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The State of Cellular Differentiation Determines the Activity of the Adenovirus E1A Enhancer Element: Evidence for Negative Regulation of Enhancer Function

R. S. HERBST,[1] M. PELLETIER,[2] E. M. BOCZKO,[1] AND L. E. BABISS[1]*

Department of Molecular Cell Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021,[1] and Department of Pathology, New York University Medical Center, New York, New York 10016[2]

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Most of the eucaryotic enhancer elements so far described consist of multiple DNA binding sites for proteins that act either synergistically or antagonistically to modulate the rate of transcription. In this report, we show that the activity of the adenovirus E1A enhancer element is suppressed in virus-infected undifferentiated rodent fetal fibroblast cells (CREF and F111 cells) and primary rat liver hepatocytes that have lost their fully differentiated phenotype (dedifferentiated). This contrasts with the results obtained for virus-infected differentiated or partially dedifferentiated rodent hepatocytes or hepatoma cell lines and human HeLa cells, in which deletion of the E1A enhancer domain greatly reduces the rate of E1A gene transcription. An in vitro quantitation of the nuclear proteins (from HeLa and CREF cells) that interact with and modulate the activity of the E1A enhancer revealed similar binding activities for the E2f and ATF proteins. However, an AP3-like (ϕAP3) activity was present at a 10- to 20-fold higher concentration in CREF cells than in HeLa cells, and removal of this ϕAP3-binding site on the viral genome resulted in an increase in the rate of E1A gene transcription in virus-infected CREF cells. Together, these results demonstrated that the factors which positively regulate enhancer function were present in CREF cells and that the ϕAP3 factor was acting to suppress the activity of the E1A enhancer. Furthermore, the level of this factor was found to increase to even higher levels in CREF cells treated with 12-*O*-tetradecanoylphorbol-13-acetate, and this induction resulted in a further suppression in the rate of E1A gene transcription. On the basis of these observations, we propose that E1A expression is negatively regulated by the ϕAP3 factor in undifferentiated rodent fetal fibroblast cells and that this could be an important mechanism that distinguishes between establishment of the differentiated cell versus transformed cell phenotypes.

The specificity and rate of transcription from a eucaryotic RNA polymerase II-dependent promoter have been shown to be dictated by multiple genetic regulatory sequences and the cellular proteins that interact with them (46a, 50). These sequences can be classified into two distinct functional units: the promoter-proximal sequences, which function in an orientation-dependent manner, and the enhancer sequences, which function in a position- and orientation-independent manner (7, 20, 38, 40, 56). The promoter-proximal elements for many unrelated genes can include a TATA box (which binds the cellular TFIID factor [7, 51]), A CAAT box (which binds a wide variety of related cellular proteins [5, 11, 19, 46]), a G+C-rich box (which binds the cellular factor Sp1 [8, 39]), and additional gene-specific *cis* regulatory domains. In conjunction with these well-conserved domains, enhancer elements are generally composed of multiple regions of DNA-protein interactions that collectively influence the efficiency of transcription complex formation (see references 38 and 45 for reviews). Interestingly, many of the factors that regulate enhancer activity can also bind to the promoter-proximal regulatory sequences, thereby increasing the total mass of a particular factor. For example, the cellular factor C/EBP (26) binds to the promoter-proximal and enhancer sequences of the mouse albumin, transthyretin, and alpha-1-antitrypsin genes to positively influence their rates of transcription (17). This finding further suggests that this cellular factor functions to regulate coordinate tissue (liver)-specific gene expression. In addition to DNA-protein interactions that occur on these *cis* regulatory sequences, many protein-protein interactions can also affect the rate of gene transcription (27, 53). Although the complexities of the factors involved in gene activation vary among genes, it is likely that in most cases these DNA-protein interactions serve to influence the efficiency of TFIID binding to the TATA element, thereby establishing a committed transcription complex (1, 36).

Many viral enhancer elements have been characterized, with the hope of understanding how they function to stimulate the rate of promoter utilization and to identify the cellular factors that regulate both viral and cellular gene expression (see references 38 and 46 for reviews). The simian virus 40 (SV40) early enhancer is probably the best-characterized viral enhancer element. It has been shown to be composed of a large number of modulatory units termed enhansons, which can functionally substitute for each other (34, 35, 48, 65). Although this enhancer functions in a wide variety of cell types, it is thought to be the combination of these various enhanson regions that determines the degree of enhancer function in any particular cell type (49, 54). The activities of several viral enhancer elements have also been shown to be regulated on the basis of the state of cellular differentiation. For example, SV40 (24, 44), polyomavirus (59, 63), and Moloney murine leukemia virus (58) are all unable to express their early genes in undifferentiated embryonal carcinoma cells. Upon induction of the fully differentiated cell phenotype, however, enhancer function ensues, and viral early-gene transcription is observed. Wasylyk et al. (63) have described a cellular factor

* Corresponding author.

(PEA2) that is present in undifferentiated embryonal carcinoma cells and seems to function to suppress polyomavirus enhancer function. It seems that during the process of cellular differentiation, the activity of the positive regulator of enhancer function (PEA1 or AP1) increases while the activity of the negative repressor decreases, both serving to increase enhancer function.

The adenovirus E1A gene enhancer has been shown to be composed of two distinctly functional elements (28, 29). Element I is present as two copies between nucleotides −330 and −289 and between nucleotides −202 and −192 relative to the E1A cap site, and at least one copy is required to maintain a high rate of E1A gene transcription. The more upstream of these sequences appears to interact with a poorly defined cellular factor called EF-1A (see Fig. 1 for position; J. T. Bruder and P. Hearing, in press). Directly adjacent to this sequence is an E2f factor-binding site (nucleotides −287 to −280 [41]), which is also duplicated at nucleotides −224 to −217. The DNA-binding activity of this factor has been shown to be influenced by the viral E1A and E4 gene products, and this factor functions to stimulate both viral E1A and E2A and cellular *myc* gene transcription (3, 42, 57, 60). Element II is present in four copies between the element I sequences. Element II sequences have been shown to modestly increase the rate of transcription from any RNA polymerase II-dependent promoter located on the viral genome. The factors that interact with these sequences have not yet been described.

Hen et al. (30) have described a region upstream of element I that can function to enhance expression from the adenovirus major late promoter on a plasmid vector after transient transfection into HeLa cells. This region contains a weak consensus binding site for the nuclear factor AP3, which has been shown to bind to the SV40 core C enhancer element and can be induced by treating growth-arrested cells with phorbol ester tumor-promoting agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4, 10). However, when this protein-binding site is removed from the viral genome, there is no effect on the rate of E1A gene transcription after virus infection of HeLa cells (29). Furthermore, this region cannot function as an enhancer to stimulate viral E1A gene transcription when both element I sequences are removed (28, 29). Therefore, the function of this protein-binding domain on the viral genome is unclear.

We have recently described the transforming characteristics of several viruses that lack all or part of the E1A enhancer element (R. S. Herbst, M. Pelletier, and L. E. Babiss, J. Cell Biochem., in press). These studies showed that a virus lacking all of the E1A enhancer region could transform cloned rat embryo fibroblast (CREF) cells (21) at an eightfold-higher efficiency than could wild-type adenovirus. This finding suggested the possibility that reducing the rate of E1A gene transcription could influence the efficiency of focus formation. However, analysis of E1A cytoplasmic mRNA levels in virus-infected CREF cells suggested that the E1A enhancer was not contributing to the rate of E1A transcription in this cell type. Therefore, the transforming characteristics of the enhancer-mutant viruses could not be attributed to the efficiency with which they expressed their E1A genes.

In this study, we have extended these early observations to demonstrate that the activity of the E1A enhancer element is dependent on the state of cellular differentiation for its function. We find that because of a lack of enhancer function, undifferentiated rodent fibroblast cell lines (CREF and F111) do not support high rates of E1A gene expression. We also show that fully differentiated primary cultures of rat hepatocytes lose their ability to support enhancer function as they lose their differentiated phenotype in culture. We demonstrate that the activity of the E1A enhancer element is suppressed in these cells by the increased abundance of the cellular factor that interacts with the viral DNA sequences between nucleotides −341 and −330 relative to the E1A cap site. We have called this protein φAP3 because our studies and the studies of Barrett et al. (4) suggest a relatedness to the previously described AP3 protein (10) but do not conclusively demonstrate they are the same. Using a virus containing a deletion of the φAP3-binding site, we can restore E1A enhancer function in CREF cells. Additional studies suggest that the activity of this factor can be increased by treating CREF cells with a phorbol ester tumor-promoting agent (TPA), which leads to an even greater suppression of E1A gene transcription. Unlike cellular AP3 however, the φAP3 factor is not affected by TPA treatment of serum-starved HeLa cells. The significance of these findings as they relate to the processes regulating cellular differentiation and cellular immortalization is discussed.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of human HeLa and 293 (25) cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% defined bovine calf serum (Hyclone). Human hepatoma (HepG2 [41]) cells were maintained as monolayer cultures and were grown in Ham F12 medium supplemented with 5% fetal bovine serum, 1× minimal essential medium essential amino acids, and 25 μg of garamycin per ml. The rat hepatoma cell lines FaO and C2 are clonal descendants of cell line H4-II-E-C3 (17, 52). Monolayer cultures of rat hepatoma cells were maintained in the medium used for HepG2 cells. Primary cultures of rat hepatocytes were prepared by perfusion of rat livers in situ with EDTA and collagenase as previously described for mouse hepatocytes (12, 13). Cells were cultured in Eagle medium containing 10% fetal bovine serum, 25 μg of garamycin per ml, 10 μg of hydrocortisone per ml, nd 10 μg of insulin per ml. Monolayer cultures of CREF (21) and F111 (22) cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum.

The viruses used (Fig. 1) included wild-type In340, which has the same genotype as *dl*309 virus but contains a duplication of the viral enhancer and packaging signal DNA sequences in the right end of the viral genome (28), and the E1A enhancer mutant viruses 340-2, 340-11, 340-A5, and 309-6, which contain various deletions of viral DNA sequences (28, 29). Viral stocks were obtained by infection of monolayer cultures of 293 cells and the preparation of crude cellular lysates by repeated cycles of freezing and thawing. Titers of viral stocks were determined on 293 cells by fluorescent focus assay (61) with antiserum raised in rabbits against cesium chloride-banded purified virions.

Assays for gene expression. Two assays were used to score the rate of viral early-gene transcription and cytoplasmic mRNA accumulation in virus-infected cells. Transcription of the viral early genes was scored by isolating infected-cell nuclei and allowing previously initiated RNA polymerase II transcripts to elongate in the presence of [^{32}P]UTP (35, 64). An equal amount of radiolabeled RNA (representing a similar number of cells) from each independent sample was then hybridized to denatured DNA probes bound to nitrocellulose filters. The following probes were on the filters:

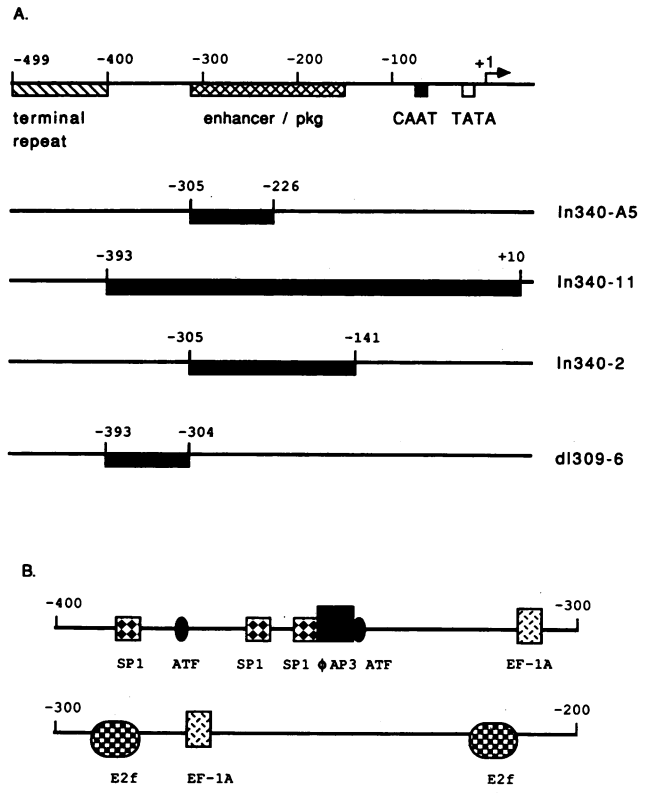

FIG. 1. Schematic representation of the adenovirus E1A gene promoter-proximal and enhancer region and the cellular proteins that interact with enhancer DNA sequences. (A) Left end of the adenovirus genome, showing the relative nucleotide locations of viral DNA regions that contribute to E1A promoter function (enhancer, CAAT box, and TATA box), viral DNA replication (terminal repeat), and DNA-packaging signal (pkg) relative to the E1A gene cap site. The viral genomes shown contain various deletions affecting E1A expression; the isolation and characterization of these viruses has been described elsewhere (27, 28). ■, DNA deletions, with the nucleotide locations shown (in base pairs) relative to the E1A cap site. (B) Diagram of the specific sites of DNA-protein interactions that occur on the E1A gene enhancer. Element I encompasses regions from −301 to −289 and from −203 to −191, and element II encompasses the region from −251 to −218 relative to the E1A cap site (29). Names of the proteins are shown below the DNA-protein binding sites; each protein has been ascribed the position on the basis of in vitro DNA-protein binding studies or similarity to known DNA-binding protein consensus sequences (38; Bruder and Hearing, in press).

E1A5′, 0 to 1,338 nucleotides; E1A3′, 1,338 to 1,538 nucleotides; E1b5′, 2,058 to 2,486 nucleotides; E1b3′, 3,328 to 3,786 nucleotides; E2A, 22,179 to 22,435 nucleotides; E4, 32,264 to 35,937 nucleotides (the adenovirus nucleotide sequences are all relative to the extreme left end of the viral genome); chicken β-actin (14); and pBR322. After hybridization, the filters were treated with RNases A and T_1 and exposed for autoradiography.

The production of stable cytoplasmic RNAs from the viral E1A and E1B genes in virus-infected cells was scored by using an RNase T_2 protection assay as previously described (3, 32). Uniformly labeled antisense RNA probes representing the E1A5′ region (both the 13S and 12S mRNAs) and E1A3′ and E1B5′ mRNAs have been previously described (3, 32) and were generated by linearizing the appropriate plasmid with the appropriate restriction endonuclease and transcribing these sequences by using SP6 RNA polymerase. The sizes of the protected RNA fragments are shown in each figure.

In vitro analysis of DNA-protein binding. Nuclear extracts were prepared from uninfected and In340 virus-infected HeLa and CREF cells essentially according to the protocol of Dignam et al. (18). Nuclear proteins were extracted by using 0.45 M NaCl and were precipitated with ammonium sulfate at a final concentration of 0.3 g/ml of extract. In vitro exonuclease III (ExoIII) stop reactions were performed, using 1 ng of a uniquely end labeled DNA probe (extending from nucleotides −393 to −45 relative to the E1A cap site) by the method of Kovesdi et al. (42). For all reactions, 30 μg of nuclear extract was used, and 2 μg of salmon sperm DNA or 4 μg of poly(dI-dC) was also included as a nonspecific competitor of DNA-binding proteins. After ExoIII digestion (100 U per reaction; Boehringer-Mannheim Biochemicals), the products were analyzed on 5% denaturing polyacrylamide gels. Gel retardation analysis was performed as previously described (31). End-labeled E1A fragment (extending from nucleotides −393 to −311 relative to the E1A cap site) was incubated with 1 to 10 μg of nuclear protein in 40 mM KCl–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–1 mM $MgCl_2$–0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.5 mM dithiothreitol–320 μg of poly(dI-dC) per ml–40 μg of pGEM1 DNA per ml–4% Ficoll (Pharmacia Fine Chemicals) in a final volume of 12.5 μl for 20 min. Protein-DNA complexes were separated from free probe on 5% polyacrylamide gels in 50 mM TBE (50 mM Tris (pH 7.4), 50 mM boric acid, 10 mM EDTA). For DNase I footprinting studies, we used two independently isolated CREF cell nuclear extracts and a HeLa extract (approximately 100 mg) that was fractionated on a P11 column by step elution using KCl. The fractions were dialyzed against BC100 buffer (3). A 1-ng sample of ^{32}P-labeled DNA probe (extending from nucleotides −393 to −141) was incubated with 5 to 30 μg of extract, 5 μg of poly(dI-dC), and 4% polyethylene glycol in a buffer containing 10 mM HEPES (pH 7.9), 7 mM $MgCl_2$, and 0.75 mM dithiothreitol at 0°C for 60 min. DNase I (10 to 50 ng) was added for 3 to 4 mm, and the reaction was terminated by the addition of proteinase K (200 μg/ml) and sodium dodecyl sulfate (0.5%). The DNA was extracted with phenol and ethanol precipitated, and the labeled fragments were analyzed on a 5% denaturing acrylamide gel.

RESULTS

The E1A enhancer element does not stimulate the rate of E1A transcription in fetal rodent fibroblast cells. We have previously observed that the rate of transcription from the adenovirus E1A gene in wild-type virus-infected undifferentiated fetal CREF cells is reduced by 5- to 10-fold compared with the rate in an equivalent number of wild-type virus-infected human HeLa cells (32). To determine whether this decrease in E1A transcription in CREF cells was the result of enhancer-independent E1A expression, we obtained several adenovirus mutants that were lacking all (340-2 and 340-11) or part (340-A5) of the E1A enhancer element (see Fig. 1 for the precise locations of the enhancer mutations; 29). CREF and HeLa cells were infected with 10 PFU of each virus or the parental wild-type In340 virus (containing a wild-type E1A enhancer) per cell for 8 (CREF) or 6 (HeLa) h. Cytoplasmic RNA was isolated and analyzed for the abundance of the viral E1A 13S and 12S mRNAs. E1A

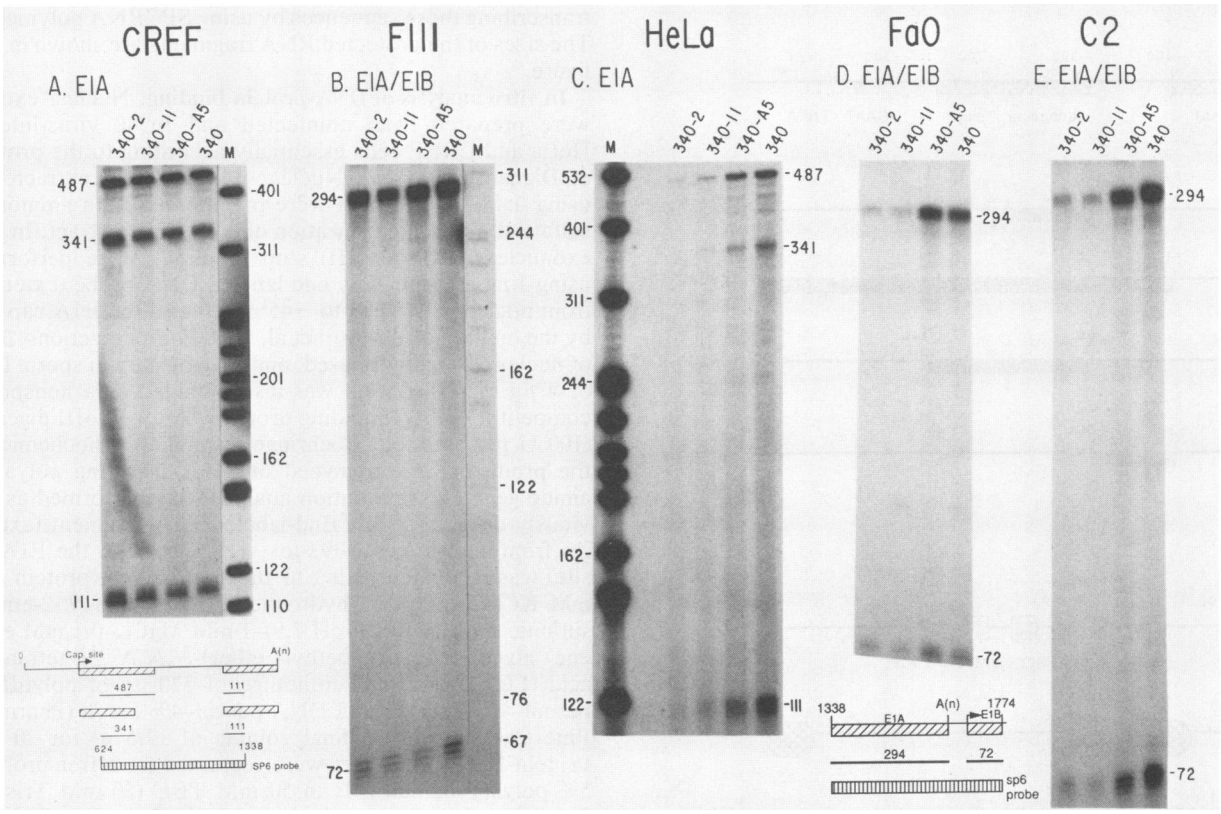

FIG. 2. Demonstration that the E1A enhancer does not contribute to the accumulation of E1A mRNA in the cytoplasm of virus-infected undifferentiated rodent cells. Each cell of the cell lines shown above the autoradiograms was infected with 15 PFU of the viruses indicated above the lanes and scored for the presence of E1A (A and C) or E1A and E1B (B, D, and E) mRNAs by RNase T_2 protection assays. CREF and F111 cells were infected for 8 h; HeLa, FaO, and C2 cells were infected for 6 h. Total cytoplasmic RNA (20 μg) was hybridized to [^{32}P]UTP-labeled antisense RNA probes (7.5×10^5 cpm) for each of the probes shown in the diagram below, and the RNase T_2 digestion products were analyzed on 5% denaturing acrylamide gels. Autoradiographic exposure times were 12 h for HeLa, FaO, and C2 cells and 40 to 50 h for CREF and F111 cells. The lengths of the protected RNAs are shown below panels A and D.

mRNA accumulation in In340-2 virus (no enhancer)-infected HeLa cells was reduced to 5% (by densitometric quantitation) of the level found in In340 virus-infected HeLa cells (Fig. 2C), as had previously been observed by Hearing and Shenk (29). A virus lacking one of the duplicated enhancer elements (340-A5) accumulated about 1/3 as much E1A mRNAs as did with In340 virus-infected cells, whereas a virus lacking the entire enhancer and E1A cap site sequences expressed 1/10 as much E1A mRNAs as did In340 virus. The latter virus was probably expressing the E1A mRNAs through the formation of a unique transcription complex, since no E1A *cis* regulatory elements were present. In these and subsequent studies, the quantitation of each RNA sample was normalized by measuring the accumulation of the cellular actin mRNA (data not shown).

When E1A mRNA accumulation was determined in CREF cells, a very different pattern emerged. All of the viruses, regardless of whether they contained the E1A enhancer element, synthesized equal amounts of E1A mRNAs (Fig. 2A). This autoradiogram was exposed 10 times longer than that from the HeLa experiment, and the results suggested that the reduction in E1A mRNA accumulation in CREF cells may have been due to the lack of E1A enhancer function.

To determine whether the E1A enhancer mutations could be affecting the accumulation of the E1A mRNAs in both cell types (even though these regulatory sequences are not transcribed), the rate of E1A transcriptional initiation was measured in nuclei isolated from the same virus-infected cells that were used to obtain the cytoplasmic RNAs used in the studies described earlier. The rate of E1A gene transcription was significantly reduced and proportional to the accumulation of the E1A mRNAs in enhancer mutant virus (340-2 and 340-A5)-infected HeLa cells (Fig. 3). Equivalent E1A transcription was found in CREF cells infected with any of the viruses, confirming the findings shown in Fig. 2 and suggesting that E1A transcription is not influenced by its enhancer in CREF cells. Again, the exposure times for the CREF and HeLa autoradiograms differed; when corrected, the results again suggested that E1A expression by wild-type virus was reduced 5- to 10-fold in CREF cells.

The state of cellular differentiation determines the activity of the adenovirus E1A enhancer element. The lack of E1A enhancer activity in CREF cells could either be a specific attribute of the CREF cell line or a general characteristic of undifferentiated rodent fibroblast cells. To address this question, another cloned rat embryo fibroblast cell line (F111) was used for a similar analysis of E1A enhancer function (22). F111 cells were infected with the three enhancer mutant viruses used to infect CREF and HeLa cells. Cytoplasmic RNA was analyzed for the relative accumulation of the viral E1A and E1B mRNAs. The results (Fig. 2B) suggested that like CREF cells, F111 cells did not support E1A enhancer-dependent transcription. There was a modest (1.5- to 2-fold)

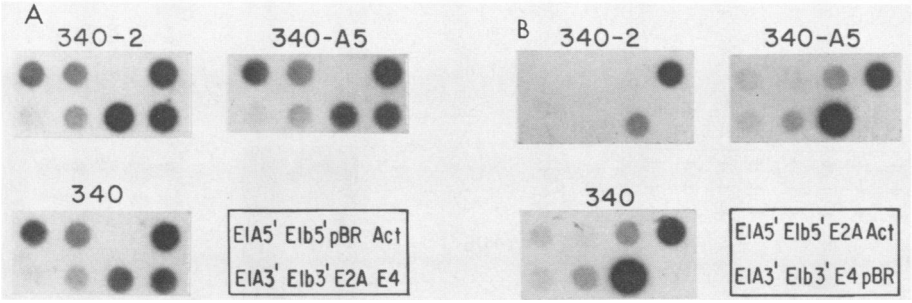

FIG. 3. Demonstration that the E1A enhancer does not contribute to the rate of transcriptional initiation in virus-infected CREF cells. CREF (A) and HeLa (B) cells were infected with each of the viruses shown above each panel as described in the legend to Fig. 2. Infected-cell nuclei were incubated with [^{32}P]UTP to allow previously initiated RNA chains to elongate. This labeled RNA was purified, hybridized (10×10^6 cpm) to excess DNA bound to nitrocellulose filters, washed, and exposed to autoradiography. The pattern of the DNA dots on each filter is diagrammed for each cell type. The DNA probes used on the filters are described in Materials and Methods.

decrease in E1A mRNA accumulation in 340-2 and 340-11 virus-infected F111 cells compared with the level of E1A mRNAs upon 340 virus infection. Nonetheless, comparison of the CREF and F111 findings suggested that in both cell types the E1A enhancer was not functioning as it did in HeLa cells.

To determine whether a characterized stage of cellular development could be correlated with the function of the E1A enhancer element, several rodent hepatoma cell lines were analyzed. FaO is a partially differentiated rat hepatoma cell line that has characteristics of the fetal stage of liver development (as determined from high expression of alpha-fetoprotein [17, 52]) but also displays many phenotypes associated with fully differentiated rodent liver hepatocytes. We consider this cell line to be a rodent equivalent of the human HeLa cell line (partially differentiated); as with HeLa cells, 340-2 virus infection led to a significant reduction in E1A mRNA accumulation compared with results for 340-virus-infected FaO cells (Fig. 2D). Therefore, the E1A enhancer element could function in this rodent cell line to increase the rate of E1A gene expression. The C2 cell line is a dedifferentiated revertant of the FaO cell line that has lost many of the phenotypes associated with adult hepatocytes, including a severe reduction in the ability to express liver gene functions. Infecting these cells with the viruses shown in Fig. 2 resulted in a similar dependence on enhancer sequences for high levels of E1A mRNA accumulation (Fig. 2E). Therefore, these cells probably had not reverted to the same undifferentiated state as the CREF cells; rather, the dedifferentiation process led to a cell type that does not manifest a suppression E1A enhancer activity.

To examine this possibility, we prepared primary cultures of adult rat liver hepatocytes that had previously been shown to lose many properties associated with fully differentiated hepatocytes during prolonged periods in culture. Using cells that were freshly plated for 2 h (fully differentiated) and cells plated for 72 h (dedifferentiated), we could determine whether the process leading to the loss of the hepatocyte-specific gene expression resulted in a loss of E1A enhancer function upon virus infection. Late (72 h) cultures of virus-infected hepatocytes accumulated roughly similar amounts of E1A mRNAs (about twofold less for enhancerless viruses than for wild-type virus), regardless of the absence or presence of the E1A enhancer element (Fig. 4). Virus infection of early (2 h) cultures of hepatocytes revealed E1A mRNA levels that suggested that the E1A enhancer was fully functioning to stimulate E1A gene transcription. In this experiment, E1A mRNA accumulation was greater for the 340-A5 virus but in subsequent studies was only about one-half that of wild-type virus (data not shown). We again confirmed that the change in the regulation of E1A gene expression observed was due to transcriptional control rather than a posttranscriptional mechanism by measuring the rate of E1A gene expression upon virus infection of both cell types (Fig. 4). These results again suggested that as the liver hepatocytes lost their fully differentiated phenotype (observe the reduction in albumin gene transcription by late hepatocytes), the viral E1A enhancer element lost its ability

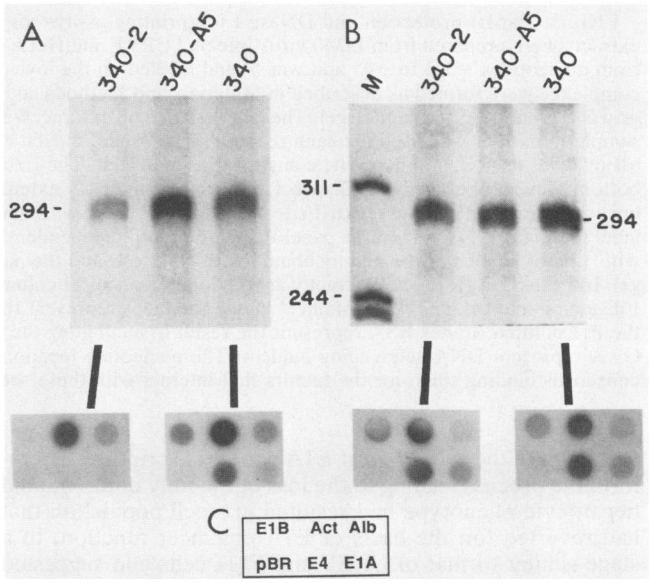

FIG. 4. Demonstration that the E1A enhancer does not fully function to stimulate E1A gene expression in cultured rat hepatocytes. Rat liver hepatocytes were cultured for 2 h (A) or 72 h (B) and then infected for 6 h at 20 PFU per cell with each of the viruses shown above the lanes. Total cytoplasmic RNA was isolated, and the accumulation of the viral E1A mRNA was determined as described in the legend to Fig. 2 and Materials and Methods. Panel A was exposed for 6 h, and panel B was exposed for 36 h. For transcription analysis, nuclei from virus-infected cells were isolated and incubated with [^{32}P]UTP to allow previously initiated RNA chains to elongate. Nuclear RNA (10×10^6 cpm) was hybridized to excess DNA bound to nitrocellulose filters, washed, and exposed to autoradiography. (C) Pattern of DNA dots on each filter. The E1A and E1B clones were both derived from the 5′ portion of these viral genes and are described in Materials and Methods.

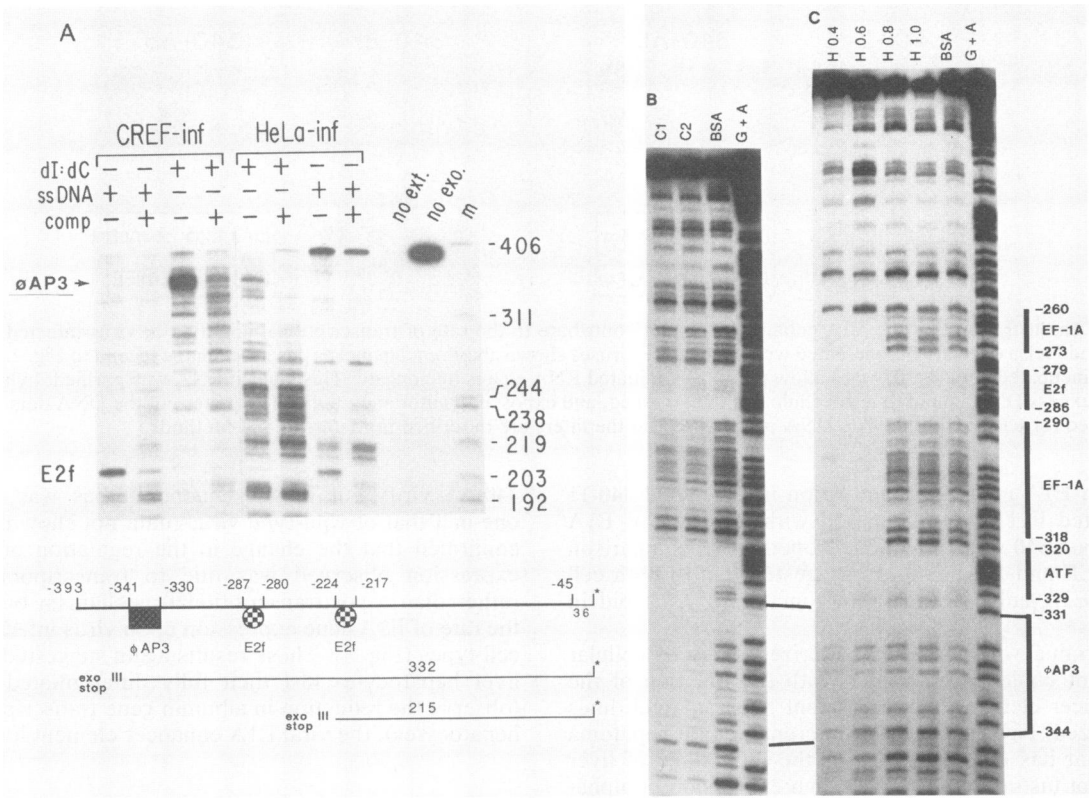

FIG. 5. ExoIII protection and DNase I footprinting assays for the binding of cellular factors to the E1A upstream sequences. Nuclear extracts were prepared from In340 virus-infected CREF and HeLa cells (6 h at 20 PFU per cell). The probe used for ExoIII analysis extends from nucleotides −393 to −45 and was 5′ end labeled on the lower DNA strand (at the *Hin*dIII site at −45) with [γ-^{32}P]ATP. DNA-protein complexes were formed as described in Materials and Methods and incubated with ExoIII, and partially digested labeled DNA was analyzed on a 5% denaturing acrylamide gel. The lanes labeled no ext. received no protein; those labeled no exo. received no protein or ExoIII. A 30-μg sample of extract was used for each reaction. The origin of each extract is shown at the top. Salmon sperm DNA (ssDNA) or poly(dI-dC) (dI:dC) was used as a nonspecific competitor as indicated. The ExoIII stops for the E2f and ϕAP3 factors are indicated on the left and at the bottom. The probe used for DNase I footprinting analysis extends from nucleotides −393 to −141 and was generated by repair of the linearized fragment (at the *Hin*dIII site at nucleotide −106) with Klenow DNA polymerase and ^{32}P-labeled deoxynucleoside triphosphates to label the lower DNA strand. In panel A, 30 μg of two independently prepared crude CREF cell nuclear extracts (C1 and C2) was incubated with 1 ng of labeled probe and incubated with DNase I, and the partially digested labeled DNAs were analyzed on a denaturing acrylamide gel. In panel B, a HeLa cell extract was fractionated on a P11 column, and 5 to 30 μg was incubated with the same DNA probe before DNase I digestion and gel analysis. Numbers above the lanes represent the molar concentration of KCl used for each step elution of protein from the P11 column. Lanes BSA represent the result of incubating the probe with bovine serum albumin before incubation with DNase I; lanes G+A represent DNA sequencing ladders. The nucleotide locations, based on this DNase I footprint analysis and the locations of known consensus binding sites, for the factors that interact with these sequences are shown on the right.

to stimulate the rate of viral E1A gene transcription. Therefore, the process leading to the loss of the fully differentiated hepatocyte phenotype had resulted in a cell population that had reverted (on the basis of E1A enhancer function) to a stage similar to that of CREF and F111 cells and suggested that the C2 revertant did in fact differ from typical dedifferentiated or undifferentiated cells.

A cellular factor (ϕAP3) binds to DNA sequences 5′ to the E1A enhancer element and is more abundant in CREF than in HeLa cell nuclei. The lack of E1A enhancer function in CREF cells could be due to a lack of synthesis or modification of those cellular factors that positively regulate enhancer function or to the presence of a factor that by some undefined mechanism suppresses E1A enhancer activity. We have previously shown that the cellular factor E2f is equally abundant in CREF and HeLa cell nuclei and that the activity of this factor is determined by viral E1A and E4 gene products (3). This factor has been shown to positively regulate the activity of the E1A enhancer element, whereas the EF-1A factor (Fig. 1) does not appear to contribute to enhancer function (29; Bruder and Hearing, in press). To get a thorough overview of the DNA-protein interactions occurring in and around the E1A enhancer sequences, an in vitro ExoIII stop analysis was performed (3, 42). This assay involves incubating a uniquely end-labeled DNA fragment, extending from nucleotides −393 to −45 relative to the start site of E1A transcription, with a nonspecific competitor [salmon sperm DNA or poly(dI-dC)] and nuclear protein (30 μg for each extract). The complexes formed are then exposed to digestion with ExoIII, and the products are analyzed by gel electrophoresis. Uninfected and wild-type virus-infected (In340 virus at 10 PFU per cell for 7 h) HeLa and CREF cell nuclear extracts were prepared as previously described (31). When we used salmon sperm DNA as the nonspecific DNA in the reaction and either HeLa or CREF cell extracts, we were able to observe an ExoIII stop at nucleotide −224 relative to the E1A cap site (Fig. 5A). This stop delineated the boundary of the more promoter proximal

of the two E2f-binding sites and demonstrated that the relative abundances of E2f-binding activity observed in the two extracts were comparable. For reasons that are not clear, we were never able to observe the ExoIII stop representing the more promoter-distal E2f-DNA binding site. When we use poly(dI-dC) as the nonspecific competitor, a very strong ExoIII stop was observed in virus-infected CREF extracts, at position −341 relative to the E1A cap site (this position corresponds to the site of DNA binding for the factor described by Hen et al. [30]). The protein(s) capable of inducing this specific ExoIII stop was present in HeLa cells at about 1/20 the level observed in CREF cells. We have called the protein responsible for generating this ExoIII stop φAP3 because its DNA-binding domain (on the basis of ExoIII analysis of both DNA strands [Fig. 5A and data not shown], DNase I footprints [Fig. 5B and C], and gel shift with a specific oligonucleotide) shares some homology to the AP3 factor DNA-binding sequence (AP3, 5′-GGGTGTGG AAG-3′; φAP3, 5′-TGTGGCAAA-3′) (4, 10). Although additional minor ExoIII stops were observed, only the E2f- and φAP3-induced ExoIII stops were specifically competed for when a 20-fold molar excess of unlabeled probe (or a DNA fragment from the viral E2A gene, which contains two E2f-DNA binding sites) was added to the reaction (Fig. 5 and data not shown).

To better define the region of φAP3 factor binding, two CREF cell nuclear extracts were prepared and used for footprint studies, using a labeled probe extending from nucleotides −393 to −141. A broad region of DNase I protection, representing the φAP3 (nucleotides −344 to −331) and ATF (nucleotides −329 to −320) DNA-binding domains, was observed (Fig. 5B). Both this assay and the ExoIII assay failed to detect EF-1A DNA binding, suggesting that this factor may have been present at a low concentration in the extract, making it impossible to detect. Crude HeLa extracts failed to protect the labeled probe from DNase I digestion (data not shown). Fractionation of the HeLa nuclear extract resulted in a distinct pattern of protected regions encompassing both EF-1A and ATF protein-binding sites and, to a partial extent, the φAP3-binding site (Fig. 5C). As in the ExoIII analysis, however, the level of φAP3 factor appeared to be much lower in HeLa cells than in CREF cells. Further purification of this protein from both extracts should result in a clearer definition of the DNA contact points and thus the relatedness of this protein in both cell types. Since poly(dI-dC) was used as a nonspecific competitor in these reactions, no E2f-footprintable region could be discerned.

To better characterize the cellular factors that were binding to the DNA sequences around position −341, a gel retardation analysis was performed. Extracts from uninfected and In340-virus-infected HeLa and CREF cells were prepared and normalized for equal amounts of protein by polyacrylamide gel electrophoresis analysis of total protein and titration of cellular AP1 factor level (Fig. 6 and data not shown). Equal amounts of cellular extracts were incubated with a radiolabeled DNA fragment (−393 to −311) that contained the binding sites for the nuclear proteins described in Fig. 1 and assayed by gel retardation (Fig. 6). Only uninfected extracts were used in Fig. 6A, and we have shown evidence for a weak ATF protein-DNA shifted complex (45) that appears to be equivalent in the two extracts (this analysis does not reveal whether both ATF sites present on the probe are occupied). No Sp1 (39)-specific shifted complexes were observed, although it should be noted that the homology of the Sp1 sites in this region to the consensus Sp1-binding site is poor. Although both CREF and HeLa cell extracts displayed a shifted complex (termed φAP3) that was specifically competed for by the SV40 core C sequence (which contains an AP3 DNA-binding site), this complex was more abundant in the CREF cell nuclei. Unlike the cellular E2f protein activity, φAP3 DNA-binding activity did not appear to be influenced by viral early-gene expression (Fig. 6B). Furthermore, when we compared the AP1 levels among the different extracts (Fig. 6C) and then used this comparison to determine the ratio of φAP3 in HeLa and CREF cells, we obtained a 1:30 ratio, which was similar to the result from experiments with ExoIII. Although this probe contains numerous DNA-protein binding domains, we have obtained similar results with use of an oligonucleotide probe containing only the φAP3-DNA binding site extending from nucleotides −349 to −331 relative to the E1A cap site (data not shown; see Fig. 8).

The φAP3 factor suppresses E1A enhancer activity. To determine whether the high level of φAP3 was contributing to the lack of E1A enhancer function in CREF cells, we obtained an adenovirus mutant that was deleted in the region that binds φAP3 (309-6; see Fig. 1 for location of deletion; 28). CREF and HeLa cells were infected at 20 PFU per cell; at the times indicated (Fig. 7), cytoplasmic RNA was isolated and the steady-state levels of the E1A 13S and 12S mRNAs were measured. In comparison with infection with 340 (wild-type) or 340-2 (no enhancer) virus infection of CREF cells with *dl*309-6 virus allowed a more rapid appearance of E1A mRNAs at 7 h postinfection and ultimately a three- to fivefold-greater accumulation at 12 h postinfection. This result demonstrates that the E1A enhancer element can contribute to the rate of E1A gene expression in CREF cells when the sequences between nucleotides −393 and −304 are deleted. Since there did not appear to be any difference in the nuclear factors that bound to these DNA sequences in HeLa and CREF cells other than the concentration of φAP3, it was most likely the removal of this DNA-binding domain that was responsible for the change in enhancer function in CREF cells. We are presently introducing point mutations into this region of the viral genome to more decisively confirm these findings. In HeLa cells, the 340 and 309-6 viruses accumulated E1A mRNAs to the same level, whereas the enhancerless virus (340-2) accumulated 5 to 10 times less. Therefore, the low amount of φAP3 was unable to influence early E1A gene expression in virus-infected HeLa cells. However, this factor did appear to be responsible for the suppression of enhancer function in CREF cells.

The φAP3 factor is TPA inducible in CREF cells and leads to a further reduction in the rate of E1A gene transcription. Chiu et al. (10) first described AP3 as being TPA inducible in HeLa TK^- cells but not HeLa S3 or HepG2 cells. An experiment was done to determine whether TPA could affect regulation of E1A gene expression via the φAP3-binding site. Serum-starved (48 h) CREF and HeLa cells were treated with TPA (100 ng/ml) or solvent (dimethyl sulfoxide) for 8 h. At 2 h posttreatment, the cells were infected with 309-6 virus (5 and 20 PFU per cell for CREF and HeLa cells, respectively) or 340 virus (20 and 50 PFU per cell for HeLa and CREF cells, respectively); after 6 h of infection, the steady-state levels of the E1A mRNAs (Fig. 8A) and rate of E1A gene transcription (Fig. 8B) were determined. We varied the multiplicity of infection of both viruses so that the rate of E1A gene transcription would be equivalent in solvent-treated cells. The accumulation of E1A mRNAs (in CREF and HeLa cells) and rate of E1A gene transcription (CREF cells only) from the 309-6 virus in both cell types did

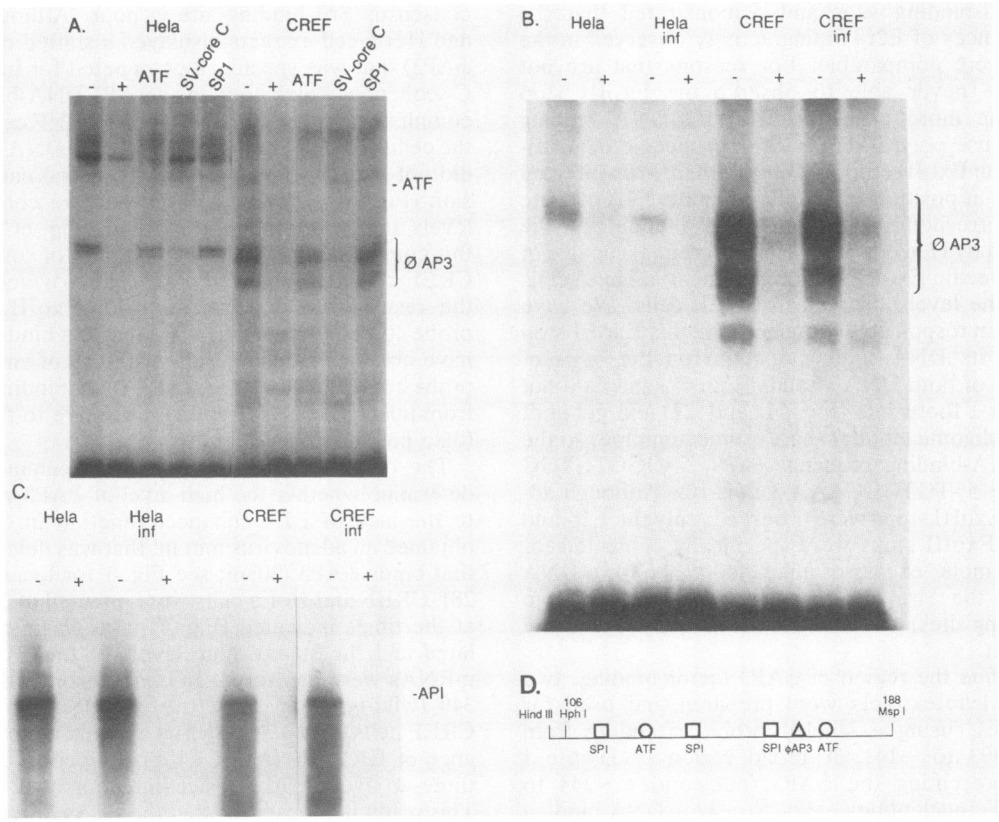

FIG. 6. (A and B) Gel retardation analysis of the nuclear DNA-binding proteins that interact with the E1A enhancer element. Nuclear extracts from In340 virus-infected and uninfected CREF cells were incubated with a labeled probe extending from nucleotides −393 (base pair 106 from the left end of the viral genome) to −311 (base pair 188 from the left end of the viral genome) to form specific complexes. For all reactions, poly(dI-dC) was used as a cold competitor. The complexes were then resolved on 6% acrylamide gels as described in the text. As indicated above each lane, binding reactions were done with no competitor (−) or in the presence of cold competitor DNA sequences at a 20-fold molar excess relative to the labeled probe (+). These sequences included ATF (from the E2A gene [44]); SV40 core C-, and Sp1 (39)-specific sequences. Positions of the ATF-DNA and ϕAP3-DNA complexes are shown to the right of panels A and B. (C) AP1 factor gel retardation analysis, using 4 μg of the extracts used in panel B. The labeled AP1 probe was derived from the SV40 core C element (32). (D) Relatives sites of factor-DNA interactions.

not appear to be influenced by TPA treatment (Fig. 8A and B). This finding was predicted, since this virus does not contain a ϕAP3-DNA-binding site. E1A mRNA levels and transcription rates (data not shown) were comparable for all four combinations of 309-6 or 340 virus-infected HeLa cells treated with solvent or TPA. In contrast, TPA-treated CREF cells infected with 340 virus accumulated 5- to 10-fold-less E1A mRNA than did solvent-treated 340 virus-infected CREF cells, suggesting that the TPA was either modifying the activity of preexisting ϕAP3 or inducing de novo expression of ϕAP3. The amount of ϕAP3-DNA-shifted complex was increased when we used TPA-treated CREF cell extracts in a gel retardation analysis, using a labeled oligonucleotide probe extending from nucleotides −349 to −331 relative to the E1A cap site (Fig. 8C). This TPA-induced increase in the in vitro DNA-binding activity of ϕAP3 was observed only in cells treated for at least 6 to 8 h with TPA, and the TPA-induced suppression of E1A expression was sensitive to cycloheximide treatment (data not shown). These findings suggested that de novo synthesis of ϕAP3 was occurring during TPA treatment, resulting in an increase in the nuclear abundance of this factor and leading to a further suppression of E1A enhancer function in wild-type virus-infected CREF cells.

DISCUSSION

Previous studies have shown that the adenovirus E1A enhancer domain is composed of multiple sites of DNA-protein interactions that collectively function to modulate the rate of E1A gene transcription (28–30). However, regulation of this specific enhancer is more complex than for most known viral and cellular enhancer elements because a protein product of the E1A gene can autoregulate E1A enhancer function in *trans* (6, 62). In this paper, we have identified still another level of regulation that can be imposed on this enhancer element: suppression of E1A enhancer function in undifferentiated fetal rodent fibroblast cells. In addition, we have demonstrated that this suppression is due to the activity of a nuclear factor (termed ϕAP3) that is more abundant in undifferentiated fetal rodent cell nuclei than in partially differentiated rodent and human cell types. Infection of undifferentiated rodent cells with a mutant adenovirus that lacked the DNA sequences necessary for ϕAP3 binding generated enhancer activity, thus demonstrating that this factor was responsible for the suppression of E1A enhancer function. These findings suggest that during the process of rodent cell differentiation, the activity of this cellular transcription factor is altered. Furthermore, if the

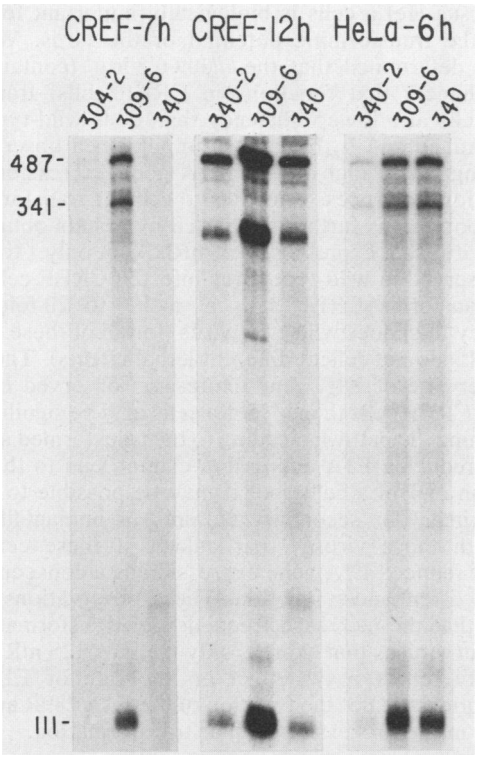

FIG. 7. Functioning of the φAP3 factor to suppress E1A enhancer activity in virus-infected CREF cells. CREF and HeLa cells were infected with 10 PFU of In340, 340-2, or 309-6 virus per cell for the times indicated at the top. The accumulation of E1A 13S and 12S mRNAs was measured by using the E1A gene SP6 riboprobe assay described in the legend to Fig. 2 and Materials and Methods.

φAP3 factor is functioning to suppress the expression of certain cellular genes, as it does for the adenovirus E1A gene, then it is possible that this factor is involved in maintaining the undifferentiated cell phenotype. An analysis of the *cis*-acting elements that regulate the expression of cellular genes which are suppressed before commitment to the terminally differentiated cell state may reveal the presence of φAP3-binding domains.

Recent evidence has suggested that the activities of many tissue or cell-specific enhancer elements are negatively regulated. For example, we have recently shown that activity of the mouse albumin enhancer element is negatively regulated in nonliver cell types (32). In this case, the factors responsible for enhancer suppression are ubiquitous. In the liver, however, the presence of the liver-enriched C/EBP protein (26) overrides the negative effect. Several other cellular genes, including the immunoglobulin heavy-chain gene (31), beta-interferon gene (23), and retinol-binding protein gene (15), have been shown to be negatively regulated either in a cell-specific manner or as a consequence of the state of cellular differentiation, suggesting the possibility that this may represent a common mechanism used to modulate tissue-specific gene expression.

In certain cases, the process of cellular dedifferentiation can lead to the suppression of cellular gene expression. The recent studies of Cereghini et al. (9) suggest that a combination of both the absence of a positive-acting cellular factor (HNF1) and the subsequent presence of a novel cellular repressor protein (vAPF) contributes to the lack of albumin expression in certain dedifferentiated hepatoma cell lines. In our studies, we do not see changes in the in vitro levels of the factors that positively regulate E1A enhancer function during the process of cellular dedifferentiation. However, in

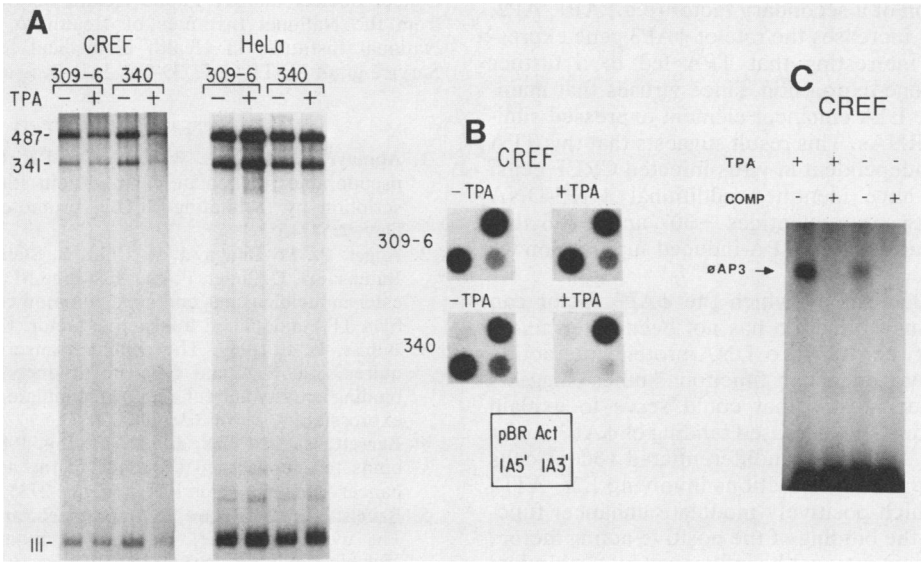

FIG. 8. Demonstration that the φAP3 factor is TPA inducible and leads to a further suppression of E1A transcription in TPA-treated and virus-infected CREF cells. CREF and HeLa cells were serum starved for 48 h, at which time TPA or solvent (dimethyl sulfoxide) was added to the medium (100 ng/ml); 2 h posttreatment, CREF cells were infected with 5 PFU of 309-6 virus or 50 PFU of 340 virus per cell. TPA- and solvent-treated HeLa cells were infected with either virus at 20 PFU per cell, and all virus infections were terminated at 6 h postinfection or after 8 h of TPA (+TPA) or solvent (−TPA) exposure. (A) Accumulation of the viral E1A 13S and 12S mRNAs; (B) comparative rate of E1A gene transcription for each virus infection, determined by nuclear run-on assay. (C) Gel retardation analysis of the φAP3 factor, using extracts derived from TPA- or solvent-treated CREF cells. Nuclear extracts were prepared at 8 h after exposure to TPA or solvent. An oligonucleotide probe extending from nucleotides −349 to −331 relative to the E1A cap site was used to score φAP3-DNA complex formation, and the specificity of this complex was confirmed by competition with a 20-fold molar excess of unlabeled oligonucleotide (cOMP +).

accord with the findings for hepatoma cell lines, we observed that during the process of cellular differentiation, the activity of a negative-acting factor is diminished. The loss of the fully differentiated phenotype of rodent hepatocytes in cultures would therefore predict that the level of the φAP3 factor would increase with time in culture, at which point a loss of enhancer function is observed. Our preliminary studies have suggested that higher φAP3 levels are present in late-cultured hepatocyte nuclei than in early-cultured hepatocytes (data not shown). However, these findings are difficult to interpret because changes in the in vitro DNA-binding activities of several common cellular factors (AP1 and Sp1) that would serve as a frame of reference are also changing.

Barrett et al. (4) have suggested that the HeLa cell factor described by Hen et al. (30) does not contribute to the rate of E1A gene transcription from a viral genome because its activity is suppressed by E1A protein. The apparent lack of φAP3 function in HeLa cells (positive or negative) compared with its suppressive function in CREF cells could simply be explained by the marked difference in factor concentrations. Alternatively, the factors that interact with this sequence could be different in the two cell types. We are presently attempting to distinguish between these possibilities by purifying the factors from both HeLa and CREF cells and determining the DNase I and methylation interference footprinting characteristics of each.

Our failure to observe an increase in the in vitro DNA-binding activity of the φAP3 factor in TPA-treated HeLa clone 2 cells is similar to the findings of Chiu et al. (10) for HeLa S3 and HepG2 cells. In addition to AP3, the cellular factors AP1 and AP2 (2, 10), and NFκB (55) have all been shown to be induced by phorbol esters. However, unlike our results with CREF cells, TPA inducibility in these cases has been shown to be rapid and independent of de novo protein synthesis. Therefore, it is possible that φAP3 may not be a direct target for TPA induction but may instead be induced by the indirect action of a secondary factor (e.g., AP1, AP2, AP3, or NFκB) that increases the rate of φAP3 gene expression. It was also interesting that TPA led to a further reduction in E1A gene expression, since viruses that maintained or lacked the E1A enhancer element expressed similar levels of E1A mRNAs. This result suggests that this TPA effect is promoter independent in virus-infected CREF cells. Barrett et al. (4) have identified additional φAP3-DNA binding regions between nucleotides −307 and −146 that may also be contributing to the TPA-induced suppression we are observing.

The precise mechanism by which the φAP3 factor can suppress E1A enhancer function has not been determined. The correlation between in vitro DNA-protein interaction analyses and in vivo enhancer function studies suggests several possible mechanisms that could serve to explain enhancer suppression. The increased binding of φAP3 factor 5′ to the enhancer domain in undifferentiated rodent cells could ablate DNA-protein interactions involving E2f, ATF, or EF-1A, all of which positively modulate enhancer function. Alternatively, the binding of the positive-acting factors to the enhancer domain may not be influenced by the binding of φAP3, but the ability to form an active committed transcription complex as the result of TFIID binding to the TATA element may become less efficient. In vivo footprinting analysis of the factors associated with the E1A enhancer in virus-infected CREF and HeLa cells should permit us to distinguish between these two mechanisms.

Last, it is important to consider whether the reduction of E1A gene expression observed in adenovirus-infected CREF cells versus HeLa cells is biologically important for determining the transforming potential of the virus. We have recently determined that the *dl*309-6 virus (containing an E1A enhancer that functions in CREF cells) transforms CREF cells at a lower efficiency than does wild-type virus (G. R. Adami and L. E. Babiss, unpublished observation), suggesting that a high rate of E1A gene expression is not favored for the processes leading to cellular transformation. This hypothesis is further supported by results obtained by using a virus that expresses E1A mRNAs at only 1 to 5% the level observed in wild-type virus-infected CREF cells. This virus transforms CREF cells at a 10- to 20-fold-higher frequency than does wild-type virus (for all of these studies, the results do not reflect different lethal effects). Therefore, the lower rate of E1A gene expression observed in virus-infected CREF cells than in HeLa cells may be significant for establishing the pathway leading to the transformed state. Of course, reducing E1A expression cannot lead to the transformation of HeLa cells, but it may be possible to immortalize primary or secondary cultures of human fibroblast cells with these viruses, particularly if these cell types manifest reduced E1A gene expression as a consequence of the lack of enhancer function. These observations further suggest that the increased frequency of transformation observed for viruses that express only the E1A 12S mRNA (47) may only reflect a *cis* effect on the rate of E1A gene transcription and not the function of the E1A 289R and 243R proteins in the process of cellular transformation.

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