell* **46**:973-982.
- Cutt, J. R., T. Shenk, and P. Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **61**:543-552.
- Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**:97-120.
- Gerster, T., and R. G. Roeder. 1988. A herpesvirus *trans*-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc. Natl. Acad. Sci. USA* **85**:6347-6351.
- Goding, C., P. Jalinot, D. Zajchowski, H. Boeuf, and C. Kedinger. 1985. Sequence-specific *trans*-activation of the adenovirus E1A early promoter by the viral E1V transcription unit. *EMBO J.* **4**:1523-1528.
- Hardy, S., D. A. Engel, and T. Shenk. 1989. An adenovirus early region 4 gene product is required for induction of the infection-specific form of cellular E2F activity. *Genes Dev.* **3**:1062-1074.
- Hardy, S., and T. Shenk. 1989. E2F from adenovirus-infected cells binds cooperatively to DNA containing two properly oriented and spaced recognition sites. *Mol. Cell. Biol.* **9**:4495-4506.
- Hiebert, S. W., M. Lipp, and J. R. Nevins. 1989. E1A-dependent *trans*-activation of the human MYC promoter is mediated by the E2F factor. *Proc. Natl. Acad. Sci. USA* **86**:3594-3598.
- Huang, M. M., and P. Hearing. 1989. The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev.* **3**:1699-1710.
- Imperiale, M. J., R. P. Hart, and J. R. Nevins. 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A induced transcription. *Proc. Natl. Acad. Sci. USA* **82**:381-385.
- Kovesdi, I., R. Reichel, and J. R. Nevins. 1986. Identification of a cellular transcription factor involved in E1A *trans*-activation. *Cell* **45**:219-228.
- Loeken, M. R., and J. Brady. 1989. The adenovirus E1A enhancer: analysis of regulatory sequences and changes in binding activity of ATF and E1F following adenovirus infection. *J. Biol. Chem.* **264**:6572-6579.
- Marton, M. J., S. B. Baim, D. A. Ornelles, and T. Shenk. 1990. The adenovirus E4 17-kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRNA. *J. Virol.* **64**:2345-2359.
- McKnight, J. L., T. M. Kristie, and B. Roizman. 1987. Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. *Proc. Natl. Acad. Sci. USA* **84**:7061-7065.
- Mudryj, M., S. W. Hiebert, and J. R. Nevins. 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation-dependent signal transduction pathway. *EMBO J.* **7**:2179-2184.
- Neill, S. D., C. Hemstrom, A. Virtanen, and J. R. Nevins. 1990. An adenovirus E4 gene product *trans*-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F. *Proc. Natl. Acad. Sci. USA* **87**:2008-2012.
- Neumann, J. R., C. A. Morency, and K. O. Russian. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* **5**:444-447.
- O'Hare, P., C. R. Goding, and A. Haigh. 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO J.* **7**:4231-4238.
- Preston, C. M., M. C. Frame, and M. E. Campbell. 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. *Cell* **52**:425-434.
- Pugh, B. F., and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**:1187-1197.
- Raychaudhuri, P., S. Bagchi, S. D. Neill, and J. R. Nevins. 1990.

- Activation of the E2F transcription factor in adenovirus infected cells involves an E1A-dependent stimulation of DNA binding activity and induction of cooperative binding mediated by an E4 gene product. *J. Virol.* **64**:2702–2710.
26. **Reichel, R., S. D. Neill, I. Kovesdi, M. C. Simon, P. Raychaudhuri, and J. R. Nevins.** 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for *trans* activation of E2 transcription and for E2F activation. *J. Virol.* **63**:3643–3650.
27. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeo-domain directs formation of a multiprotein-DNA complex with the HSV *trans*-activator VP16. *Nature (London)* **341**:624–630.
28. **Thalmeier, K., H. Synovzik, R. Mertz, E. L. Winnacker, and M. Lipp.** 1989. Nuclear factor E2F mediates basic transcription and trans-activation by E1A of the human MYC promoter. *Genes Dev.* **3**:527–536.
29. **Yee, A. S., P. Raychaudhuri, L. Jakoi, and J. R. Nevins.** 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Mol. Cell. Biol.* **9**:578–585.