The Adenovirus E4 Gene, in Addition to the E1A Gene, Is Important for *trans*-Activation of E2 Transcription and for E2F Activation†

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Previous experiments have demonstrated that adenovirus infection of human and mouse cells leads to an E1A-dependent activation of the DNA-binding capacity of a cellular transcription factor termed E2F. E2F binds to two sites in the adenovirus E2 early promoter which have been shown to be critical for E1A-dependent E2 early transcription, and the E2F-binding sites can confer E1A-induced transcription to a heterologous promoter. In addition, under a variety of circumstances, the increase in E2F-binding activity coincides with the activation of E2 transcription. We now find that, in addition to the E1A gene, another early viral gene, the E4 gene, is necessary for the activation of E2F-binding activity. Extracts prepared from human 293 cells, which express the E1A and E1B genes, had low levels of E2F activity, whereas infection of 293 cells with the E1A mutant *dl312* increased E2F activity. This increase did not occur when 293 cells were infected with *dl366*, an E4 deletion mutant, nor was there an increase in E2F activity in HeLa cells infected with either *dl366* or *dl312*; however, a coinfection with the two mutants yielded the normal wild-type increase in E2F. Furthermore, infection of HeLa cells with a high multiplicity of *dl312*, conditions that allow E4 gene expression in the absence of E1A, did not yield an increase in E2F activity. Thus, it appears that both the E1A gene and the E4 gene are directly involved in E2F activation. Measurements of E2 RNA production in a *dl366* infection as compared with a wild-type or *dl312* infection demonstrate that the E4 gene is essential for full E2 transcription. Furthermore, transfection assays of the E2 promoter demonstrate that, although E1A alone can *trans*-activate the E2 promoter, it is not as effective as the combination of E1A and E4 in the induction of the E2 promoter. We therefore conclude that the activation of the E2F factor leading to the activation of E2 transcription requires the combined action of both the E1A 289-amino-acid protein and an E4 product.

The efficient initiation of transcription by RNA polymerase II is dependent upon the interaction of protein factors with the DNA-regulatory elements of promoters and enhancers. Although some genes are transcribed in all cells with little or no variation, other genes are subject to regulation that restricts transcription to specific cell types or at particular times of development or in response to extracellular factors. Of interest, then, is an elucidation of the molecular mechanisms for gene-specific transcription control. Many of the transcription factors that recognize and bind to promoter and enhancer elements may not be rate-limiting components; thus, although necessary for transcription, they do not define the time or place for transcription activity. However, in several cases factors have been identified by DNA-binding assays that fluctuate in parallel with changes of transcription of the gene to which they bind (see reference 23 for a review). Such DNA-binding proteins thus become candidates for rate-limiting transcription factors that determine the regulatory properties of the system. The identification of these factors is critical in allowing the next step to be taken in the elucidation of the regulatory pathway, namely, the

basis for the control of the activity of the factor as well as the activities that mediate this control.

A particularly useful system for the study of the role of inducible transcription factors in gene control is the set of early adenovirus genes whose transcription is dependent on the viral E1A gene (2, 28). A variety of experiments have demonstrated that transcription of the early viral genes is dependent on the action of cellular transcription factors (28). One such example is the E2F transcription factor. E2F interacts with E2 promoter sequences that are critical for E2 transcription (20, 48), and an E2F-binding site can confer E1A inducibility to a heterologous promoter (47). The increase in E2F DNA-binding activity coincides with the activation of E2 transcription (26, 36), and the presence of an E1A-like activity in F9 teratocarcinoma cells and the down-regulation of this activity upon differentiation are accompanied in parallel by a loss of E2F activity (18, 35). Finally, our recent experiments demonstrate that the purified E2F factor can stimulate E2 transcription *in vitro*, dependent on E2F binding (47). Our previous experiments have shown that E2F DNA-binding activity was induced in an adenovirus infection, dependent on the expression of the 289-amino-acid E1A gene product and coincident with the activation of E2 transcription (36). However, these studies did not rule out the possible involvement of other early viral genes in the activation process. Numerous previous experiments have shown that E1A can function alone as a *trans*-activator of the E2 promoter. However, we now report that the combined action of E1A and E4 is necessary for full E2 tran-

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scription induction and that the activation of E2F requires the expression of the viral E4 gene.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa cells were grown in minimal essential medium (Joklik) with 5% calf serum. Monolayer cultures of HeLa cells (S3 line; American Type Culture Collection) or Vero cells were maintained in Dulbecco modified Eagle medium (DME) with 10% fetal calf serum. Wild type (WT) adenovirus type 5 (Ad5) was grown in HeLa cells and purified as described previously (25). The *dl312* mutant was grown in 293 cells, and the *dl366* mutant was grown in the W162 cell line (46). Mutant viruses were purified as described previously for the WT (25). Infectious virus assays were performed as described previously (39).

Preparation of extracts. Nuclear extracts were prepared from HeLa cells or 293 cells as previously described (19). Whole cell extracts were prepared as described previously (34).

E2F assays. Assays for E2F-binding activity were performed essentially as described before, by employing either the gel retardation assay procedure or exonuclease III protection (19). The DNA probe used for binding was a reduced version of the E2 promoter fragment, which contains sequences from positions -21 to -98 (35).

Preparation and analysis of RNA. HeLa cells were lysed in 5% Nonidet P-40 at pH 8.6 to prepare cytoplasmic RNA (24). RNA samples were isolated 4 to 6 h after infection and analyzed by hybridization to ³²P-labeled SP6 riboprobes synthesized in vitro (24). A Northern analysis of E4 RNA was performed with poly(A)⁺ whole cell RNA prepared as described previously (43). Samples were fractionated in a formaldehyde-agarose gel and blotted to nitrocellulose, and the blot was probed with a ³²P-labeled E4-specific DNA probe (91.9- to 98.3-map-unit fragment).

Transfection assays. HeLa monolayer cultures were maintained in the presence of cytosine arabinoside (40 µg/ml of culture medium) and infected with 20 focus-forming units of Ad5, *dl366*, or *dl312* per cell 8 h before transfection with DNA-calcium phosphate precipitates. DNA precipitates were removed 12 h later, incubation was continued for another 12 h, and the cell extracts were prepared and assayed for chloramphenicol acetyltransferase (CAT) activity (6, 38). For cotransfection assays, Vero cells grown in DME with 10% fetal calf serum were employed. Cells were transfected with calcium phosphate-DNA precipitates. After 12 h, precipitates were removed, and incubation was continued for an additional 36 h. Extracts were prepared and assayed for CAT activity.

Plasmids. The plasmid termed pE2CAT is the previously described plasmid pEC113 (12), which contains E2 promoter sequences from positions -285 to +40 cloned upstream of the CAT gene in pCAT3M. The pE4 plasmid contains Ad5 sequences from 84.8 to 100 map units cloned in pBR322. The pE1A plasmid has been described before (12).

RESULTS

E1A is not sufficient for induction of E2F. The study of transcription activation of the adenovirus E2 gene has advanced rapidly over the past few years such that there is now considerable detail available concerning the DNA sequences and transcription factors involved in the control. It appears that three distinct cellular transcription factors interact with the E2 early promoter to allow maximal transcription (Fig.

FIG. 1. Schematic diagram of the early adenovirus transcription units and the E2 promoter. The elements in the E2 promoter that bind factors functional in transcription are depicted.

1). One is an ill-defined TATA binding protein (15), and another is the factor termed ATF (48) that also interacts with several other early adenovirus promoters (21). Both factors are critical for E2 transcription, as judged from mutagenesis of their binding sites (14, 40, 49). Finally, there are also two binding sites for the E2F factor (19). Purified E2F recognizes and binds to the E2 promoter via the duplicated sequence TTTTCGCGC (48) and as a result of this interaction can stimulate transcription from the E2 promoter (47). Various experiments have demonstrated that fluctuations in the level of E2F, as measured by DNA-binding assays, correlate with E2 transcription (19, 20, 35, 36, 41). This fact, coupled with mutagenesis experiments that define the E2F-binding sites as critical positive elements for E2 promoter function (14, 21a), argues strongly that the E2F induction is important for the activation of E2 transcription.

We have previously shown that adenovirus infection of HeLa cells leads to a dramatic increase in E2F levels and that this does not occur in an E1A mutant (*dl312*) infection, including a high-multiplicity *dl312* infection that allows expression of the various early genes (19). However, this does not prove that E1A expression is sufficient for E2F induction, only that it is necessary; it remains possible that other early viral genes may be involved in this induction. Our first real indication that E1A might not be sufficient for E2F induction was from an experiment as shown in Fig. 2. By analyzing extracts from 293 cells, an adenovirus-transformed human cell line that expresses E1A and E1B but no other early viral gene (1), we could not detect E2F-binding activity. That this was not just a peculiarity of this cell line, such as a poor extraction of the E2F factor, was suggested by the fact that Ad5 infection of 293 cells substantially increased the E2F levels. This increase in E2F could be the result of two possibilities. First, the E1A gene product might be limiting in 293 cells with respect to E2F activation, and the WT Ad5 infection would introduce additional copies of the E1A gene, thus allowing increased E1A expression. Alternatively, an additional early viral gene, which would also be provided by the Ad5 infection, might be responsible for enhanced E2F induction. In fact, the latter appears to be true, since an infection with *dl312*, which is deficient in E1A but provides the other early viral genes, induced E2F activity in 293 cells to a level nearly equivalent to that seen in an Ad5 infection. We therefore conclude that E1A is not sufficient for an induction of E2F.

E2F induction requires expression of the E4 gene as well as the E1A gene. Although there are many variables in a viral infection that might contribute to the activation of E2F, one obvious possibility is the requirement of another viral gene product. In considering possible candidates for other early viral genes involved in E2F induction, we could readily rule out several possibilities. The E1B gene was unlikely since it

FIG. 2. Assay for E2F in 293 cells. Nuclear extracts of uninfected 293 cells (U) and 293 cells infected with WT Ad5 or *dl312* were prepared as described in Materials and Methods. Gel retardation assays for E2F were performed by using a probe spanning the E2F-binding sites. Assays were carried out in the absence (–) or presence (+) of specific competitor DNA. The complex of E2F with E2 promoter is indicated (E2F).

was already expressed in 293 cells, E3 was not likely involved since this gene is defective in *dl312* (17), and we viewed the E2 gene as an improbable candidate simply based on the fact that E2F induction can take place in WT-infected cells where protein synthesis has been inhibited from 1 h postinfection, a condition that would prevent expression of the E2 gene product (34). This left the product of the L1 region as well as the E4 gene as candidates. Although mutants are not available for L1, they are available for E4; we thus directed our attention to the potential role of the E4 gene in activation of E2F. The mutant *dl366* harbors a large deletion of the E4-coding sequence (91.9 to 98.3 map units) and is defective for growth in HeLa cells (7). 293 cells were infected with WT Ad5, *dl312*, and *dl366*, and nuclear extracts were prepared at 8 h postinfection to assay for E2F levels. There was no increase in E2F activity after infection of 293 cells with *dl366* (Fig. 3), thereby indicating that the E4 gene was necessary, in addition to E1A, to induce E2F.

Infections of HeLa cells with the various viruses confirmed that both the E1A gene and the E4 gene were necessary for E2F activation. Infection of HeLa cells with WT Ad5 produced a large increase in E2F activity over the level found in mock-infected cells when assayed by exonuclease III protection (Fig. 4A). In contrast, infection with *dl312* or *dl366* alone yielded little or no increase in E2F activity. However, coinfection of HeLa cells with *dl312* and *dl366* resulted in a large induction in E2F activity, equivalent to that seen in WT-infected cells. Thus, as suggested by the results in 293 cells, both E1A and E4 are necessary for E2F activation.

The results shown in Fig. 3 and 4A demonstrate that an E4 gene product is essential for activation of E2F. The E1A requirement in this process is less clear, since E1A is necessary for E4 expression and one might imagine that the requirement of E1A in E2F activation could simply be to activate E4 expression, with the E4 product being the sole requirement for E2F activation. However, we have previously shown that infection with a high multiplicity of *dl312*, conditions that allow expression of the various early genes

FIG. 3. E4 gene is required for E2F induction. Nuclear extracts were prepared from uninfected 293 cells (U) and 293 cells infected with Ad5, *dl312*, or *dl366*. E2F assays were as described in the legend to Fig. 2.

including E4 (26, 27), did not result in activation of E2F (19). This result is repeated in the experiment shown in Fig. 4B. At an input multiplicity of 12,000 particles of *dl312* per cell, the level of E4 RNA was equal to that in a WT infection at 350 particles per cell (Fig. 4C). (The majority of the E4 RNAs migrated at 2 kilobases as indicated; the larger RNA was probably unspliced precursor RNA.) When E2F was assayed in extracts of such infections, there was clearly an increase in the WT infection but no evidence of an increase in the *dl312* infection (Fig. 4B). Of course, there are multiple E4 gene products produced from alternatively spliced mRNAs (44), and at the present time we do not know which is responsible for the activation of E2F. Thus, although the level of the total E4 RNA population was equivalent in the *dl312* and WT infections shown in Fig. 4C, it is possible that there was less of the particular E4 RNA and protein in the *dl312* infection that was responsible for the activation of E2F. With this caveat in mind, it is nevertheless true that we did not detect an activation of E2F under these conditions; we thus conclude that E1A most likely plays an active role in the process of E2F activation.

Full E2 transcription requires E4 expression. The analysis of E2F activation demonstrating a dependence on E4 gene expression suggested a role for the E4 gene in early transcription control. Although previous experiments employing virus infections have not provided evidence for such (7), there has been a report of a *trans*-activating function attributed to E4 (5). In particular, cotransfection of an E2 promoter-driven plasmid with an E4-expressing plasmid resulted in a 5- to 15-fold stimulation of the E2 promoter. In view of this result and the apparent requirement of E4 in the activation of E2F, we reinvestigated the role of the E4 gene in the activation of early E2 transcription. HeLa cells were infected with WT Ad5, *dl312*, or *dl366*; RNA samples were prepared at various times postinfection, and E2 RNA production was measured by using an SP6 RNA protection assay as described previously (39). The results of two such experiments are shown in Fig. 5. In each case, the level of E2A RNA in a *dl312* infection was greatly reduced, amounting to no more than 1 to 2% of the WT level in the 6-h sample. In the *dl366* infection there was an increase in E2A RNA as compared with that in *dl312*-infected cells; thus, the E1A gene is sufficient to achieve an activation of E2 tran-

FIG. 4. Requirement for E1A and E4 expression in E2F induction in HeLa cells. (A) Whole cell extracts were prepared from mock-infected HeLa cells, Ad5-infected HeLa cells, *dl312*-infected HeLa cells, *dl366*-infected HeLa cells, and cells coinfecting with *dl312* and *dl366*. E2F activity was measured by an exonuclease III protection assay as described in the text with a DNA probe containing sequences from positions -21 to -98 of the E2 promoter. The exonuclease III stop induced by E2F binding is verified by the competition with a DNA fragment containing the E2F-binding site (lane 3). (B) Whole cell extracts were prepared from mock-infected HeLa cells, HeLa cells infected with Ad5 at 350 particles per cell, and HeLa cells infected with *dl312* at 12,000 particles per cell. E2F activity was assayed by exonuclease III protection by using the probe described above. (C) Northern analysis of E4 RNA produced in a WT Ad5 infection at a multiplicity of infection of 350 particles per cell and a *dl312* infection at a multiplicity of infection of 12,000 particles per cell. Whole cell poly(A)⁺ RNA was prepared, and 10 μ g of each sample was analyzed by formaldehyde-agarose gel electrophoresis as described in Materials and Methods.

scription. However, the level of E2A RNA in the *dl366* infection was significantly reduced (about 10-fold) compared with that in the WT infection, indicating that expression of the E4 gene is necessary for full activation of E2. In addition, assays for E1A RNA sequences by using an SP6 riboprobe specific for E1A 3' sequences common to all E1A RNAs revealed a small reduction in the level of the E1A RNAs in *dl366*-infected cells as compared with that in Ad5-infected cells in one experiment (Fig. 5A) and a somewhat greater reduction in the *dl366* sample compared with the WT in the second experiment (Fig. 5C). A limited role for the E4 gene in E1A transcription is not altogether unexpected, since we have shown previously that E2F binds to duplicated sequences within the E1A enhancer (20). From these results we conclude that the action of E1A alone is sufficient to achieve a stimulation of E2 transcription (*dl366* infection versus *dl312* infection) but that full activation of E2 transcription requires the action of an E4 gene product(s) (WT infection). Since in at least one experiment there was an E4-dependent effect on both E1A and E2 RNA expression, one might interpret the results to mean that E4 does not affect E2 expression directly. However, the E4-dependent

FIG. 5. Full E2 transcription requires E4 gene expression. (A and B) HeLa cells were infected with Ad5, *dl312*, or *dl366* at a multiplicity of 20 focus-forming units per cell. Samples were harvested at the indicated times postinfection, RNA was prepared, and then 10 μ g of each was assayed for E2A RNA (A) by an SP6 protection assay. Also shown (B) is an assay for E1A RNA (3' sequences) in the 4-h samples. (C) HeLa cells were infected with Ad5, *dl312*, or *dl366*, and RNA was prepared at 6 h postinfection. A sample of each was assayed for E2A RNA, E1A 3' sequences, and γ -actin by SP6 protection.

effect on E1A RNA levels was not as severe as the effect on E2 RNA levels, and further experiments described below (see Fig. 7) clearly demonstrate a direct role for E4 in the activation of E2 transcription. A role for the E4 gene in E2 transcription contrasts with the previous study of Halbert et al. (7) in that these authors concluded that the E4 gene did not play a role in the control of early gene expression, including the E2 gene. The reason for the discrepancy between the two studies is not clear, although several possible explanations may be provided. First, the result could relate to the sensitivity of the assays employed in the two experiments as well as the timing within the infection. Second, the cell type could have an influence; Halbert et al. employed HeLa spinner cells for the experiments, whereas we utilized HeLa monolayer cells, and it is clear from a variety of experiments that the cell type can influence the requirements for *trans*-activation (13).

Another approach to defining the role of the E4 gene in the process of E1A *trans*-activation was to assay a plasmid containing the E2 promoter driving the CAT gene after transfection into HeLa cells infected with either WT Ad5 (E1A⁺ E4⁺), *dl366* (E1A⁺ E4⁻) or *dl312* (E1A⁻ E4⁻). In fact, one of our earlier suspicions that full *trans*-activation might involve more than just the E1A gene derived from the observation that expression of pE2 was reproducibly higher in a transfection with Ad5 coinfection than in a cotransfection with pE1A. Infection of cells with either *dl312*, *dl366*, or Ad5 followed by transfection with pE2CAT demonstrated that, although there was induction of E2 by *dl366*, there was a much greater induction with WT Ad5 (Fig. 6). The increase in E2 CAT expression with *dl366* was approximately 8-fold, whereas the increase with WT Ad5 was 30-fold. We there-

FIG. 6. Full activation of the E2 promoter requires E4 expression. HeLa cells were infected with either Ad5, *dl312*, or *dl366* for 8 h in the presence of cytosine arabinoside and then transfected with pE2CAT. Cells were then harvested for CAT assays 24 h later as described in Materials and Methods.

fore conclude that the combined action of E1A and E4 is necessary for the full activation of E2 transcription.

The experiments shown in Fig. 5 and 6 clearly demonstrate a role for the E4 gene in the activation of E2 transcription, but they are complicated by the presence of E1A and the question as to whether E4 functions directly in the activation of E2 or rather indirectly through an effect on the E1A gene. To address this problem, we assayed for the ability of the isolated E1A and E4 genes to *trans*-activate the E2 promoter in cotransfection assays. In this way we could assess the ability of the E4 gene to *trans*-activate the E2 promoter independent of E1A. Vero cells were used for these experiments because we have found that they yield the best and most reproducible *trans*-activation. Cotransfection of pE2CAT with pE1A resulted in a 25-fold stimulation (Fig. 7A), as reported in many previous experiments (11, 18, 47). Clearly, E1A alone can function as a *trans*-activator of the E2 promoter. Likewise, cotransfection of pE2CAT with pE4, a plasmid containing the E4 transcription unit, also

resulted in an approximately 15-fold stimulation, in agreement with a previous study (5). Thus, it is clear that the E4 transcription unit does, indeed, encode a *trans*-activating function that can directly stimulate the E2 promoter independent of E1A action. Further insight into the *trans*-activation of E2 was gained by an experiment in which pE2CAT was cotransfected with pE1A or pE4 or a combination of pE1A and pE4 (Fig. 7B). Assay of a titration of pE1A or pE4 with a constant amount of pE2CAT demonstrated that the *trans*-activation potential of either one was not linear with increasing dose but gradually leveled off. Furthermore, cotransfection of pE2CAT with both pE1A and pE4 yielded higher levels of CAT activity than that obtained with either *trans*-activator alone; this was true at all input levels (Fig. 7B). One simple explanation for the enhanced expression of E2 CAT in the presence of both E1A and E4 is that the two *trans*-activators together boost their own synthesis. However, this is not compatible with the fact that E1A-mediated or E4-mediated *trans*-activation of E2 is not significantly increased upon further addition of E1A or E4, despite the fact that E1A or E4 expression is linear through this range (data not shown). Thus, the greater activation of E2 CAT with both *trans*-activators cannot be due to increased amounts of E1A and E4 but rather appears to be due to a cooperative effect of the two *trans*-activators. We therefore conclude that E1A and E4 most likely perform different functions in the *trans*-activation of E2 transcription.

DISCUSSION

Previous experiments have clearly documented the role of the adenovirus E1A gene in *trans*-activation of early viral and cellular transcription (2, 28). However, since a functional E1A gene, and in particular a functional 289-amino-acid E1A gene product, is required for transcription of all early genes, it remained possible that in some instances a cascade of activation was involved. That is, E1A might activate one early gene, the product of which was then

FIG. 7. E1A-mediated and E4-mediated *trans*-activation of the E2 promoter. (A) Vero cells were transfected with pE2CAT alone (5 μ g) or together with pE1A (5 μ g) or pE4 (5 μ g). Cells were harvested for CAT assays as described in Materials and Methods. (B) Vero cells were transfected with pE2CAT alone (10 μ g) or with pE2CAT together with increasing amounts of pE1A, increasing amounts of pE4, or increasing amounts of pE1A plus pE4 as indicated. The pE4 plasmid is approximately twice the size of pE1A; thus, twice the mass of pE4 relative to pE1A represents equal molar amounts. Cells were harvested for CAT assays as described in Materials and Methods. CAT activity was quantitated by scintillation counting of appropriate regions of the thin-layer chromatography plate.

responsible for the activation of another early gene. This was particularly relevant for the activation of the E2 gene, since maximal E2 transcription occurs somewhat later in the early phase of infection than is the case for most of the other early genes (29). Furthermore, the lag in E2 transcription induction is paralleled by the appearance of E2F DNA-binding activity, suggesting that the two are indeed linked (36). The results presented here suggest that a product of the E4 transcription unit is necessary for the full activation of E2 transcription as well as the activation of E2F. Thus, the lag in E2F activation and consequent E2 transcription activation may be accounted for by the necessity to first express the E4 gene. However, several lines of evidence suggest that the activation of E2F and E2 transcription is not a simple cascade whereby E1A activates E4 gene expression, the product of which then activates transcription of the E2 gene with E1A performing no role in the actual *trans*-activation of E2 once the E4 gene was expressed. Rather, it appears more likely that E1A and E4 both actively participate in the activation of E2F. First, it is clear that E1A acting alone, in the absence of E4, can activate E2 transcription. This was evident in the virus experiments in which E2 transcription was increased in a *dl366* (E1A⁺ E4⁻) infection as well as in numerous previous transfection experiments in which cotransfection with an E1A-expressing plasmid could induce E2 transcription (11, 18, 47). Thus, the E1A gene product appears to play a direct role in the activation of E2 transcription. The converse is also true; cotransfection with an E4-expressing plasmid was shown to induce E2 expression (5). Thus, it would appear that either the E1A gene product or the E4 gene product can independently *trans*-activate the E2 gene and that a combination of the two together is more effective.

We could envision at least three possibilities for the combined action of two regulatory gene products in the *trans*-activation process. First, the 289-amino-acid E1A protein and the critical E4 gene product could be functional as a physical complex. This possibility would appear unlikely, given the observation, as discussed above, that either gene alone can effect an inefficient activation of E2 transcription. It is of course conceivable that a cellular protein equivalent is recruited in the absence of the other viral protein, but it is also true that there is no evidence for a complex between an E4 protein and the E1A 289-amino-acid protein in virus-infected cells; therefore we view this possibility as unlikely.

A second possibility is that the E1A protein and an E4 protein are both required to fully stimulate E2 transcription but that they do so by affecting different events in the process, only one of which is the activation of the DNA-binding capacity of E2F. If, for instance, the activation of E2 transcription involved an alteration in the transcription-stimulatory activity of a factor as well as the DNA-binding activity of a factor, then one could envision a mechanism whereby the E1A protein and an E4 protein performed these roles separately. The role of one of the *trans*-activators might be to alter the E2F factor, or a different E2 promoter-binding factor such as the ATF factor, to convert it to a transcriptionally active form with no change in the capacity of E2F to bind to DNA. Thus, there would be a stimulation of E2 transcription but no increase in E2F DNA-binding activity. If the E2F factor was also limiting with respect to the available factor active in DNA binding, then the role of the other *trans*-activator could be to increase the binding activity of the factor, which would then produce a further stimulation of transcription. However, this mechanism would predict that either E1A or E4 acting alone would

stimulate E2F to nearly full WT levels, a result which we did not observe.

Finally, E1A and E4 might act through different mechanisms to achieve the same final result: an increase in the DNA-binding activity of E2F. Our recent experiments have shown that the activation of E2F likely involves a phosphorylation event converting an inactive form of the factor to an active form (1a). However, there is no indication that either the E1A or E4 gene product is a protein kinase, either experimentally or through an analysis of the sequence of the proteins which shows no homology to the conserved sequence of the ATP-binding site found in all known kinases (8). We thus presume that the activation of E2F is mediated by a cellular kinase that is stimulated by either the E1A protein or an E4 protein. The involvement of a cellular kinase in E2F control suggests the presence of a phosphatase that reverses the activation and, thus, a regulated system. An effect on either the activation process (stimulation of the kinase) or the inactivation process (inhibition of the phosphatase) would lead to the same end result, an increase in active factor. More to the point, if E1A were to affect one step and E4 affected the other step then the two together would produce a greater activation. This mechanism would predict that each activator alone would give a small stimulation; although we observed no evidence of an increase in E2F activity with either E1A alone or E4 alone, we suspect that small increases in E2F activity may well go unnoticed in the preparation of extracts. Based on all of the available data, we believe that the most likely mechanism is this last scenario. Proof of this will likely only come from an analysis of the *in vitro* activation of E2F and a direct determination of the role of E1A and E4 in this process.

Past studies have clearly demonstrated the role of the E2F factor in E2 transcription and *trans*-activation of E2 transcription. The data presented here now demonstrate a role for the E4 gene in the *trans*-activation process. This is not the first indication of a gene-regulatory function for E4. Previous experiments have demonstrated that the E4 gene was essential for the adenovirus helper role in adenovirus-associated virus replication (16, 37). The mechanism by which E4 functioned in this regard was not defined, but clearly one aspect of the adenovirus helper effect is an activation of adenovirus-associated virus transcription. Our experiments do not define the particular E4 gene product responsible for E2F activation, and since the *dl366* deletion affects all of the E4 open reading frames it could be any of the seven possible proteins encoded in this region (44). A series of E4 deletion and substitution mutants was generated; these mutants define each of the open reading frames, and the analysis of these should lead to an identification of the E4 gene product responsible for E2F activation (7). Obviously, the identity of the E4 protein mediating this activity and its mechanism of action are of some considerable interest.

The apparent requirement of two adenovirus genes for the full *trans*-activation of E2 transcription is reminiscent of previous studies with other viral systems. Analogous to E1A, the herpes simplex virus immediate-early ICP4 gene product is defined through the analysis of viral mutants as essential for early gene activation (32, 45). However, in various other assay systems, the ICP0 immediate-early gene product can also *trans*-activate and in cotransfection experiments can augment the induction by ICP4 (3, 4, 30, 32, 33). A similar situation is also true for human cytomegalovirus, in which two immediate-early genes have been shown to have *trans*-activating activity (10, 42). Furthermore, previous

experiments have demonstrated that simian virus 40 T antigen could activate the adenovirus E2 promoter and that, when E1A and T antigen were combined, there was an enhanced activation of E2 transcription, analogous to the combined effects of E1A and E4 (22). This activation by T antigen required the same E2 promoter sequences as activation by E1A, thus suggesting that the effect was mediated through the E2F factor. We might therefore conclude that the viruses that have acquired an ability to stimulate transcription have often done so through the use of multiple regulatory genes. Even in the instances where there appears to be a single *trans*-activating immediate-early gene, such as in pseudorabies virus, the results presented here for the E4 gene show that there clearly could be a second gene that must first be induced by the immediate-early gene. Why, then, the need for multiple regulatory genes that control the transcriptional activity of viral genes through the action of cellular transcription factors? Perhaps more than one gene simply extends the range of factors that can be regulated. Alternatively, as discussed above, a second gene may simply augment the action of the first and thus is required, depending on the cellular environment. If, for instance, many of the cellular transcription factors utilized by the viral genes are regulated normally in response to cell proliferation, then levels may be quite low in the differentiated cells that these viruses normally infect. Thus, under these more stringent conditions additional *trans*-activation may be important.

In vivo experiments like those described here provide the framework for understanding complex systems and are useful in defining the participants in the process. However, the precise roles of the E1A protein and the E4 protein in the activation of E2F will likely only be determined through *in vitro* assays of their activities. As discussed above, it is encouraging that recent experiments demonstrate that the activation of E2F can be reproduced *in vitro* by utilizing cell extracts (1a). We would hope that such an assay system will allow an elucidation of the role of the viral proteins in this process.

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