Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 387–390, January 1988
Biochemistry

Activation of a preexisting cellular factor as a basis for adenovirus E1A-mediated transcription control

(*E1A*/transcription activation/E2F modification/gene regulation)

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Communicated by James E. Darnell, Jr., September 28, 1987

ABSTRACT **Transcription of the set of early adenovirus genes is subject to positive control by the viral *E1A* gene. For one early viral gene, the *E2* gene, this induction involves an increase in a cellular promoter-specific factor termed E2F. We have analyzed the kinetics for this induction and find that E2F is present at only very low levels in extracts of uninfected cells or cells infected for up to 3 hr with adenovirus type 5. The factor increases rapidly at 5 hr and reaches a maximal level at 7–8 hr. The kinetics of induction of the factor are thus coincident with the induction of *E2* transcription. The 13S *E1A* gene product (289-amino acid protein), which is required for the efficient activation of *E2* transcription in a productive infection, is also responsible for the activation of E2F, because infection with mutant strain pm975 ($13S^+$, $12S^-$) induces the factor, whereas no increase of E2F occurs in cells infected by mutant strain dl1500 ($13S^-$, $12S^+$). Finally, increase in the factor does not involve synthesis of any new protein, because extracts prepared from cells infected with adenovirus type 5 and treated with cycloheximide from 1 hr after infection contain approximately the same level of E2F as extracts from infected but untreated cells. From these results, we conclude that activation of E2F, as a posttranslational event, is responsible for the stimulation of *E2* transcription by E1A.**

The analysis of transcription control in the eukaryotic cell is now well advanced. Promoter and enhancer sequences critical for transcription have been defined (1). Proteins that interact with these sequences have been identified, and in several instances, such proteins have been purified to homogeneity (2–4). It is also clear from a number of studies that such protein DNA interactions can be complex, involving several different proteins. Due to this complexity of the transcriptional regulatory region, the determination of the relative roles of interacting proteins has been difficult. Many of the proteins, undoubtedly, are not involved in gene regulation (i.e., are not limiting factors) but rather are common, abundant factors that are required for formation of the active transcription complex. Should formation of a stable complex be the basis for transcriptional activation of a gene, one might expect to find a change in a limiting factor or factors in extracts as measured by DNA binding. Indeed, one such example is the E1A-mediated activation of the adenovirus *E2* promoter (5–7). Previous experiments demonstrated that stable complex formation on the promoter inside virus-infected cells coincided with activation of transcription from the *E2* promoter (8). Consistent with this observation was the finding that extracts from uninfected cells or *E1A* mutant-infected cells contained only very low levels of an *E2* promoter-binding factor termed E2F, whereas extracts of wild-type virus-infected cells contained high levels of the factor (9). To gain further insight into the mechanism for induction and control of transcription by E1A, we have analyzed the kinetics and requirements for activation of E2F.

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MATERIALS AND METHODS

Cells and Virus. HeLa cells were used throughout and were maintained in Joklik's modified minimal essential medium containing 5% calf serum. Growth and preparation of adenovirus type 5 (Ad5) has been previously described. The mutant strains pm975 and dl1500 were provided by A. Berk (UCLA), and mutant strain dl312 was provided by T. Shenk (Princeton).

Preparation of Extracts and Binding Assays. Procedures for the preparation of nuclear or whole-cell extracts have been described (9, 10). Conditions for assay of protein binding with labeled probes have been detailed previously, as have the conditions for analysis by exonuclease III protection (9, 10).

RESULTS

Time Course of E2F Induction. As a rapid and quantitative assay for E2F binding activity in extracts, we used an exonuclease III protection assay. We have previously shown that exonuclease III digestion of complexes formed with an endlabeled *E2* promoter probe and E2F yields exonuclease III-resistant fragments with end points at −71 and −33 (10). For experiments described here, we used an *E2* probe labeled at the downstream end, thus mapping the upstream border of protection—i.e., −71. In such assays, we measured the levels of E2F in extracts prepared from uninfected or Ad5-infected HeLa cells at various times after infection. Fig. 1 *Upper* shows that barely detectable levels of E2F were found in uninfected cells and that this amount did not increase through 3 hr of infection. Then a sharp increase in the E2F level was evident in the 5-hr extract, followed by a slight increase in the 7-hr extract and finally a slow decline through 13 hr. In other experiments (see Fig. 3), activation was more gradual, beginning at 3–5 hr after infection. The experiments show control of the level of E2F during an early viral infection, and the increase of E2F closely corresponds to the activation of *E2* transcription (7, 12).

As shown in Fig. 1, a slight decrease in E2F level appeared in the 13-hr extracts; this change might indicate an actual decrease in the factor in the cell, or alternatively a sequestration of factor by the increase in replicated viral DNA. To distinguish these possibilities, we prepared high-salt, whole-cell extracts from 8-hr-infected cells and 23-hr-infected cells.

Abbreviations: Ad5, adenovirus type 5; pfu, plaque-forming units; E2F, *E2* promoter binding factor; EIIA-EF, EIIA promoter early factor.

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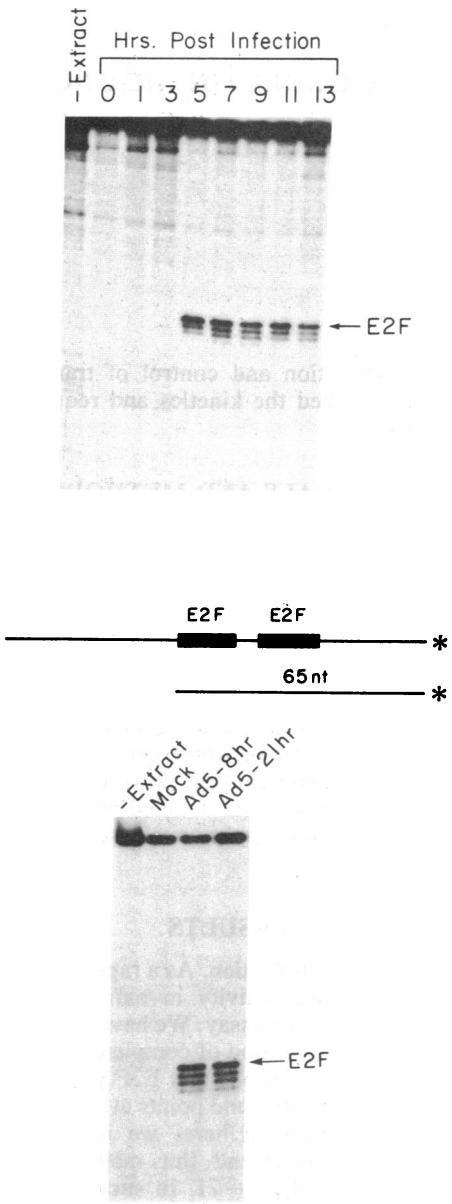

FIG. 1. Kinetics of E2F activation during adenovirus infection. (*Upper*) HeLa cells were infected with Ad5 [10 plaque-forming units (pfu) per cell] and at the indicated times postinfection, nuclear extracts were prepared as described (9). Conditions for the E2F binding assay, exonuclease III digestion, and analysis of products have been described (9, 10). Each assay mixture contained 30 μg of nuclear extract, 1 ng of ^{32}P probe, and 1 μg of salmon sperm DNA used as the nonspecific competitor, in a volume of 25 μl. Positions of the two E2F binding sites (11) within the end-labeled probe (solid boxes) are depicted at bottom, as well as the 65-nucleotide exonuclease III product. (*Lower*) HeLa cells were infected with Ad5 (10 pfu per cell) and at the indicated times postinfection, whole-cell extracts were prepared as described (10). Assays with exonuclease III were as described above using 30 μg of whole-cell extract.

These conditions should ensure efficient extraction of proteins bound to the DNA, as previously demonstrated in other such studies (13). Indeed, there were equal levels of E2F in extracts prepared in this manner. Thus, once the factor was activated and reached peak level at about 7 hr after infection, a time coincident with the peak of *E2* transcription (7, 12), this level was maintained throughout the course of infection. Although the kinetics of activation of E2F correlates with the activation of *E2* transcription, the subsequent decline in transcription is not paralleled by a decrease in E2F. Thus the transcription decline is apparently not due to any loss of E2F but rather probably results from an active shut-off of the *E2* promoter. Such a possibility is suggested by previous experiments that indicated a requirement for protein synthesis for *E2* transcription to decrease (12).

Induction of E2F Requires the 13S E1A Product. The *E1A* gene encodes at least three distinct gene products (14) and, as has been shown in numerous studies, it is the product of the 13S E1A mRNA, a 289-amino acid protein that is responsible for the efficient stimulation of early viral transcription, including *E2* gene transcription (15–17). Therefore, we have determined the E1A requirements for activation of E2F. Extracts were prepared from cells infected for 7 hr with mutants dl1500 (13S$^-$, 12S$^+$), and pm975 (13S$^+$, 12S$^-$), and then each extract was assayed for E2F levels by an exonuclease III protection assay. Fig. 2 shows a large increase in E2F levels in extracts prepared from mutant pm975-infected cells, equivalent to the increase that occurs during a wild-type infection. In contrast, little, if any, increase in E2F occurred in cells infected with mutant dl1500. Thus, the 13S E1A product was much more efficient in inducing E2F than the 12S E1A product. From these results, as well as previous experiments indicating a strict concordance between E2F induction and *E2* transcription, we conclude that E2F is likely the limiting factor for *E2* transcription, and that the control of this factor by the E1A 289-amino acid protein is responsible for the activation of *E2* transcription by E1A.

Induction of E2F Does Not Require Protein Synthesis. The results of Figs. 1 and 2, as well as our previous experiments, clearly demonstrate an increased level of the E2F factor in extracts of infected cells as compared with uninfected cells. Furthermore, as shown before and in Fig. 2, this increase is dependent on the *E1A* gene and more specifically, the 13S E1A product. However, as the assay for E2F is DNA-binding activity, we cannot define the actual mechanism for increasing the level of E2F. There might be an increase in the actual amount of protein, or there might be modification of a pre-existing factor that activated binding capacity of the protein. One approach to distinguish between such possibilities is to determine whether activation can occur in the

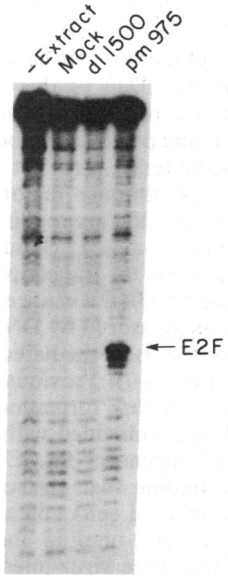

FIG. 2. Requirement of the 13S E1A product (289-amino acid protein) for induction of E2F. HeLa cells were mock-infected or infected with mutants dl1500 or pm975 (each at 10 pfu per cell) and incubated for 6 hr. Nuclear extracts were prepared and assayed for E2F binding as described for Fig. 1.

absence of protein synthesis. If so, it would suggest a posttranslational modification as the mechanism. This experiment is complicated in the case of induction by adenovirus infection, however, because the synthesis of the inducer (E1A) must be allowed. To circumvent this problem we initiated infection and then after 1 hr added cycloheximide to block subsequent protein synthesis. This protocol assumes sufficient E1A protein is made during the first hour of infection so as to then allow efficient activation of E2F. Results of such an experiment are shown in Fig. 3 *Upper*. In this experiment we assayed for binding with poly(dI·dC) rather than with salmon sperm DNA as the nonspecific competitor. Under these conditions we detected an additional protein binding to the E2 promoter, just upstream of E2F (11). This factor, termed EIIA promoter early factor (EIIA-EF), is present in uninfected cells and does not change with infection (11, 18), a result evident in the experiment of Fig. 3 *Upper*. When the E2F level is examined in this experiment, the kinetics of activation without cycloheximide are similar to those seen in Fig. 1. Lanes marked + in this experiment are extracts prepared from cells to which cycloheximide was added at 1 hr after infection. Clearly, E2F factor was still induced in the absence of ongoing protein synthesis. Cycloheximide did cause some difference, most notably a delay as evinced by the 5-hr time point. However, we interpret this change as most likely due to the timing of the experiment and the probability that adding cycloheximide at 1 hr reduces the level of E1A production. Indeed, if cycloheximide is added 2.5 hr after infection, E2F is induced to the same level as without cycloheximide (data not shown). Thus, from these results the activation of E2F appears to take place without protein synthesis and thus represents a posttranslational event. Because infection proceeded for 1 hr before cycloheximide addition, it is conceivable that some of the factor was also newly synthesized during this time, in addition to being activated posttranslationally as must occur after 1 hr of infection. We view the possibility of new synthesis as highly unlikely, however, because of the short time period remaining after allowance for the time lag necessary for E1A protein synthesis.

Fig. 3 *Lower* shows that cycloheximide alone does not result in an induction. We can therefore conclude that E1A is prerequisite for E2F increase but that the increase does not require any new protein synthesis. At first glance, the requirement for E1A in the presence of cycloheximide for activation of E2F might appear to be at odds with previous experiments that indicated cycloheximide treatment alone could replace E1A in activating early viral transcription (7). However, that result was true for *E4* and *E1B* transcription, but only marginally so for *E2* and *E3*. Thus, the requirement for E1A in the activation of E2F in the presence of cycloheximide is not inconsistent with the data on transcription of *E2* under these circumstances. Furthermore, the data suggest a distinction in the mechanism for activation of *E2* and *E4* transcription. The most straightforward interpretation would be that different factors are involved in the activation of these promoters and that they are affected differently by cycloheximide. Certainly this is true in the sense that E2F is not involved in *E4* transcription (19), but whether an *E4*-specific factor does respond to cycloheximide treatment remains to be shown. Finally, the behavior of EIIA-EF in the presence of cycloheximide is noteworthy. As seen in Fig. 3 *Upper*, soon after cycloheximide addition a rapid decrease occurs in EIIA-EF levels to near undetectable levels. From this result we conclude that the half-life of the active form of this protein must be extremely short, no more than 20–30 min.

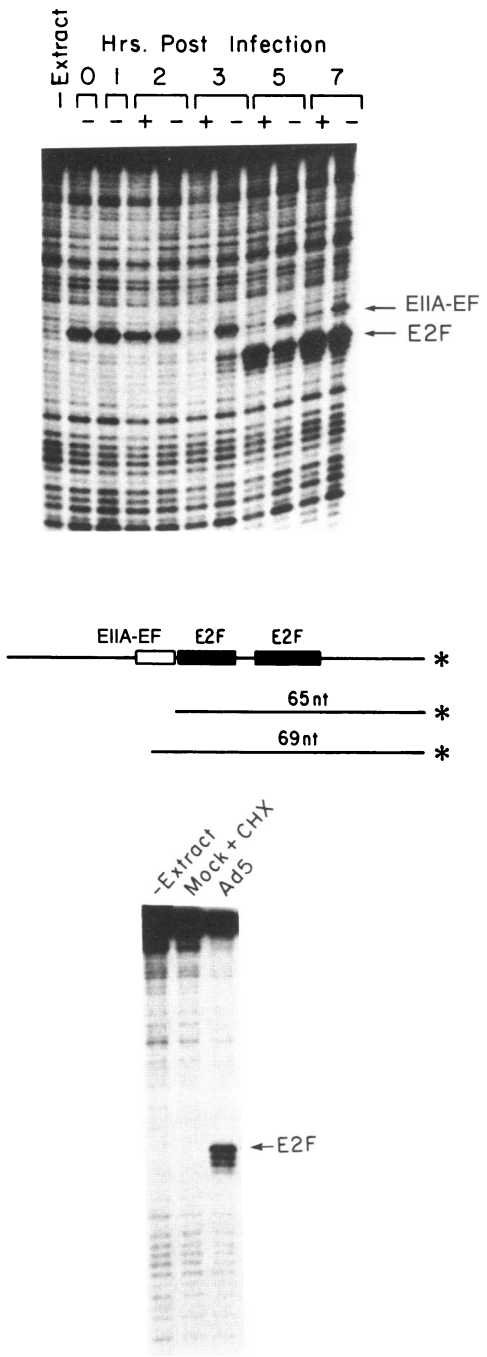

FIG. 3. Effect of cycloheximide on the activation of E2F. (*Upper*) HeLa cells were infected with Ad5 (10 pfu per cell) and at the indicated times after infection, nuclear extracts were prepared. Some cells of the culture received cycloheximide (50 μg/ml) at 1 hr after infection (+). Assays were similar to those in Fig. 1 except that poly(dI·dC) was used as nonspecific competitor at an assay concentration of 1 μg per 25 μl. As shown beneath the gels, the 69-nucleotide-containing band is due to binding of an additional factor termed EIIA at a site (open box) just upstream of the distal E2F site (11, 18). (*Lower*) HeLa cells were infected with Ad5 or were mock-infected. Cycloheximide was added to the mock-infected culture, and then both were incubated for 9 hr. Nuclear extracts were then prepared and assayed for E2F binding as described for Fig. 1.

DISCUSSION

By a number of criteria, the E1A-mediated activation of the E2F factor appears to be the crucial event in the stimulation of adenovirus *E2* transcription. The level of the factor in

extracts correlates with the transcription of the gene within the virus-infected cells. This was originally observed in wild-type virus-infected HeLa cells (9), it has been found in uninfected F9 teratocarcinoma cells that are able to complement an *E1A* mutant (10), and we now demonstrate that the kinetics of activation coincide with the kinetics of *E2* transcription activation. Furthermore, the induction is mediated by the E1A 13S product, the E1A product necessary for the efficient stimulation of *E2* transcription (15–17). We thus conclude that E2F is limiting in HeLa cells with respect to the *E2* promoter and that the increase in E2F levels is likely responsible for the induction of *E2* transcription.

The experiments reported here also address the mechanism for this activation and indicate that the activation is not increased synthesis of the E2F protein. Rather, it appears that E1A catalyzes an alteration (modification) of a preexisting protein that activates its binding ability, although the nature of this alteration is thus far unclear. This finding has significance in obviously focusing our attention on the nature of this modification, which could serve as a molecular key for E1A transcription control. From these results, we can speculate on the mechanism for coordinate stimulation of transcription by E1A. First, we have previously shown that E2F binds to the *E1A* enhancer, as well as to the *E2* promoter, but that it does not interact with regulatory sequences of any of the other E1A-inducible genes (*E1B, E3, E4*, and *hsp70*) (19). Thus, activation of the E2F factor is probably responsible for a large stimulation of *E2* transcription and the smaller stimulation of *E1A* transcription, but very likely other proteins are important for stimulation of the other promoters. One candidate protein is a TATA-binding factor because the critical sequences for E1A stimulation in the *E1B* promoter (20) and the *hsp70* promoter (Simon, M. C., Fisch, T., Benecke, B., Nevins, J. R. & Heintz, N., unpublished data) appear to be the TATA element. In contrast, stimulation of the *E4* promoter appears to require sequences upstream of the TATA (21). Finally, E1A also stimulates polymerase III transcription (13, 22, 23), possibly via the TFIIIC transcription factor. Thus, E1A may effect a stimulation of transcription of a group of genes through the action of at least four promoter-binding factors. The data presented here indicates that, in at least one case (E2F), the activation involves a modification.

Thus, possibly the same mechanism is involved in the activation of each factor. For instance, one could imagine that each protein possessed an identical regulatory domain that was the site of E1A action. If each protein possessed a specific DNA sequence-recognition domain, then through a single mechanism of action, E1A could activate the binding ability of a group of factors that would, in turn, activate a group of genes; thus, coordinate control would be achieved. Clearly, a final understanding will require the isolation of several factors coregulated by E1A and a determination of their common aspects. Isolation of one of these factors and demonstration of the mechanism for its control are a start in the direction of that understanding.

We thank Dr. A. Berk for the E1A mutants dl1500 and pm975 and Dr. T. Shenk for dl312. R.R. held a fellowship from the Deutsche Forschungsgemeinschaft, and I.K. held a fellowship from the National Sciences and Engineering Research Council (Canada). This work was supported by a grant from the National Institutes of Health (GM26765).

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