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Biochemistry

Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system

(*E1A* trans-activation/*E2* promoter/activated kinase)

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ABSTRACT **Adenovirus infection induces a large increase in the DNA binding activity of a cellular transcription factor that is utilized by the viral *E2* promoter and termed E2F. Using cell-free extracts, we have developed an assay for the *in vitro* activation of DNA binding activity of E2F. E2F activity is undetectable in HeLa extracts but upon incubation with a fraction from adenovirus-infected cells, there is an ATP-dependent increase in E2F DNA binding activity. This increase does not occur using an equivalent fraction from dl312 (E1A$^-$)-infected cells. Incubation of E2F with phosphatase inactivates E2F binding activity. Incubation of the phosphatase-inactivated E2F with an infected cell fraction restores E2F activity as does incubation with a known protein kinase. In contrast, incubation with an extract from mock-infected cells does not restore activity. We conclude that the DNA binding activity of E2F is regulated by phosphorylation in an E1A-dependent manner.**

The study of the transcriptional activation of the early genes of adenovirus has proved useful in defining details of transcriptional control. Transcription of the early viral genes is dependent on the interaction of cellular transcription factors with regulatory regions of the viral promoters (1, 2). Through the action of the 289-amino acid E1A protein, there is a marked stimulation of early viral transcription (3–6). Since there is no evidence for a direct interaction of the E1A protein with DNA (7), it would appear that E1A mediates this activation in an indirect manner, influencing transcriptional activity by way of the cellular factors that interact with the viral promoters.

Numerous studies have defined the regulation of the viral *E2* gene (6, 8), including the sequences required for transcription (9–14). An analysis of *E2* promoter–protein interactions *in vivo* demonstrated that the transcription of the gene coincided with the formation of stable promoter complexes (15), consistent with the prediction from the earlier experiments of Gaynor and Berk (16). These results implicated an E1A-dependent effect on the DNA binding capacity of a protein or proteins that interacted with the *E2* promoter, and an analysis of proteins that recognized the promoter identified such a protein. A cellular DNA binding protein termed E2F was found to interact with *E2* promoter sequences and the level of E2F binding activity rose dramatically upon adenovirus infection, dependent on E1A function (17). Since the E2F binding sites can confer inducibility on a heterologous promoter and the affinity-purified E2F factor can stimulate transcription *in vitro* that is dependent on E2F binding to the promoter (18), it appears that E2F is a transcription factor involved in E1A-dependent control of *E2* transcription.

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The activation of E2F DNA binding activity does not require new protein synthesis, suggesting a modification of a pre-existing factor (19). The nature of the modification, thus implicating the mechanism of viral-mediated trans-activation by E2F, is clearly of importance to a final understanding of the basis for trans-activation in this viral system as well as in similar cellular systems. To this end, we have assayed for E2F activation *in vitro*, measured as the conversion of an inactive factor to an active state by incubation with an extract of adenovirus-infected cells. We find that under appropriate conditions we can detect *in vitro* activation of E2F binding activity in cell-free extracts. We further demonstrate that this activation probably involves the phosphorylation of a pre-existing inactive form of E2F.

EXPERIMENTAL PROCEDURES

Cells and Virus. HeLa cells, grown in suspension in Joklik's modified minimum essential medium containing 5% (vol/vol) calf serum, were used throughout. The procedures for growth and purification of adenovirus type 5 (Ad5) have been described (20).

Nuclear Extracts. Nuclear extracts were prepared from HeLa cells or Ad5-infected HeLa cells 6 hr after infection as described (17, 21).

Fractionation of Extracts. HeLa nuclear extract (250 mg; from 5 liters of Ad5-infected HeLa cells or mock-infected HeLa cells) was fractionated on a 30-ml heparin-agarose column. After loading, the column was washed with buffer [20 mM Tris·HCl, pH 7.5/0.1 M KCl/0.2 mM dithiothreitol/0.2 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol]. The heparin-agarose flow-through and 0.1 M KCl wash contained >90% of the E1A protein as determined by Western blot immunoassay using monoclonal antibody against E1A. Fractions were pooled and diluted with an equal volume of buffer B (20 mM Tris·HCl, pH 7.5/0.2 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/10% glycerol) containing no KCl and applied to DEAE-Sephacel column equilibrated with buffer B containing 50 mM KCl. The DEAE-Sephacel column was washed with buffer B containing 0.15 M KCl and material was eluted with buffer B containing 0.3 M KCl.

Phosphatase Treatment. Heparin-agarose-purified E2F or oligo-affinity-column-purified E2F was treated with alkaline phosphatase (12 units/ml) bound to agarose (Sigma) in a buffer containing 50 mM Tris·HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, and 300 mM KCl at 30°C for the indicated period of time. The enzyme-containing beads were then removed by centrifugation at 4500 × *g* for 30 sec. The supernatant was centrifuged at 10,000 × *g* for 10 min to remove any remaining agarose-bound enzyme and then assayed for E2F activity using a gel retardation assay or an exonuclease III assay.

Abbreviations: Ad5, adenovirus type 5; ATP[γ-S], adenosine 5'-[γ-thio]triphosphate.

Partial Purification of E2F. Purification of E2F both from mock-infected nuclear extracts and Ad5-infected nuclear extracts by heparin-agarose chromatography was performed essentially as described (22). The heparin fractions from mock-infected nuclear extract were assayed for E2F activity after *in vitro* activation with partially purified E1A. The purification of E2F by DNA affinity chromatography was done essentially as described (18).

***In Vitro* Activation of E2F Activity.** The activation assay was carried out in two steps: (*i*) incubation of infected-cell nuclear extract with mock-infected nuclear extract to activate the inactive E2F molecule present in the mock nuclear extract and (*ii*) assay of newly activated E2F molecules by using an exonuclease III protection analysis. Nuclear extract (30 μg) prepared from uninfected HeLa cells was incubated with 50 μg of heparin-agarose flow-through fraction in a total reaction volume of 30 μl in the presence of 1 μg of salmon sperm DNA and 1 ng of 5'-^{32}P-labeled E2 probe. The reaction mixture also contained 12 mM Hepes (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 1.2 mM dithiothreitol, and 1 mM ATP. After a 1-hr incubation at 30°C, the reaction mixture was digested for 15 min at 30°C with 200 units of exonuclease III for assay for E2F–*E2* DNA complex as described (17).

Activation with cAMP Kinase. Phosphatase-inactivated E2F, prepared as described above, was incubated with either 40 picomolar units or 100 picomolar units of the catalytic subunit of cAMP-dependent protein kinase (Sigma), under conditions described above for activation of E2F in infected cell extracts. After incubation at 30°C for 30 min, E2F activity was measured by a gel retardation assay (17).

RESULTS

Using the probe depicted in Fig. 1 that spans the two E2F binding sites, one can readily measure the activation of E2F binding activity that occurs in a virus infection. Extracts from mock-infected HeLa cells or adenovirus-infected HeLa cells were assayed for E2F levels by exonuclease III protection. Using increasing concentrations of extract for the assays, a substantial increase in the level of E2F was evident in the adenovirus-infected cells as compared to the uninfected cells. This difference was observed at all three protein levels and at the highest extract concentration a modest amount of E2F in the uninfected cell could be detected.

Assay for *in Vitro* E2F Activation. Past experiments, as well as that depicted in Fig. 1*B*, have shown that the E2F binding activity is a cellular factor, as it can be detected in extracts of uninfected cells (17, 26). Furthermore, since the activation process does not involve *de novo* synthesis of a protein factor (19), we presume there must be a significant pool of inactive factor that can be activated in the uninfected cell. One might expect, therefore, that the incubation of an extract from uninfected cells containing the inactive E2F with an extract from adenovirus-infected cells might activate the inactive E2F under the appropriate conditions. We have, therefore, prepared extracts from uninfected HeLa cells, which contain low levels of the active E2F factor (Fig. 1), and used these extracts as the substrate for activation. We then prepared extracts from adenovirus-infected HeLa cells as a source of E1A protein and any other activity that may be necessary for the activation to determine if such extracts could mediate an activation of the presumably inactive E2F in the uninfected cell extracts. Of course, the infected cell extracts also contain a substantial amount of already activated E2F (Fig. 1) and thus cannot be used directly because of the very high background that would exist in the assay. However, when infected-cell extracts are fractionated on a heparin-agarose column, all of the E2F activity is bound to the column and is not eluted until 0.3 M KCl is applied to the column (data not shown). Thus, we have employed the heparin-agarose flow-through fraction as the source of factors that might mediate the activation of E2F.

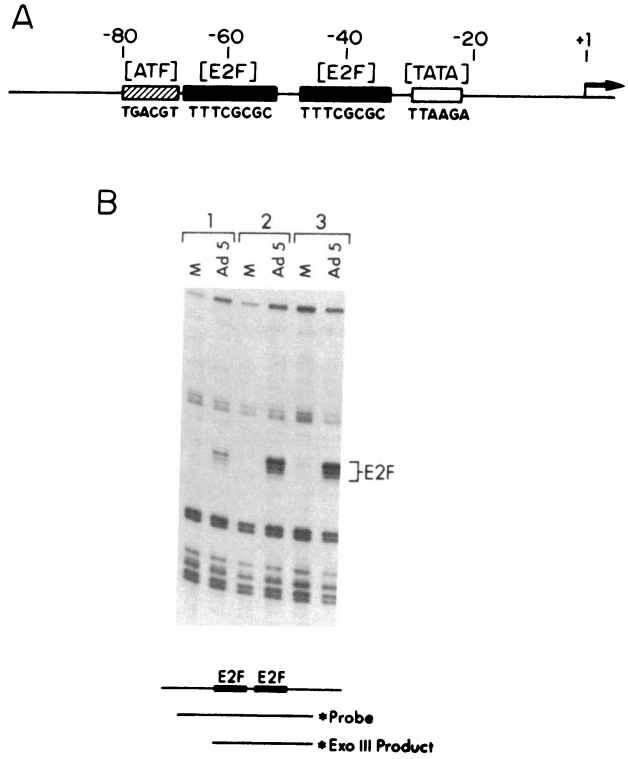

FIG. 1. (*A*) *E2* promoter depicting the two E2F binding sites (17, 22) as well as the site for activating transcription factor (ATF) binding (23, 24) and a pseudo TATA site (25). Various mutagenesis experiments have defined the region downstream of position −80 as being sufficient for *E2* promoter activity (9, 10, 12, 14). (*B*) *In vivo* activation of E2F. Nuclear extracts prepared from Ad5-infected HeLa cells or mock-infected HeLa cells were assayed for E2F activity by exonuclease III protection (17). Increasing amounts of each extract were assayed such that lanes 1 contain 10 μg of extract, lanes 2 contain 20 μg, and lanes 3 contain 30 μg. Also shown is a schematic of the two E2F binding sites, the end-labeled probe, and the 69-nucleotide protected fragment after exonuclease III digestion.

Exonuclease III protection assays of the extract from uninfected HeLa cells showed no evidence of E2F activity (Fig. 2). In addition, the assay of the heparin-fractionated Ad5-infected extract also showed no evidence of E2F activity thus indicating the effective removal of the active E2F from these extracts. In this particular assay, there were nonspecific exoIII stops above the E2F site but these are variable depending on the sample and are unrelated to E2F binding. Since this heparin fraction was devoid of E2F activity, it served as the activator in the assay for E2F activation. The addition of increasing amounts of the heparin-agarose flow-through fraction from the infected extract to the extract of uninfected cells followed by incubation at 30°C produced a substantial amount of active E2F (Fig. 2*A*). A similar nuclear extract from uninfected HeLa cells was fractionated by heparin-agarose chromatography and used for the assay of E2F activation, and incubation of this extract with the uninfected cells resulted in no increase in E2F activity. Thus, we conclude that an activity in extracts of adenovirus-infected cells, when incubated with an extract containing inactive E2F, is capable of *in vitro* activation of previously inactive E2F. We have tried to determine if the E1A protein is directly involved in the activation through the use of an E1A monoclonal antibody. If added directly to the reaction mixture, the antibody has no effect on the activation process. If the infected extract is first incubated with the antibody to clear the preparation of E1A protein, we have observed an

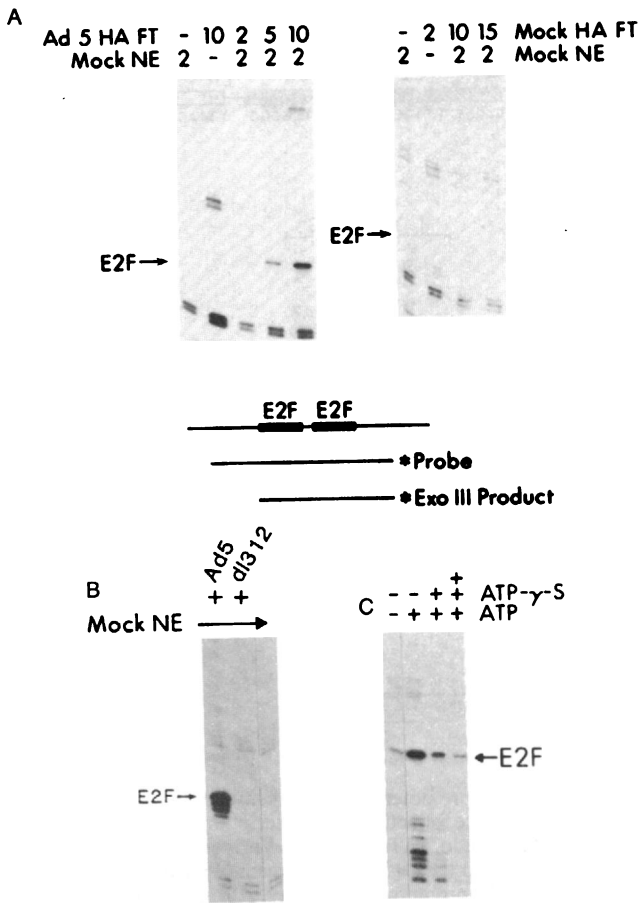

FIG. 2. *In vitro* activation of E2F. (*A*) (*Left*) E2F activity, assayed by exonuclease III protection, was measured in 2 μl of a nuclear extract (10 mg/ml) from mock-infected HeLa cells (mock-NE), in 10 μl of the heparin-agarose flow-through fraction (5 mg/ml) from Ad5-infected nuclear extracts (Ad5 HA FT), or in a mixture of both preparations. The incubations were at 30°C for 60 min. Exonuclease III was then added and incubation continued for 15 min. (*Right*) A heparin-agarose flow-through fraction (5 mg/ml) from a mock-infected extract (Mock HA FT), prepared in the same way as the Ad5 HA FT, was used for the assays. (*B*) Activation assays (as in *A*) were carried out using 3 μl of nuclear extract (10 mg/ml) from mock-infected HeLa cells incubated alone (lane to the right) or with 2.5 μl of the heparin-agarose flow-through fraction (5 mg/ml) from Ad5-infected nuclear extract or from d1312-infected nuclear extract. (*C*) ATP hydrolysis is required for E2F activation. An activation assay was carried out using 25 μg of mock nuclear extract and 10 μg of the heparin flow-through fraction from an Ad5-infected cell extract further purified through a DEAE-Sephacel column. E2F activation was assayed by exonuclease III protection in the absence of any added ATP (lane 1), in the presence of 200 μM ATP (lane 2), in the presence of 200 μM ATP plus 200 μM ATP[γ-S] (lane 3), or in the presence of 200 μM ATP plus 400 μM ATP[γ-S] (lane 4).

inhibition of activity (data not shown). However, this has not been reproducible and may reflect the inefficiency of the antibody treatment or the more likely possibility that the E1A effect is indirect. For instance, E1A might activate another component and once this has occurred, the E1A activity would no longer be required. Nevertheless, the E1A protein appears to be required for the activation since extracts prepared from cells infected with a high multiplicity of the E1A mutant d1312, conditions that allow expression of other early gene products (6), showed no evidence of activation of E2F (Fig. 2*B*).

The *in vitro* activation of E2F is an ATP-dependent process (Fig. 2*C*). E2F activation was enhanced ≈8-fold in the presence of ATP. Furthermore, there is a requirement for ATP hydrolysis in the activation since the nonhydrolyzable ATP analog adenosine 5'-[γ-thio]triphosphate (ATP[γ-S]) blocks the activation process. The addition of a concentration of ATP[γ-S] equal to that of ATP reduced activation by a factor of ≈2 whereas a further 2-fold increase in ATP[γ-S] reduced activation to nearly that in the absence of ATP. We thus conclude that the activation of E2F binding activity requires hydrolysis of ATP.

E2F Activation Involves Phosphorylation of the Inactive Factor. Although several possibilities exist for mechanisms involving ATP, one obvious possibility is a kinase-dependent reaction in which the γ-phosphate is transferred from ATP to the inactive factor, which then induces its capacity to bind specifically to DNA. As an initial step to determine the possible role of a phosphorylation in the activation of E2F, we have tested the sensitivity of virus-induced active E2F to phosphatase treatment. A preparation of active E2F was incubated with agarose-immobilized calf intestinal phosphatase for various lengths of time and then assayed for binding after removal of the phosphatase. There was a rapid and near complete loss of E2F activity after treatment with the phosphatase, suggesting that phosphorylation of E2F is indeed important for its activity (Fig. 3).

We next determined whether E2F activity could be restored to the factor that was inactivated by phosphatase treatment. That is, is the inactive factor generated by phosphatase treatment essentially the same as the inactive factor present in extracts of uninfected cells (i.e., could it serve as a substrate for the virus-specific activation reaction)? To be certain that we were measuring the restoration of phosphatase-inactivated E2F activity rather than the activation of inactive E2F molecules already present in the sample, we have used an E2F preparation for phosphatase treatment that was purified through an E2F affinity column (18). In that way, we have selected for active E2F (as defined by DNA binding) and have eliminated E2F molecules that are in the inactive state. Indeed, when an uninfected HeLa nuclear extract is passed through an E2F affinity column, inactive E2F that can serve as a substrate for activation is found only in the flow-through (data not shown). Phosphatase treatment once again completely abolished E2F binding activity as measured by exonuclease III protection (Fig. 4*A*, lane 5). When this material was then incubated with the infected cell extract, there was a restoration of E2F activity (lane 6) not back to the original level but to a substantial degree. Assay of the infected cell extract again showed no evidence for contaminating E2F (lane 1). Therefore, since the starting material was active E2F, we conclude that the appearance of active E2F after the incubation must be due to the reactivation of E2F that had been rendered inactive by phosphatase treatment.

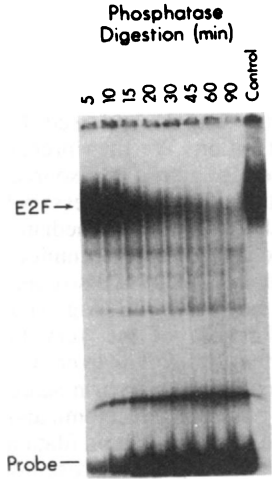

FIG. 3. Phosphatase inactivation of E2F activity. A 0.4-ml sample of affinity-purified E2F from Ad5-infected cells was incubated with 5 units of agarose-bound calf intestinal phosphatase at 30°C as indicated. The phosphatase was then removed by centrifugation and 5 μl of the supernatant was assayed for E2F by a gel retardation analysis. Control is E2F incubated without phosphatase.

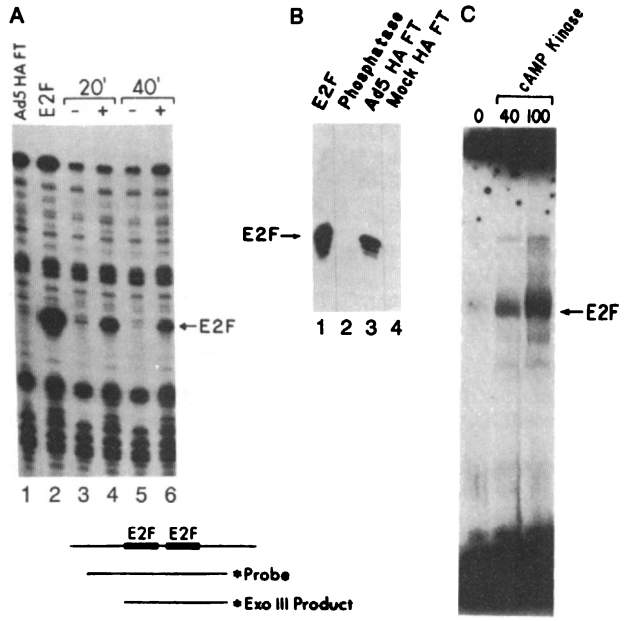

FIG. 4. Reactivation of phosphatase-inactivated E2F. (*A*) A sample of E2F purified through an E2F affinity column was incubated with agarose-bound calf intestinal phosphatase for either 20 min (lanes 3 and 4) or 40 min (lanes 5 and 6). The phosphatase was removed and the supernatants were incubated with the heparin-agarose flow-through fraction from an Ad5-infected cell extract. After 60 min at 30°C, the incubations were assayed for E2F activity by exonuclease III protection. Assay of the heparin flow-through fraction alone is shown in lane 1; E2F prior to phosphatase is assayed in lane 2. −, E2F after phosphatase but without further incubation; +, further incubation with heparin flow-through fraction from Ad5-infected cells. The relationships among the E2F binding sites, the probe, and the exonuclease III product are shown below. (*B*) A sample of affinity-purified E2F was incubated with (lane 2) or without (lane 1) agarose-bound calf intestinal phosphatase for 20 min. The phosphatase was removed and the supernatant was incubated with the heparin flow-through fraction from an Ad5-infected cell extract (lane 3) or a heparin flow-through fraction from a mock-infected cell extract (lane 4). (*C*) Reactivation of E2F by cAMP-dependent protein kinase. A sample of affinity-purified E2F was incubated with phosphatase. After removal of phosphatase, the inactivated E2F was incubated alone (lane 0) or with 40 units (lane 40) or 100 units (lane 100) of catalytic subunit of cyclic AMP-dependent protein kinase at 30°C for 30 min. E2F activity was measured by gel retardation.

The results shown in Figs. 3 and 4*A* demonstrate that phosphorylation is important for E2F activity. However, it remained possible that the reactivation of the phosphatase-treated E2F was not due to an action related to normal trans-activation; that is, phosphorylation of E2F was important but was not the basis for E1A-dependent trans-activation. To address this, we have assayed for reactivation of E2F using extracts from mock-infected cells as compared to Ad5-infected cells. As shown in Fig. 4*B*, phosphatase-inactivated E2F was reactivated with extracts from Ad5-infected cells whereas incubation with mock-infected extract had little effect. We thus conclude that the reactivation of E2F after phosphatase-inactivation, which most likely involves a phosphorylation, is dependent on components in the infected cell. This result strongly suggests that the E2F activation process involves transfer of phosphate from ATP to the inactive E2F molecule.

Based on the sensitivity of E2F to phosphatase, we conclude from the data of Fig. 4 *A* and *B* that phosphorylation of an inactive E2F converts the factor to a form capable of binding to the promoter. To further substantiate this point, we have attempted to reactivate the phosphatase-treated E2F with a known protein kinase. Such an experiment with partially purified protein kinase C was negative, yielding no increase in E2F (data not shown). However, incubation of phosphatase-treated E2F with the catalytic subunit of cAMP-dependent protein resulted in an increase in E2F activity (Fig. 4*C*). Incubation with 40 units of kinase resulted in a 4-fold increase and 100 units stimulated E2F activity 10-fold. Thus, a known cellular protein kinase is capable of reactivating E2F activity using phosphatase-inactivated factor as the substrate. We do not wish to imply from this result that the kinase responsible for E2F activation in extracts of virus-infected cells is the cAMP kinase. However, we do conclude from these results that the activity of E2F can indeed be regulated by a phosphorylation process.

DISCUSSION

Transcription of the E2 gene appears to require the interaction of four protein molecules with the promoter (Fig. 1). In addition to the binding of the activating transcription factor (ATF) (23, 24) and a "TATA" factor (25), two molecules of E2F bind to the promoter (17, 22), and studies employing site-directed mutagenesis demonstrate that the E2F sites are important for promoter activity (27). Furthermore, since the E2F binding sites can confer E1A-inducibility to a heterologous promoter and the purified E2F factor stimulates transcription dependent on specific binding (18, 28), it appears likely that E2F is involved in E1A-dependent trans-activation of E2 transcription. An *in vivo* analysis of protein interaction at the E2 promoter, by exonuclease III protection, suggested an E1A-dependent stimulation of stable complex formation (15), thus implicating enhanced factor–DNA binding as a basis for the activation of E2 transcription. A variety of studies now implicate the activation of the DNA binding activity of the E2F factor as responsible for this process. For instance, there is a large increase in E2F DNA binding activity upon adenovirus infection, the kinetics of which coincide with activation of *E2* transcription (6, 8, 19). The activation of E2F requires expression of the 289-amino acid E1A protein (19) as does the activation of *E2* transcription (5, 29, 30). E2F DNA binding activity is present at high levels in undifferentiated F9 cells but then drops to undetectable levels upon differentiation (26), again coincident with the transcription of the *E2* gene in these cells (31). We, therefore, suggest that the E1A-dependent activation of E2F DNA binding activity is critical to the stimulation of *E2* transcription. Recent experiments have suggested an additional complexity to the *E2* trans-activation process since a product of the adenovirus *E4* gene appears to be necessary, in addition to E1A, for E2F activation (R. Reichel, S. Neill, I. Kovesdi, M. C. Simon, P.R., J.R.N., unpublished results). Numerous previous experiments have shown that E1A alone can effect an activation of *E2* transcription (10, 13, 14, 32). However, it is also true that the *E4* gene alone can trans-activate the *E2* promoter, dependent on the same promoter sequences as required for E1A trans-activation (33), and that *E2* transcription in a viral infection is not maximal in the absence of *E4* since there is an additional 8- to 10-fold increase with *E4* (R. Reichel *et al.*, unpublished results). Thus, the *E4* gene does appear to participate in the activation of E2F and *E2* transcription and as such the activation of E2F binding activity is clearly part of the trans-activation of *E2*. We do not mean to suggest, however, that activation of E2F binding activity is solely responsible for trans-activation of *E2* transcription and certainly other events may contribute, including alterations in E2F transcription function or possibly effects on other factors.

The experiments reported here do not define the identity of the kinase responsible for E2F activation. We believe that it is unlikely that the E1A 289-amino acid protein or one of the *E4* gene products is the kinase since all protein kinases

studied to date possess a conserved sequence that includes the ATP binding site (34); neither the 289-amino acid E1A protein nor any of the possible E4 proteins possess this sequence. We favor the possibility that a cellular kinase, which normally controls the activity of E2F within the cell, is activated during the viral infection leading to increased levels of active E2F. It is also of course possible that part of the increase in E2F involves the inhibition of a specific phosphatase, which could explain the involvement of two viral trans-activators.

We identified (35) a factor with specificity to the *E4* promoter that possessed properties analogous to E2F. This factor, termed E4F, binds to *E4* sequences critical for transcription and E1A stimulation, the level of the factor increases significantly in virus-infected cells and the increase coincides with the induction of *E4* transcription (8, 35). E4F activity is also controlled by phosphorylation (36), and, based on these findings, we speculate that the coordinate control of *E1A*, *E2*, and *E4* transcription may involve a common mechanism of phosphorylation of the E2F and E4F factors. Furthermore, at least two other cellular transcription factors appear to be mediators of the E1A-dependent trans-activation of transcription. Studies of both the adenovirus *E1B* promoter (37) and the human 70-kDa heat shock protein promoter (38) have defined the TATA element as the target for E1A control, implicating a TATA factor in the regulation. In addition, E1A-mediated induction of polymerase III transcription appears to involve transcription factor IIIC (39–42). Thus, at least four distinct transcription factors appear to be targets and eventual effectors of E1A trans-activation. Given the apparent involvement of a kinase mechanism in the activation of E2F and E4F, it is not unreasonable to suggest that this is the basis for activation of the other factors as well. Indeed, studies with transcription factor TFIIIC suggest a role for phosphorylation in an adenovirus-mediated alteration of the factor, although there was no indication of a functional consequence of the phosphorylation (43).

The results in this report bear on the general considerations of transcriptional regulatory pathways within the cell. By defining the basis for E2F activation, we have begun to elucidate the steps in a pathway of transcriptional regulation. Since it is clear that cellular activities analogous to the E1A system can be demonstrated (26, 29), we presume it is likely that the activation process assayed here will be informative for these cellular events of gene control.

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