Domains of the Adenovirus E1A Protein Required for Oncogenic Activity are also Required for Dissociation of E2F Transcription Factor Complexes

Stephen Devoto
Wesleyan University, sdevoto@wesleyan.edu

Follow this and additional works at: http://wesscholar.wesleyan.edu/div3facpubs

Part of the Life Sciences Commons

Recommended Citation
Devoto, Stephen, "Domains of the Adenovirus E1A Protein Required for Oncogenic Activity are also Required for Dissociation of E2F Transcription Factor Complexes" (1991). Division III Faculty Publications. Paper 55.
http://wesscholar.wesleyan.edu/div3facpubs/55

This Article is brought to you for free and open access by the Natural Sciences and Mathematics at WesScholar. It has been accepted for inclusion in Division III Faculty Publications by an authorized administrator of WesScholar. For more information, please contact dschnaidt@wesleyan.edu, ljohson@wesleyan.edu.
Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes.

P Raychaudhuri, S Bagchi, S H Devoto, et al.

*Genes Dev.* 1991 5: 1200-1211
Access the most recent version at doi:10.1101/gad.5.7.1200

This article cites 57 articles, 33 of which can be accessed free at: [http://genesdev.cshlp.org/content/5/7/1200.refs.html](http://genesdev.cshlp.org/content/5/7/1200.refs.html)

Article cited in: [http://genesdev.cshlp.org/content/5/7/1200#related-urls](http://genesdev.cshlp.org/content/5/7/1200#related-urls)

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](http://genesdev.cshlp.org/subscriptions)
Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes

Pradip Raychaudhuri, Srilata Bagchi, Stephen H. Devoto, Virginia B. Kraus, Elizabeth Moran, and Joseph R. Nevins
Howard Hughes Medical Institute, Section of Genetics, Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710 USA; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA

Recent experiments have shown that the cellular E2F transcription factor is found in complexes with cellular proteins and that one such complex contains the cyclin-A protein. Isolation of a cellular activity, which we term E2F-BF, can reconstitute the E2F–cyclin-A complex and has permitted a more detailed analysis of the mechanism of E1A dissociation. Through the analysis of a series of E1A mutants, we find that sequences in conserved region 1 (CR1) and conserved region 2 (CR2) are important for dissociation of the E2F complex, whereas amino-terminal sequences are not required. In contrast to the requirements for dissociation, only the CR1 sequences are required to block formation of the complex if E1A is added when the components are combined. We have also identified an activity, termed E2F-I, that inhibits E2F binding to DNA, again apparently through the formation of a complex with E2F. This inhibitory activity is also blocked by E1A, dependent on the same elements of the E1A protein that disrupt the interaction with E2F-BF. Because the E1A sequences that are important for releasing E2F from these interactions are also sequences necessary for oncogenesis, we suggest that this activity may be a critical component of the transforming activity of E1A.

[Key Words: E2F-BF; Cyclin A adenovirus E1A protein; oncogenic activity; E2F transcription factor]

Received January 16, 1991; revised version accepted May 9, 1991.

The control of transcription initiation of RNA polymerase II-transcribed genes is a complex process involving the interaction of multiple DNA-binding proteins with specific regulatory sequences within promoter and enhancer elements (for review, see Mitchell and Tjian 1989). Moreover, it is now clear that the proteins that bind to these elements do not always do so as simple monomers, rather, multicomponent complexes are often the active form. For instance, during a herpes simplex virus infection, the virus-encoded VP16 protein associates with the cellular Oct-1 transcription factor to direct transcription from various immediate early herpesvirus promoters (McKnight et al. 1987; Gerster and Roeder 1988; O’Hare and Coding 1988; Preston et al. 1988). As another example, the active form of the serum response factor (SRF), which mediates a stimulation of fos transcription following serum stimulation, appears to be a multicomponent complex (Shaw et al. 1989). Finally, recent studies have suggested the involvement of accessory factors that may bridge the interactions between various upstream binding factors as well as with basal factors such as TFIID (Pugh and Tjian 1990).

A series of recent experiments have demonstrated that a product of the adenovirus early E4 transcription unit forms a complex with the cellular E2F transcription factor to allow cooperative binding at the E2 promoter (Hardy et al. 1989; Huang and Hearing 1989; Reichel et al. 1989; Marton et al. 1990; Neill et al. 1990; Raychaudhuri et al. 1990). The binding of E2F/E4 complexes at adjacent promoter sites generates a very stable DNA complex and ultimately a stimulation of E2 transcription. The observation that adenovirus E4 protein can interact with E2F to alter its function suggests that there may be cellular proteins that also interact with E2F. Indeed, recent experiments have shown this to be the case (Bagchi et al. 1990). Although the precise function of E2F complexes within the cell is not clear, they are significant for a viral infection in that they prevent interaction with the E4 protein. Perhaps the most intriguing aspect of these recent studies is the finding that the adenovirus E1A protein can dissociate E2F from these cellular complexes (Bagchi et al. 1990). This dissociation then allows
the E4 protein to interact with E2F and form the stable promoter complex.

In the absence of a viral infection, we believe that the E2F interactions may influence the activity of E2F in relation to cell proliferation controls. E2F-binding sites can be found in regulatory regions of several genes that respond to serum addition (Mudryj et al. 1990), and mutagenesis has shown the importance of the E2F sites in the c-myc promoter [Lipp et al. 1987; Hiebert et al. 1989; Mudryj et al. 1990], as well as the dihydrofolate reductase [DHFR] promoter [Blake and Azizkhan 1989]. E2F-binding activity changes dramatically following serum stimulation. There is an initial rise in the overall level of E2F as cells progress from a quiescent state to a growing state [Mudryj et al. 1990]. As the cell proceeds through G1 and into S phase, there are changes in the interactions involving E2F, as judged by gel mobility-shift assays [Mudryj et al. 1991]. Clearly, an alteration in the normal interaction involving this cellular transcription factor could alter normal cellular gene expression. This becomes a particularly relevant issue considering that the initial assays indicated that the 243-amino-acid E1A product, which is sufficient for E1A-dependent cell transformation, was capable of this activity. We have now explored this question further by analyzing a series of E1A mutants to precisely define the essential sequences. The results define a requirement for separate regions of the protein in distinct events and suggest a correlation between this activity and E1A-mediated transformation.

Results

E1A protein sequences within conserved regions 1 and 2 are required for dissociation of the E2F complex

Our recent experiments have demonstrated that the activity of the adenovirus E1A protein that mediates a dissociation of complexes containing the E2F transcription factor is independent of E1A conserved region 3 (CR3) but dependent on conserved region 2 (CR2) [Bagchi et al. 1990]. To define more precisely the E1A protein sequences necessary for the activity and, therefore, to provide information as to the relationship with other known activities of the E1A protein, we have made use of a collection of mutants that target various portions of the 243-amino-acid protein product of the 12S E1A mRNA. As depicted in Figure 1, these mutants alter the amino terminus of the protein, conserved region 1 (CR1), CR2, and the sequence between the two conserved regions. Each mutant was constructed in the form of a cDNA in a pGEM vector that could be transcribed in vitro by SP6 RNA polymerase. The RNA products were then translated in a reticulocyte lysate to produce the corresponding proteins. Each reticulocyte lysate was then assayed for E2F-dissociating activity by incubation with an L-cell whole-cell extract, as described in previous assays [Bagchi et al. 1990].

As we have reported previously [Bagchi et al. 1990] and as shown in Figure 2, lane 3, the wild-type 12S E1A product was able to dissociate the E2F complex (E2Fc), as indicated by the disappearance of the slowly migrating gel complex (E2Fc) and the appearance of the faster mobility complex characteristic of the interaction of a single E2F molecule with the DNA [E2F]. In contrast, addition of a reticulocyte lysate programmed with a control bromo mosaic virus (BMV) RNA had no effect on E2Fc [lane 2]. The two E1A amino-terminal mutants were also capable of dissociating the complex [lanes 4 and 5], as were two point mutants in CR1 [lanes 8 and 9]. Furthermore, two deletion mutants that removed sequence between CR1 and CR2 [lanes 10 and 11] also showed full wild-type activity. In contrast, the two deletion mutants within CR1 [lanes 6 and 7], as well as the two point mutants within CR2 [lanes 12 and 13], were inactive in dissociating E2Fc. An analysis of the in vitro translation products demonstrated that there was roughly equal synthesis of each of the mutant proteins [data not shown]. We thus conclude that sequences within both CR1 and CR2 are required for the E2F-dissociating activity, whereas sequences at the amino terminus of the protein, as well as sequences between CR1 and CR2, are not required.

CR1 and CR2 are required for trans-activation

Our previous experiments have shown that a plasmid expressing the 12S E1A product can trans-activate an
Raychaudhuri et al.

Figure 2. Dissociation of E2F by wild type and mutants of E1A. E2F-binding activity was measured in the L-cell whole-cell extract (lane 1) or after the addition of 1 μl of a 1:5 dilution of reticulocyte lysate programmed with 12S E1A mRNA (WT E1A) or the mRNAs derived from the mutants as described in Fig. 1. Reticulocyte lysates programmed with BMV RNA were used as control.

E2F-dependent promoter but that this activity is dependent on the cell line (Bagchi et al. 1990). That is, there was little if any trans-activation in HeLa cells, whereas the trans-activation in L cells was only slightly less efficient than that of the 13S E1A product. This result correlates with the presence or absence of E2F in complexes, thus providing an explanation for variable results of many past experiments measuring E1A trans-activation because most of these experiments utilized HeLa cells, cells in which E2F is largely in a free, uncomplexed state. These results would also suggest that complexed E2F is inhibitory for E2 transcription.

Although these experiments demonstrated a requirement for CR2 sequence for this activity, because the point mutation 928 was defective, they did not define precisely the E1A sequence requirements. Because the results of Figure 2 clearly demonstrate a role for CR1 in addition to CR2 in the dissociation of E2F complexes, we have now assayed several of these additional mutants for trans-activating function. L cells were transfected with the pA10CAT-E2F plasmid alone or in combination with wild-type 12S E1A or the mutants indicated. Extracts were prepared after 48 hr and assayed for chloramphenicol acetyltransferase (CAT) activity (Fig. 3). Clearly, both of the domain 1 mutants were completely defective for trans-activation. The domain 2 mutant 961 was also impaired, although reproducibly not as much as the domain 1 mutants, a result observed previously for one other CR2 mutant, 928 (Bagchi et al. 1990). In contrast, an amino-terminal mutant [Δ15-35], as well as a mutant that deleted sequence between domains 1 and 2 [Δ86-120], had no effect on the trans-activating function. We therefore conclude that both E1A domains 1 and 2 are required for dissociation of the E2F complexes, as well as for full trans-activation of a promoter dependent on E2F for activity. In the absence of CR2, some trans-activation persists, although it is severely reduced.

Isolation of an E2F-binding factor that reconstitutes a cyclin A-containing E2Fc

Although the results presented thus far provide insight into the E1A requirements for dissociation of E2Fc, a better understanding of the mechanism of E1A action clearly requires the ability to manipulate the system using purified components. We have thus sought to isolate the factor that forms a complex with E2F so as to be able to reconstitute the association of the components in vitro.

Because virtually all of the E2F detected in extracts of mouse L cells appeared to be complexed to a cellular factor(s) (Bagchi et al. 1990), we reasoned that there might be an excess of the factor that forms the complex with E2F in these cells. Indeed, fractionation of an L-cell extract by heparin-agarose chromatography revealed an activity in a 0.25 M KCl eluate that could interact with E2F to generate a complex that migrated at a position typical of the complex detected in crude extracts (Fig. 4A). The 0.25 M fraction possessed no E2F-binding activity of its own, and the endogenous E2F was found to elute in the 0.75 M KCl wash (data not shown). It thus appears that an E2F-binding factor [E2F-BF] can be detected in the 0.25 M eluate. We have further purified the E2F-BF by chromatography of the heparin-agarose 0.25 M

Figure 3. Trans-activation by wild-type E1A and E1A mutants. The pA10CAT-E2F plasmid, which exhibits an E2F-dependent response to E1A, was used as a reporter gene for measuring E2F-dependent trans-activation. L cells were transfected with the pA10CAT-E2F plasmid, either alone or together with the wild-type 12S E1A-expressing plasmid, or the 12S E1A plasmids bearing the various mutations. Extracts were prepared and CAT activity was measured as described in Materials and methods.
Dissociation of E2F complexes

Figure 4. Identification of E2F-BF in L-cell extracts. (A) L-cell extracts were fractionated on a heparin-agarose column as described in Materials and methods. A 0.25 M eluate (2 μg) from the column (HA 0.25 M), which is free of any E2F-binding activity, was assayed alone (lane 3) or after incubation with a sample of E2F (lane 2). Assay of the E2F sample alone is shown in lane 1. (B) Glycerol gradient sedimentation. Peak fractions from hydroxyapatite chromatography of the E2F-BF preparation [see Materials and methods] were concentrated and analyzed by sedimentation in a 12–35% glycerol gradient as described. Individual fractions were assayed for E2F-BF activity. A gradient containing the indicated marker proteins was run in parallel.

fraction on DEAE-Sepharose followed by hydroxyapatite. Sedimentation of the hydroxyapatite-purified E2F-BF in a glycerol gradient yielded a single peak of activity with an estimated molecular mass of ~180 kD (Fig. 4B). Whether this represents a single polypeptide or multiple components is not yet clear.

Our recent experiments have identified the cyclin-A protein as a component of an E2F complex found during S phase in 3T3 cells, as well as in asynchronously growing cells [Mudryj et al. 1991]. As shown in Figure 5, E2Fc reconstituted with the partially purified E2F-BF does contain the cyclin-A polypeptide. Addition of cyclin-A antiserum to the mixture abolished E2Fc but had no effect on the free E2F. Moreover, addition of a cyclin B-specific antiserum did not alter E2Fc. Although we do not know as yet whether the source of the cyclin-A protein derives from E2F-BF preparation or the extract that provides E2F, it does appear from this analysis that E2Fc reconstituted with the E2F-BF is authentic, as judged by the gel mobility as well as the involvement of cyclin A.

Reconstitution of E2Fc with E2F-BF reveals different roles for E1A CR1 and CR2

The availability of a partially purified factor that can form a complex with the E2F transcription factor has allowed us to further investigate the properties of this interaction and the mechanism by which E1A is able to dissociate the complex. As shown in Figure 6A, the complex generated with E2F and E2F-BF was sensitive to dissociation by the wild-type 243-amino-acid 12S E1A product (lane 5). Moreover, utilization of the various E1A mutants again revealed a requirement for CR1 and CR2 for the dissociation of the complex—results identical to those seen for the dissociation of E2Fc in L whole-cell extracts. We therefore conclude that the reconstituted system exhibits the same requirements for E1A, thus indicating the authenticity of the assay system and the likelihood that the purified E2F-BF is that which is found in the complex with E2F in the crude lysates.

The availability of E2F-BF allows the effects of E1A to be assayed in a somewhat different manner. As shown by the experiment in Figure 6B, we have assayed for the ability of E1A to block the formation of E2Fc rather than to dissociate the already formed complex. In this experiment, E1A protein was added at the same time as E2F-BF is added to the free E2F rather than after formation of the complex. After incubation, E2Fc formation was measured by native gel electrophoresis, as shown in Figure 6B. Similar to the dissociation assay, we find that the wild-type E1A protein is capable of preventing the formation of the complex. Moreover, it is also clear that this activity requires CR1, as did the dissociation activity seen in Figure 6A. However, in contrast to the assays
Figure 5. Reconstituted E2Fc contains the cyclin-A polypeptide. E2F-BF was incubated with E2F to allow formation of E2Fc. Dilutions of the cyclin-A or cyclin-B antiserum were then added, incubation was continued, and E2F DNA binding was assayed by gel retardation. (Lanes a) 1:256 dilution of the antiserum; (lanes b) 1:64 dilution; (lanes c) 1:16 dilution. The sample in the first lane received no antiserum.

Detection and isolation of a factor that inhibits E2F DNA binding

During the purification of E2F-BF, we also noticed an activity that appeared to reduce or eliminate E2F-binding activity. Because the formation of E2Fc naturally results in the loss of free E2F, the presence of a separate inhibiting activity was not evident in the assays of crude extracts. However, as the purification proceeded, it became apparent that there must be an additional activity. For instance, there was an inhibition of E2F-binding activity in fractions of a DEAE-chromatography step that were separate from those containing E2F-BF (data not shown). That this was indeed a distinct activity, which abolishes the ability of E2F to bind to DNA, was established by further purification. Chromatography of the DEAE pool on a hydroxyapatite column and sedimentation in a glycerol gradient revealed a distinct peak of activity that sedimented consistent with a molecular mass of ~220 kD [Fig. 7A]. We have termed this activity E2F-I for an activity that inhibits the binding of E2F to DNA.

Although it is possible that the inhibition was the result of an irreversible inactivation of the protein, for instance, a protease that resulted in the destruction of E2F, several observations suggest that this was not the case but, rather, involved the formation of an inactive complex. First, whereas E2F-I inhibited E2F DNA binding, this activity had no effect on the binding of the E4F transcription factor [Fig. 7B]. Thus, there is specificity to the inhibition. Second, E2F-binding activity can be recovered after the inhibition is complete by treatment with deoxycholate (data not shown). Third, addition of wild-type E1A protein can regenerate E2F-binding activity following the incubation with the E2F-I activity as shown in Figure 8A. Thus, E2F-binding activity was not destroyed by incubation with E2F-I but, rather, was masked in some manner. An analysis of the E1A mutants for the ability to reverse the inhibition by E2F-I revealed the same sequence requirements as those for the dissociation of E2Fc formed with E2F-BF [Fig. 8A]. That is, a mutant in the amino terminus retained activity as did a mutant in the region between CR1 and CR2. In contrast, the two CR1 deletion mutants, as well as a domain 2 mutant, were inactive. An assay for the ability of E1A to block the inhibitory action of E2F-I rather than to reverse it is shown in Figure 8B. Similar to the results shown in Figure 6B, CR1 was required but CR2 was not required. It thus appears that two activities that alter the binding of the E2F transcription factor—in one case through the formation of a larger complex and in the other case through the inhibition of DNA binding—are affected by E1A in a similar manner.

Discussion

We believe that the experiments we describe here are of significance in three ways: (1) They establish a biochemical mechanism for the activation of transcription by a viral regulatory protein, the 243-amino-acid product of the E1A 12S RNA; (2) they define the molecular requirements for this event and describe the interplay between the viral protein and the cellular components; and (3) they demonstrate that the transcriptional activation involves E1A sequences that are also important for oncogenesis.

A biochemical mechanism for transcription activation mediated by the 12S E1A product

A series of experiments have clearly defined the role of the 289-amino-acid product of the adenovirus 13S E1A mRNA in transcriptional activation [Berk et al. 1979; Jones and Shenk 1979; Nevins 1981; Ricciardi et al. 1981]. This activity is dependent on sequence in CR3,
Dissociation of E2F complexes

Figure 6. Effects of E1A on the reconstitution of E2Fc. [A] E1A requirements for the dissociation of the reconstituted complex. Glycerol gradient-purified E2F-BF (2 μl) was incubated alone or with free E2F for 20 min at room temperature in the DNA-binding reaction mix. The reconstituted complex was then incubated further for 20 min with 1 μl of a 1 : 5 dilution of reticulocyte lysates programmed with the various mutant E1A mRNAs or BMV RNA. The DNA–protein complexes were then analyzed by gel electrophoresis. The pattern of E2Fc, formed by E2F alone is also shown (lane 2). [B] E1A requirements for the inhibition of complex formation. The ingredients and protocol of this experiment are the same as described in A, except that the reticulocyte lysates were added before the addition of E2F-BF to the reaction mixture and the incubation was carried out in one step for 20 min at room temperature.

and several mechanisms have been attributed to this function (Bagchi et al. 1989; Lillie and Green 1989; Raychaudhuri et al. 1989; Liu and Green 1990). It is also true that the 243-amino-acid product of the E1A 12S mRNA can function as a trans-activator, although this phenomenon has received less attention. In part, this is likely due to a variability of results. In particular, some experiments have indicated that the 12S product does not trans-activate at all (Lillie et al. 1986, 1987; Moran et al. 1986a, b), whereas others have described activity that is reduced compared to that of the 13S product and possibly was only significant for certain cellular targets (Winberg and Shenk 1984, Simon et al. 1987; Zerler et al. 1987). Other studies demonstrated that the 12S E1A activation was nearly as strong as the 13S activation and that early viral genes, including the E2 gene, could be the targets of activation (Leff et al. 1984; Ferguson et al. 1985). Our recent experiments have provided a rational explanation for these variable results and, more importantly, have provided a biochemical mechanism for the activation process. Specifically, we have shown that the cellular transcription factor E2F can be found in complexes with cellular factors (Bagchi et al. 1990). We have also shown that such complexes are inhibitory for transcription of the early E2 gene that normally makes use of the E2F factor. However, through the action of the E1A protein, independent of CR3, there is a dissociation of these complexes, relieving the inhibitory effect. This inhibitory effect can be explained most easily in light of the results presented here identifying a factor that blocks E2F binding. Clearly, the E1A-mediated dissociation of this complex would stimulate E2 transcription. It is also possible, however, that the complex containing E2F that is still capable of binding DNA is also inhibitory for E2 transcription.

In a lytic viral infection, the release of E2F from these complexes allows the 19-kDa product of the early E4 gene to interact with E2F and facilitate the formation of a stable complex on the E2 promoter (Hardy et al. 1989; Huang and Hearing 1989; Raychaudhuri et al. 1990), a process that further enhances E2 transcription (Babiss 1989; Reichel et al. 1989; Neill et al. 1990). We believe that the variation from experiment to experiment in many past reports is probably a function of the target promoter. For instance, we have found that the 12S E1A product cannot trans-activate the E3 or E4 promoters (V. Kraus and J. Nevins, unpubl.). It is also clear that the cell type used for the trans-activation assays influences the results. That is, it is apparent that the 12S E1A-mediated stimulation will only be observed in circumstances
Raychaudhuri et al.

Figure 7. Identification of E2F-I activity. (A) DEAE fractions containing an activity that inhibited E2F DNA binding were pooled and further purified by hydroxyapatite chromatography. Peak fractions from the hydroxyapatite column were pooled and sedimented in a 15–32% glycerol gradient. Each fraction was assayed for activity that could inhibit E2F binding to DNA. Markers were run in a parallel gradient. The band running below the indicated E2F band is a degradation product of E2F. (B) Specificity of E2F-I. Increasing amounts of glycerol gradient-purified E2F-I were incubated with E2F (lanes 3–5) or with E4F (lanes 8–10). Assays of E2F alone or E4F alone are shown in lanes 1 and 6, respectively. Specificity of binding was demonstrated by specific competition (lanes 2 and 7).

where E2F is complexed to other factors. This situation may be the normal one, but it is not found in HeLa cells, a cell system utilized for many studies of E1A trans-activation. Although not proved, it seems a likely possibility that this property of HeLa cells is due to the expression of the human papillomavirus (HPV) E7 gene in this cell line. The E7 product trans-activates the E2 promoter (Phelps et al. 1988); the E7 protein shares sequence with E1A corresponding to these regions we have shown to be important for E2F dissociation, and recent experiments have now demonstrated that the E7 protein possesses an activity that disrupts E2F complexes [W. Phelps, S. Bagchi, P. Raychaudhuri, K. Munger, P. Howley, and J. Nevins, in prep.].

The results presented in this study now clearly demonstrate a requirement for E1A sequences in CR1 and CR2 in altering the interactions of cellular factors with the E2F transcription factor. Moreover, through the partial purification of a factor (E2F-BF) that can regenerate E2Fc, we have been able to partially dissect the requirements of E1A for altering the E2F interactions. As presented in Figure 6, sequences within CR1 are required both to dissociate E2F complexes and to block the formation of E2Fc upon addition of E2F-BF. In contrast, sequences within CR2 are only required for the dissociation of E2Fc and are not required for blocking the formation of the complex. Because an E1A protein containing a functional CR1 can block the formation of the complex, and this does not require the presence of CR2, we suggest that sequences upstream of CR2, dependent on CR1, are sufficient to recognize and bind to E2F-BF in such a way that the interaction with E2F is blocked. In simplest terms, it appears that a region of the E1A protein involving the CR1 sequence can compete with E2F for binding to E2F-BF. If sequences upstream of CR2, dependent on CR1, interact with a domain of the binding factor that recognizes E2F, it is not surprising that these sequences alone do not dissociate E2Fc, as this site would already be bound by E2F.
How, then, might CR2 contribute to the dissociation? In considering the dissociation process, we suggest that E1A may simply alter the normal equilibrium of the dissociation–association event. That is, depending on the binding constants, the E2F/E2F-BF complex will be in equilibrium with the dissociated components. If this dissociation is slow, and the affinity of the E1A protein (in the absence of CR2) for the E2F-BF protein is low, then only very high concentrations of the protein would alter the equilibrium. If, however, the presence of an intact CR2 domain either increased the affinity of these sequences for E2F-BF or brought the E1A protein to the complex by virtue of recognition of a second site on E2F-BF, the effective concentration of the protein would be greatly increased such that any dissociated E2F-BF would be prevented from reassociating with E2F; thus, the equilibrium between E2F and the component parts would be shifted toward dissociation.

Perhaps it is also useful to consider the issue of E1A-mediated repression in light of the role of E1A in dissociating transcription factor complexes, because various experiments have suggested a link between E1A repression and interaction with cell proteins. For instance, recent results have shown that interaction of E1A with a 300-kD polypeptide requires sequence at the extreme amino terminus of the E1A protein (Egan et al. 1988; Whyte et al. 1989) and correlates with the ability of E1A to repress transcription driven by the insulin enhancer (Stein et al. 1990). These events are independent of sequence in CR2 and, thus, independent of interaction with proteins such as the Rb protein, as well as the E2F interactions we describe here. Nevertheless, other studies have provided evidence for a CR2 requirement in transcription repression (Lillie et al. 1986). Possibly, the requirement for the amino terminus in one case or CR2 in another case is a function of the enhancer target, reflecting the specificity of protein interactions. That is, one complex may be important for the insulin enhancer, but another is important for the SV40 enhancer. With respect to a mechanism for repression, we believe that there is a strong analogy to the events we describe here for E2F. Our experiments suggest that E2F is complexed with a factor that inhibits its activity and the release of E2F thus stimulates transcription. The precise same mechanism could achieve the opposite result, a repression, if the target for dissociation was a positive-acting complex for an enhancer. That is, an E1A-mediated dissociation would leave the DNA-binding protein void of a transcriptional activating cofactor. This would then lead to a loss of transcription and, consequently, a repression.

Role of transcription factor dissociation in oncogenesis

A series of experiments have defined the sequence requirements for the oncogenic transformation activity of the E1A protein. From these studies it is clear that CR3 of E1A is not required or only exerts a subtle change in the phenotype of the transformed cell; in general, the 12S E1A gene product is fully capable of transformation. Within the 12S product, sequences at the amino terminus, sequences within CR1, and sequences within CR2 have been shown to be important for transformation (Haley et al. 1984; Lillie et al. 1986; Moran et al. 1986b; Schneider et al. 1987; Zelder et al. 1987; Smith and Ziff 1988; Subramanian et al. 1988; Whyte et al. 1988; Jelsma et al. 1989). A summary of all of these experiments sug-
gests that residues 1–25, 36–77, and 121–139 are required for full transforming activity [Fig. 9]. Our results have shown that CR1 and CR2 sequences are required for dissociation of E2F complexes; thus, those sequences important for E2F dissociation are also necessary for oncogenic activity. Moreover, the two point mutants in CR1 [EV55 and LP49] that were wild type for E2F dissociation are also wild type for transformation (E. Moran, unpubl.). This E2F-directed activity cannot be sufficient for transformation because the amino-terminal sequences were dispensable for E2F dissociation but clearly are required for transformation. We presume that transformation mediated by the 12S E1A product must be the result of targeting more than one cellular component. The immunoprecipitation assays for cellular proteins associated with E1A indicate that the interaction of a 300-kD protein with E1A is dependent on amino-terminal sequences but not CR2 sequence, whereas other proteins such as p105 Rb do not require the amino terminus but do require CR2 (Egan et al. 1988; Whyte et al. 1989, Stein et al. 1990). Interestingly, CR1 appears to be required for stable interactions with most of the associated cellular proteins, including Rb and the 300K protein.

A careful comparison of the transforming activity of certain mutants to the E2F-dissociating activity does reveal some inconsistencies. For instance, the activity of the Δ51-116 deletion is only mildly reduced in some transformation assays (Stein et al. 1990), yet it is clearly impaired in E2F dissociation and trans-activation. Nevertheless, other assays have shown that a deletion of E1A sequence between residue 62 and 85, a region totally deleted by the 51-116 mutant, is defective for transformation (Whyte et al. 1989). In addition, the 961 mutant is not completely defective for transformation (Moran et al. 1986a,b), and in other assays a deletion to this point has exhibited some transforming activity (Whyte et al. 1988). We interpret these variable results to simply reflect assay-dependent differences in transforming function that, depending on contributions from the host cell, influence the extent to which a particular region is required in generating the final phenotype.

If the E2F-dissociating activity of E1A does coincide with oncogenic activity of E1A, how might this specific biochemical activity have an impact on the regulation of cell growth? E2F-binding sites have been found in a number of cellular genes, including c-myc, N-myc, c-myb, DHFR, and epidermal growth factor (EGF) receptor (Hiebert et al. 1989; Mudryj et al. 1990); and at least for the c-myc and DHFR genes, the E2F sites have been shown to be important for transcriptional activity (Blake and Azizkhan 1989; Thalmeier et al. 1989). Recent experiments have shown that there are changes in the nature of the E2F-containing complexes that appear to reflect transitions between stages of the cell cycle (Mudryj et al. 1991). In particular, one complex accumulates during S phase and then disappears at the end of S phase. This complex contains the cyclin-A protein and thus appears to be the same as the complex that is reconstituted with E2F-BF. Although the precise functional role of these interactions is not yet clear, the tight control evident during the cell proliferative cycle and the observation that various genes regulated during cell proliferation utilize E2F suggest a functional significance. The ability of E1A to alter these interactions, dependent on E1A sequence, which is also important for oncogenic transformation, is consistent with a role for this E1A activity in disrupting cell proliferation control.

Materials and methods

Preparation of E2F

E2F was isolated from L-cell extracts as was described previously (Bagchi et al. 1990). Extracts of undifferentiated F9 teratocarcinoma cells were used as the source of free E2F in the assays of E2F-BF.

Assay of E4F

The isolation of E4F and the conditions for assay of binding have been described previously (Raychaudhuri et al. 1987; Rooney et al. 1990).

E1A mutants

Construction of the E1A mutant plasmids designated d186-120 (referred to previously as E1A-NCdl), pm928, and pm961, has been described previously [Moran et al. 1986b]. The plasmids designated 15-35, 2-36, 51-116, 73-120, 76-120, and 81-120 in this study are 12S cDNA versions of genomic plasmids also described previously [Stein et al. 1990]. The single-residue substitution mutants, p12S.DA21 (changing Asp21 to Ala), p12S.LP49 (changing Leu49 to Pro), and p12S.EV55 (changing Glu55 to Val), were constructed by standard oligonucleotide-directed mutagenesis techniques. A restriction fragment containing the point mutation was subcloned into a 12S WT parent plasmid, and the presence of the single-residue substitutions was verified by dideoxy sequencing through the entire restriction fragment. The plasmid designated p12S.38-67 was con-

![Figure 9](image-url)
structured by removing the intervening fragment between the XhoI linkers in the linker-insertion mutants pSVXL132 (deleting residues 38-44) and pSVXL124 (deleting residues 64-67) [Velcich and Ziff 1988; obtained from Ed Ziff and Roland Stein]. An appropriate restriction fragment of the resulting plasmid was subcloned into a 12S.WT background. As a result of the linker insertion, this plasmid encodes a deletion of residues 38-67 with an insertion of three residues—Ser-Ser-Arg—between residues 37 and 68.

In vitro translation of E1A proteins

EcoRI-PstI DNA fragments of cDNAs encoding wild-type or mutant sequences of the 12S gene products of E1A were cloned at the EcoRI-PstI sites of pGEM-4. The pGEM plasmids containing E1A sequences were linearized with PstI, and capped RNAs were made as described previously [Bagchi et al. 1990]. RNAs were translated in vitro using the reticulocyte lysate translation kit from Promega Biotechnology. [35S]Metionine-labeled full-length translation products were assayed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Transfection assays

Transfection assays were performed as described previously using DEAE-dextran procedures [Cullen 1987]. Dishes (60 mm) of mouse L cells at 70% confluence were transfected with a total of 3 μg of DNA. pA10CAT-E2F plasmid (1 μg) and 1 μg of E1A or mutant E1A-expressing plasmids, along with pGEM-4 DNA, were used in these experiments. Cells were harvested 48 hr post-transfection, and CAT assays were performed as described previously [Simon et al. 1988]. The pA10CAT-E2F plasmid has been described [Yee et al. 1989] and consists of the two E2F sites from the E2 promoter cloned upstream of the early SV40 promoter in the plasmid pA10CAT2.

Purification of E2F-BF

The heparin–agarose 0.25 m fraction, obtained by fractionating whole-cell extracts prepared from 24 liters of L cells, was dialyzed against 3 liters of buffer A containing 50 mM KCl for 4 hr. Buffer A contained 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.2 mM PMSF, and 10% (vol/vol) glycerol. The dialyzed material was then applied to a DEAE–Sepharose column (100 ml for 400 mg of protein), which was equilibrated previously with buffer A containing 50 mM KCl. The column was washed successively with 300 ml of buffer A plus 50 mM KCl and 300 ml of buffer A plus 100 mM KCl and then eluted with a linear KCl gradient of 800 ml, ranging from 100 to 400 mM KCl concentration. A sample of 3 μl of the alternate column fractions (10 ml) was assayed for E2F-BF.

The DEAE column fractions between 30 and 35 were pooled (32 mg of total protein), adjusted to 10 mM potassium phosphate (pH 7.0), and applied to a hydroxyapatite column [8 ml]. The column was first washed with 80 mM potassium phosphate (pH 7.0) containing buffer B and then eluted with a 10 bed volume gradient from 80 to 400 mM potassium phosphate containing buffer B. Buffer B contained the indicated amount of potassium phosphate (pH 7.0), along with 0.2 mM DTT, 0.2 mM PMSF, and 10% (vol/vol) glycerol. One-milliliter fractions were collected, and 1 μl of the indicated fractions was assayed for E2F-BF.

The hydroxyapatite-purified material was concentrated using a Centricon apparatus. An aliquot [100 μl, ~0.5 mg of protein] was applied to a 4.2-ml glycerol gradient [12-35% (vol/vol)] and centrifuged using a SW60 rotor at 50,000 rpm for 18 hr. The gradient tube was punctured at the bottom, and 0.2-ml fractions were collected. A sample of 2 μl from each fraction was assayed for E2F-BF. A gradient containing molecular mass marker proteins (catalase, aldolase, and bovine serum albumin, 100 μg each) was centrifuged in parallel. The marker proteins were assayed by SDS–gel electrophoresis.

Antiserum

The cyclin-A and cyclin-B antisera were generous gifts of J. Pines and T. Hunter [Salk Institute] [Pines and Hunter 1989, 1990].

Acknowledgments

We thank Mary Corrigan Carter and Lynne Dengler for their excellent technical assistance. E.M. is the recipient of an American Cancer Society Junior Faculty Research Award (JFRA-206). This work was supported by grants from the National Institutes of Health to E.M. (CA-46436) and to J.N. (GM-26725).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References


Dissociation of E2F complexes


