The Cellular Transcription Factor E2f Requires Viral E1A and E4 Gene Products for Increased DNA-Binding Activity and Functions To Stimulate Adenovirus E2A Gene Expression

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Whereas a wide variety of cellular proteins interact with the *cis*-regulatory elements of the adenovirus E1A and E2A genes, only the DNA-binding activity of the cellular E2f factor is modulated by viral early-gene expression. An analysis of cellular E2f protein levels and adenovirus early-gene expression in a panel of independently cloned virus-transformed rodent cell lines and in virus-infected rodent cells has established that both the E1A 289-amino-acid (289R) protein and a yet-to-be-defined E4 gene product are required for maximal E2f DNA-binding activity. To distinguish between the multiple roles the E1A protein could serve in this process, the E2f DNA-binding activity was determined in a virus-transformed cell line which contains a conditional-lethal mutation affecting the 289R protein. Since E4 gene expression was not altered by the incubation conditions, the observation of reduced cellular E2f activity at the nonpermissive temperature suggests a direct role for the E1A 289R protein in E2f activation. When a virus containing a deletion in the E4 gene was introduced into cell lines which can complement the E4 gene defect, a correlation between high cellular E2f levels and increased rates of E2A gene transcription was observed. A time course analysis of the viral infection revealed that E2f functions catalytically to stimulate viral E2A gene transcription. These observations have led to several hypotheses concerning possible mechanisms by which elevated E2A gene expression, which leads to cytotoxicity, might be avoided in the transformed cell.

Expression of the adenovirus type 5 (Ad5) E1A proteins in a mammalian cell initiates a cascade of events resulting in a variety of changes in the transcriptional control of both viral and cellular genes (for a review, see reference 23). Since there is little evidence to suggest that these changes are brought about by the E1A protein(s) directly interacting with specific promoter-proximal DNA sequences (21, 24, 34, 47), it is thought that the E1A proteins must either induce the production of cellular transcription factors or interact with or modify preexisting cellular transcription factors (17, 26, 27, 46, 48). This hypothesis is supported by recent studies of Hoefler et al. (17), which suggest that the modification of the cellular transcription factor TFIIC by the E1A 289-amino-acid (289R) protein results in the stimulation of viral VAI gene expression. It has also been suggested by the studies of Wu et al. (46) that E1A modulation of viral E1B expression is brought about by E1A modification of the cellular transcription factor TFIID (TATA-binding protein). That the viral early proteins are directly modifying or interacting with preexisting cellular factors and not inducing the synthesis of new factors is supported by the observations of Spangler et al. (42), which demonstrate that supplementing HeLa cell extracts with purified E1A protein results in an E1A-dependent stimulation in the rate of viral E2A gene transcription. More recently, Green et al. (12) have shown that a peptide representing domain 3 of the E1A 289R protein (31) can *trans* activate viral early promoters in cells pretreated with protein synthesis inhibitors, again supporting the idea that E1A does not induce the synthesis of these cellular transcription factors.

Another cellular factor has been described which appears to be required for viral E1A, E2A, E3, and E4 gene expression (5, 20, 29, 40). Unlike TFIID and TFIIC, however, this factor, ATF-E4F, does not require viral E1A gene expression for its activation. Taken together, these examples

suggest that the interplay between factors that require activation by both viral early-gene products and generalized cellular transcription factors represent the means by which a wide variety of unrelated promoter elements can be modulated by the adenovirus E1A gene products. Since many of the cellular transcription factors described appear to regulate both viral and cellular gene expression, the virus becomes a model system for understanding developmental and tissue-specific cellular gene regulation.

The regulatory elements that are essential for viral E2A gene expression have been well studied (5, 22, 24, 34, 40). While numerous cellular transcription factors have been shown to interact with the DNA sequences 5' to the E2A cap site (ATF, E2f, and TFIID), only E2f appears to bind to these sequences, by both *in vivo* and *in vitro* analysis, as a consequence of viral early-gene expression (26, 27, 41). While there is no evidence for E2f DNA binding to the E1A-responsive E1B, E3, and E4 promoter elements, this factor has been shown to also bind to two DNA sequences in the E1A enhancer element (28, 47). Hearing and Shenk (15, 16) have shown that viruses containing mutations in the E1A enhancer that remove these E2f DNA-binding sites initiate E1A transcription at reduced rates. Furthermore, the localization of the E1A-E2f DNA-binding sites 5' to the cap site of the mouse β -globin gene, leads to an E1A-dependent increase in β -globin gene expression (28). Taken together, these results suggest that E2f exerts a positive role in transcription initiation.

From the work that has emerged on the cellular transcription factor E2f, two fundamental questions remain. First, if in fact the viral early-gene products are responsible for the activation of endogenous E2f factor in the infected cell, it is still not clear which of these viral early genes are required. While E1A (most likely the 289R protein) has clearly been implicated (26, 27, 38), it could either be directly responsible

for E2f activation or it could function indirectly by increasing the expression of another viral early gene(s). Second, while E2f has been shown to interact with the promoter-proximal sequences of the E2A gene and E1A enhancer, it is not clear whether this DNA-protein interaction leads to increased transcription initiation from the E2A cap site.

In this paper, I have used several well-defined In340 virus (15)-transformed cloned rat embryo fibroblast (CREF) (8) cell lines and an Ad5hr1 virus-transformed CREF cell line that displays a cold-sensitive transformation phenotype as a result of an E1A mutation (2, 14, 39), to begin to correlate the *in vitro* DNA-binding function of the cellular E2f factor with its biological role in regulating viral early-gene transcription. The basic conclusions that arise from these studies are that E1A protein is functioning in a direct manner and in concert with a still-undefined E4 gene product to activate the cellular E2f factor. Furthermore, the modified form of the E2f factor that interacts with the E2A promoter-proximal sequences does lead to increased rates of E2A transcription. Previous studies have suggested that the product of the E2A gene, the 72-kilodalton single-stranded DNA-binding protein, is toxic to cells (25; L. E. Babiss and H. S. Ginsberg, unpublished results). Since the activated form of E2f increases E2A mRNA levels, and since no transformed cell line was characterized which expresses high levels of both activated E2f and E2A mRNAs, some mechanism could exist by which E2A gene expression is suppressed during the generation of the transformed cell. While this might involve the loss of E2A or E4 gene sequences or both during the process of viral DNA integration, I discuss various mechanisms by which the transformed cell avoids high rates of E2A expression.

MATERIALS AND METHODS

Cells and viruses. A panel of independently cloned In340 virus-transformed CREF (a single-cell clone of Fisher rat embryo fibroblast cells) (8) cell lines was obtained by infecting CREF cells with 10 PFU of virus per cell and allowing foci to develop at 37°C in medium reduced in calcium, as previously described (2). Monolayer cultures of these established cell lines were grown in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum. In340 virus (this virus was derived from the parental virus *dl309* and has replicative and transforming characteristics similar to those of Ad5wt) (15) and Ad5wt virus stocks were obtained by the infection of monolayer cultures of HeLa cells (maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum) and the preparation of crude cellular lysates by repeated cycles of freezing and thawing. The E1A mutant viruses *pm975* (expresses only the E1A 13S mRNA in a *dl309* virus genetic background) (33) and *dl1500* (expresses only the E1A 12S mRNA in an Ad2wt genetic background) (32) were propagated in the E1A-complementing 293 cell line. Virus titers were obtained by fluorescent-focus assay, using antisera raised in rabbits against cesium chloride-banded purified virions. The E4 mutant virus *dl366* (13) was obtained from Tom Shenk.

Assays for gene expression. Two assays for the expression of the viral early genes were used. Transcription of the viral E2A gene was scored directly by infecting the CR-A and CR-G cell lines with 50 PFU of the desired virus per cell for 5.5, 11, and 22 h postinfection, isolating cell nuclei, and allowing chain elongation to continue in the presence of [³²P]UTP (18, 44). This labeled RNA was purified and hybridized to excess DNA bound to nitrocellulose filters,

was washed, and was exposed to autoradiography. The following DNA clones were used as probes on filters: 2A5' (69 to 63.6 map units), 2A3' (63.6 to 60.2 map units), E3 (82.3 to 83.4 map units), chicken β -actin, and pBR322.

The production of stable mRNAs from the viral early genes was scored by their ability to protect from nuclease digestion a uniformly labeled RNA probe prepared from an SP6 subclone of a portion of the viral gene. Total cytoplasmic RNA (20 μ g) was hybridized to a specific probe (7.5×10^5 total cpm), and after RNase T₂ digestion (55 U/ml at 30°C for 2 h), the products were sized on denaturing polyacrylamide gels. The SP6 protection assays for the E1A, E1B, E2A, E4, and actin genes have been previously described (3, 7, 9). The size of the protected RNAs, indicative of early viral gene exons, is shown (see Fig. 1).

Preparation of nuclear extracts in exonuclease III (exoIII) analysis. Nuclear extracts were prepared from cultured In340 virus-transformed CREF cells according to the protocol of Dignam et al. (6). Nuclear proteins were extracted by using 0.4 molar KCl and were precipitated with ammonium sulfate at a final concentration of 0.3 g/ml of extract. The extracts were dialyzed against a buffer containing 100 mM KCl, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 20% glycerol, and 0.1 mM EDTA and were stored at -70°C. *In vitro* exoIII stop (45) reactions were performed, using 1 ng of a uniquely end-labeled probe (extending from nucleotides -285 to +40 of the E2A early gene) by the method of Kovessdi et al. (26). For all reactions, 30 μ g of nuclear protein extract was used, as was 2 μ g of salmon sperm DNA as a nonspecific competitor of DNA-binding proteins. Following exoIII digestion, the products were analyzed on 5% denaturing polyacrylamide gels.

RESULTS

Analysis of viral early-gene expression in a panel of In340 virus-transformed CREF cell lines. To understand the relationship between adenovirus early-gene expression and transformation and the role E2f plays in these processes, two criteria had to be met. First, a series of 10 independently cloned In340 virus-transformed CREF cell lines were analyzed for viral early-gene expression from the integrated viral genomes. Cytoplasmic RNA was isolated from the individual cell lines and was analyzed for the presence and abundance of the viral E1A, E2A, E1B, and E4 mRNAs, using SP6-generated ³²P-labeled antisense RNA probes. All of the cell lines expressed the E1A 13 and 12S mRNAs (Fig. 1A) and the E1B 22 and 13S mRNAs (Fig. 1B), as was expected since these gene products are necessary for the morphological phenotypes of the transformed cell (11, 19, 43). The variations in the amounts of these mRNAs are most likely a consequence of differing amounts of integrated viral genomes or of differing rates of viral E1A and E1B gene transcription, perhaps caused by the local environment at the site of DNA integration. Expression of the E4 mRNAs was observed in 7 of the 10 cell lines shown (Fig. 1D) and, again, was variable among the cell lines. The lack of E4 gene expression (determined by densitometric quantitation) by the CR-J, CR-G, and CR-C cell lines was due to the lack of E4 gene sequences integrated into the host DNA chromosomes, as determined by Southern blot hybridization analysis (data not shown). E2A expression could be scored only in the CR-F, -D, -J, -C, and -I cell lines. However, the level of E2A mRNA relative to that of the other early-gene regions was greatly reduced, since the E2A gel was exposed for 80 h, compared with 8 h for the other early genes. As has been

FIG. 1. Early viral gene expression in In340 virus-transformed CREF cell lines. Cytoplasmic RNA was isolated from 10 independently cloned In340 virus-transformed CREF cell lines, and 20 μ g was hybridized to 32 P-labeled antisense RNA probes (7.5×10^5 cpm) representing the viral E1A (A), E1B (B), E2A (C), and E4 (D) genes. After RNase T_2 digestion, the T_2 -resistant hybrids were analyzed on 5% denaturing acrylamide gels. The lengths (in nucleotides) of the protected RNA probes for each transcription unit were determined by comparison with labeled DNA markers (lanes M) and are shown at the bottom of each panel and on the sides of each exposure. Cell lines are indicated above the lanes.

suggested earlier (25), these results are consistent with the notion that high levels of E2A mRNA (and the 72-kilodalton protein) may not be tolerated by the transformed cell. Since we have measured the kinetics of nuclear run-ons and these correlate with the mRNA levels shown in Fig. 1, it appears that the suppression of the E2A genes compared with that of other early-gene promoters is transcriptional (data not shown).

Analysis of E2f DNA-binding levels in In340 virus-transformed cell lines. A second matter that needed to be settled was whether E2f modification or association with viral early-gene products occurs during a virus infection of CREF cells. Previous studies using virus-infected mouse F9 teratocarcinoma cells had established that E2f activation can occur in this rodent cell type (37). To define the location of E2f protein-E2A DNA interactions, an *exoIII* protection test (45) was designed, based on the experiments of Kovcsdi et al. (26). This assay involves incubating a uniquely end-labeled DNA fragment, extending from nucleotides -285 to +40 relative to the start site of E2A transcription (Fig. 2, bottom), with a nonspecific competitor (salmon sperm DNA) and nuclear protein (30 μ g for each extract). The complexes that are formed are then exposed to digestion with *exoIII*, and the products are analyzed by gel electrophoresis. As previously shown, *exoIII* stops 252 and 138 bases in length represent E2f binding to the E2A sequences between nucle-

otides -71 and -33 relative to the start site of E2A transcription, respectively. These stops delineate the boundaries of two E2f-binding sites extending from nucleotides -71 to -53 and -49 to -33 (47). Uninfected HeLa and CREF cell nuclear extracts had either barely detectable or no E2f-DNA-binding activity (Fig. 2). In contrast, In340 virus infection of both cell types (12 h at a multiplicity of 20 PFU per cell in the presence of cytosine arabinoside to prevent viral DNA replication) led to an increase in the activity of cellular E2f binding by at least 50-fold (see Table 1 for quantitative analysis). Therefore, it appears that the levels of activated cellular E2f observed in virus-infected HeLa and CREF cells are the same. To attempt to correlate activated E2f levels with the specific expression of viral early-gene products, nuclear extracts were prepared from the 10 cell lines described in Fig. 1. To control for variations among the extracts, total nuclear proteins were resolved on sodium dodecyl sulfate-polyacrylamide gels to assure correct protein quantitation and protein integrity, as has been described by Lichtsteiner et al. (30).

The levels of E2f-DNA-binding activity varied greatly among the cell lines (Fig. 2). Cell lines CR-C, -D, -E, -G, -I, and -J all displayed the basal level of E2f activity (within twofold of the level observed in uninfected CREF cells; see Table 1 for quantitative analysis). This contrasts with the findings for the CR-A, -B, -F, and -H cell lines, for which

FIG. 2. exoIII protection assay for the binding of cellular E2f factor to the E2A promoter-proximal sequences. Probes to be used for binding were 5' end labeled on the upper DNA strand (position -285 for the strand equivalent to the mRNA, panels B and C) or lower DNA strand (position +67 of the strand complementary to the mRNA, panel A) of the probe. After incubation of 1 ng of probe with 2 μ g of salmon sperm DNA and 30 μ g of nuclear extract, the DNA-protein complexes were incubated with exoIII, and partially digested DNA was analyzed on 5% denaturing acrylamide gels. Abbreviations: no ext, no protein; no exo, no exoIII; CR-, CREF cells; H-, HeLa cells; comp, unlabeled probe DNA in a 20-fold molar excess relative to the labeled probe. Extracts obtained from virus-infected cells (+) are indicated. The exoIII stop fragments are indicated by their position relative to the E2A transcriptional start site (+1), and the exact positions of the E2f DNA-binding sites are shown in the line drawing. Lengths of DNA fragments (in nucleotides) are indicated on the sides of the gels.

high levels of E2f were observed (at least 12-fold above the basal level). Several repeats of this assay revealed that the CR-H cell line did in fact contain higher levels of the activated form of E2f, compared with the CREF cell extract (Fig. 3).

Is E4 gene expression required for E2f activation? The results of the E2f-binding data and viral early-gene expression for the cell lines are summarized in Table 1. Analysis of these findings reveals several important conclusions. Transformed cell lines that failed to express any E4 mRNA (CR-C, -G, and -J) did not display activated E2f DNA-binding levels (Table 1). This demonstrates that E4 gene expression is required for E2f activation. However, since the RNase T₂ protection assay shown in Fig. 1 scored the 3' end of all the E4 mRNAs, a direct correlation between E4 expression and E2f activation is not always observed. For example, the CR-D, -E, and -I cell lines expressed E1A and E4 mRNAs (at variable levels) but no activated levels of cellular E2f factor. However, E2f activation was restored in the CR-I and -E cell lines after In340 virus infection, suggesting some defect in endogenous viral E1A or E4 gene expression (Fig. 3; data not shown). Virus infection of the CR-D cell line resulted in a subtle increase in activated E2f, even though virus uptake and early-gene expression were comparable among all the cell lines (Fig. 3; data not shown). This

FIG. 3. exoIII protection assay for the binding of E2f to DNA sequences, using extracts derived from virus-infected CREF cells and transformed CREF cell lines. CREF cell cultures and the CR-D and -I cell lines were infected (20 PFU per cell) with the appropriate virus, indicated above each lane (dl366, pm975, and dl1500), for 12 h in the presence of 1- β -D-arabinofuranosylcytosine, prior to nuclear extract preparation. In340 virus-infected cell extracts (+) are indicated. The identification of the location and abundance of E2f DNA-binding was determined as described in the legend to Fig. 2. no exo, No exoIII. Lengths of DNA fragments (in nucleotides) are indicated at the right.

TABLE 1. Quantitation of viral early cytoplasmic mRNA levels and cellular E2f DNA-binding levels in adenovirus-transformed CREF cell lines^a

Cell line	E2f DNA-binding level ^b	mRNA level ^c	
		E2A	E4
CREF	1	ND	ND
CREF + In340 ^d	52	ND	ND
CR-A	32	—	0.09
CR-B	15	—	2.25
CR-C	1	1.3	—
CR-D	1	2.4	0.02
CR-E	2	—	0.01
CR-F	12	0.05	0.07
CR-G	2	—	—
CR-H	30	—	0.5
CR-I	1	1	1
CR-J	1	0.3	—

^a The numbers shown in each column represent comparative quantitative analysis of the data presented in Fig. 1, 2, and 3 and two additional duplicate studies (not shown), as determined by densitometric scanning of the appropriate lanes.

^b The relative levels of in vitro E2f DNA-binding activity were determined by comparison with the basal level of activity observed in uninfected CREF cells.

^c E2A and E4 mRNA levels are all relative to the amounts of these mRNAs measured in the CR-I cell line. The autoradiographic exposure for the E2A mRNA gel was 80 h, while E1A, E1B, and E4 gel exposures were for 8 to 10 h. —, mRNA species that could not be detected; ND, not determined.

^d Extract prepared from virus-infected (20 PFU per cell for 12 h) CREF cells.

suggests that the CR-D cell line might contain some defect in the E2f activation process. The absolute requirement for E4 gene expression for E2f activation was established by infecting CREF cells with an E4 mutant virus (H5dl366) (13) and demonstrating no increase in E2f DNA-binding activity, which contrasts with the results obtained after In340 virus infection (see exoIII stop studies presented in Fig. 3). I additionally confirmed the previous observation of Reichel et al. (38) that the E1A 13S mRNA gene product is necessary for maximal E2f activation, since extracts prepared from dl1500 (32) virus (no E1A 13S mRNA)-infected CREF cells displayed no activated E2f, while pm975 (31) virus (no E1A 12S mRNA)-infected cell extracts had a 50-fold increase in E2f DNA-binding activity (Fig. 3). E1B RNA expression also positively correlates with E2f activity; however, E1B proteins do not appear to play a role in E2f modification, since infection of CREF cells with an E1B mutant virus (dl118) (1) still leads to E2f activation (data not shown). An examination of the stimulation (fold) of E2f DNA-binding activity showed that the level of E2f observed in the transformed cell lines never approached that observed in virus-infected CREF cells. This may be due to either the temporal regulation of viral early-gene expression or a greater accumulation of early viral gene products in virus-infected cells.

Do E2f activation and cellular E2A mRNA levels correlate in the transformed cell lines? An examination of the cellular E2A mRNA levels and activated E2f levels in the panel of transformed cell lines shown in Table 1 revealed that E2A mRNA accumulation occurred only in those cells which did not express appreciable levels of the activated form of E2f. The one exception is the CR-F cell line, which expressed very low levels of E2A mRNA. However, it should be noted that this does not necessarily reflect negative gene control by E2f, since the rate of E2A gene expression in all the transformed cell lines was so low. Furthermore, the product of the E2A gene, the 72-kilodalton single-stranded DNA-

FIG. 4. Early viral gene expression in the Ad5hr1 virus-transformed CREF cell line at 32 and 37°C. Cytoplasmic RNA was isolated from monolayer cultures of the Ad5hr1 virus-transformed CREF cell line maintained at either incubation temperature and was analyzed for the presence of the viral E1A (A), E1B (B), and E4 (C) gene mRNAs and cellular actin mRNAs (D), as described in the legend to Fig. 1. There was no E2A gene expression in these cells, regardless of the incubation temperature (data not shown). The E1A SP6 assay detects the 5' end of the E1A mRNAs, resulting in protected RNAs 613, 467, and 111 nucleotides in length. The actin SP6 assay has been described elsewhere (7).

binding protein, could be only weakly detected in the nuclei from the CR-C, -D, and -I cell lines, by indirect immunofluorescence studies (data not shown). The question of the role that activated E2f factor plays in the formation of an E2A transcription complex will be addressed later.

E1A protein is required for E2f modification. To distinguish between the two roles E1A protein could serve in E2f activation, I took advantage of an Ad5hr1 virus-transformed CREF cell line that displays a cold-sensitive transformed cell phenotype (2). This virus has a point mutation in the E1A 289R protein that results in the expression of a conditionally stable truncated product (39). This cell line was established at the permissive temperature of 37°C, as a focus of transformed cells. Upon expansion and growth at 32°C, the cells failed to pile up and reverted to characteristics resembling those of the untransformed parental CREF cell line. This cell line and additional Ad5hr1-transformed CREF cell lines have the interesting property that while E1A protein stability can be rendered cold sensitive, the rate of E4 gene transcription remains constant (2) (data not shown; Fig. 4). This property, then, allows one to determine the role E1A protein plays in E2f activation. Extracts were prepared from these cells, which had been maintained at 32 or 37°C for a period of 30 days. An exoIII stop characteristic of the activated form of the E2f factor could be detected only in the cell line maintained at 37°C (Fig. 5). The lack of E2f activity at 32°C was not due to the incubation temperature, since the CR-B cell line maintained at 32°C for 30 days displayed constitutive levels of activated E2f (Fig. 5, lane CR-B). To establish the time course of E2f activation and the stability of activated E2f in the absence of continued E1A gene expression, temperature shift studies were performed. While many changes contribute to the cold-sensitive nature of this cell line, I have shown that the increased DNA-binding activity associated with E1A and E4 gene expression is similar in

FIG. 5. exoIII protection assay for the binding of E2f to E2A sequences, using extracts derived from an Ad5hr1 virus-transformed CREF cell line. Monolayer cultures of the Ad5hr1 virus-transformed CREF cell line were maintained at 32 or 37°C for 30 days prior to nuclear extract preparation. Plates were shifted to either temperature for the periods of time indicated above each lane (numbers in parentheses represent days). Incubation conditions, exoIII reactions, and product analysis were as described in the legend to Fig. 2. Lanes: CR-B, nuclear extract prepared from this cell line maintained at 32°C for 30 days; no ext, no protein; no exo, no exoIII. Lengths of DNA fragments (in nucleotides) are indicated on the right.

virus-infected CREF cells and the transformed CREF cell lines. Upon shifting cells from 32 to 37°C, activated E2f levels could be detected by 1 day but were maximal at 5 days of incubation. Conversely, shifting from the permissive to the nonpermissive incubation temperature resulted in a slight decrease in E2f-binding activity by 1 day and in a complete absence of activity by 5 days. These observations could suggest that the activated form of E2f is relatively stable in the absence of E1A gene expression but that E4 gene expression alone is not sufficient to maintain this level indefinitely. Alternatively, the activity of the conditionally stable E1A protein could also explain the kinetics of E2f stability. The shift-up studies also suggest the possibility that high levels of E1A 289R protein must be maintained within the cell to allow E2f activation to occur at the maximum efficiency.

While these studies demonstrated that E2f activity could be rendered conditional relative to viral E1A protein expression, they did not yet distinguish whether E1A was functioning in a direct manner to activate the E2f factor. It has previously been shown that the rate of transcription initiation from the viral early-gene promoters is not affected in several Ad5hr1 virus-transformed cell lines maintained at 32 or 37°C (2). I reconfirmed these findings by measuring the steady-state levels of the E1A, E1B, and E4 mRNAs as shown in Fig. 4 (E2A mRNA is not expressed in this cell line; data not shown). To control for variations in RNA quantitation, the relative level of actin RNA expression was determined by an RNase T₂ protection assay, resulting in an RNA product 69 nucleotides in length (7). Since E4 expression is not altered by the incubation temperature, it is likely that E1A is playing a direct role in the modification of or interaction with the E2f factor or both and is not simply serving to increase the rate of viral E4 gene expression.

E2f activity associated with an increased rate of E2A transcription initiation. An inspection of the E2f activities

FIG. 6. E2f functions to increase the rate of viral E2A gene transcription. The CR-A (expresses E1A, E4, and activated E2f) and CR-G (expresses E1A and no E4 or activated E2f) cell lines were infected with 50 PFU of either Ad5wt virus or *d*/366 virus (contains a deletion in the E4 gene) per cell for 5.5, 11, and 22 h. Cytoplasmic RNA was isolated and analyzed for E2A mRNA (both endogenous and exogenous) as described in the legend to Fig. 1. Infected cell nuclei were incubated with [³²P]UTP to allow previously initiated RNA chains to elongate. This labeled RNA was purified and hybridized (10 × 10⁶ cpm) to excess DNA bound to nitrocellulose filters, washed, and exposed to autoradiography. The following DNA clones were used as probes on filters; 2A5' (69 to 63.6 map units), 2A3' (63.6 to 60.2 map units), E3 (82.3 to 83.4 map units), chicken β-actin (Act), and pBR322 (pBR). The rate of E3 gene expression was scored only at the 11-h time point, so this dot is omitted in the 5.5- and 22-h assays. Length of RNA fragments (in nucleotides) is indicated at the right.

and E2A mRNA levels in the cell lines shown in Table 1 revealed a correlation between high levels of E2f activity and no or low levels of E2A gene expression. While this may suggest that E2f exerts a negative effect on viral E2A transcription, another interpretation could be that levels of E2A expression resulting from E2f stimulation cannot be tolerated by the transformed cell. To investigate this possibility, a complementation assay involving the CR-G (which expresses no E4 or E2f) and CR-A (which expresses both E4 and E2f) cell lines and the viruses *d*/366 (deletion in the E4 gene) (13) and Ad5wt was performed. If E2f was acting to increase transcription initiation from the E2A cap site, then the lack of E4 expression in *d*/366 virus-infected CR-G cells would result in a decrease in E2A gene transcription compared with that in cells infected by wild-type virus. In contrast, *d*/366 virus-infected CR-A cells would express wild-type levels of E2A mRNA because of the constitutive expression of activated E2f. If E2f was acting to decrease transcription from the E2A promoter, then *d*/366 virus infection of CR-G cells (with no E2f present) would lead to increased levels of E2A mRNA, which would contrast with the result obtained in CR-A cells (which have constitutive activated E2f).

When CR-G cells were infected with *d*/366 virus at 50 PFU per cell for 5.5 and 11 h, decreases in the rate of E2A transcription and in mRNA accumulation were observed, compared with those of In340 virus-infected CR-G cells (Fig. 6). In the complementing CR-A cell line, E2A transcription from both viral genomes was comparable. These findings, therefore, demonstrate that E2f association with the E2A gene sequences between -71 and -33 bases relative to the E2A cap site leads to increased rates of E2A transcription. Both cell types were equally infected by virus, since the rate of E3 gene transcription from the Ad5wt genome was

equivalent (Fig. 6, dot labeled E3). Only a cell line without activated E2f when infected with a virus incapable of inducing E2f shows reduced E2A gene expression, so E2f is a positive factor.

At 22 h postinfection, the relationship between E2f activity and E2A transcription could no longer be observed, since *dl366* virus-infected CR-G and CR-A cells expressed similar levels of E2A mRNA. This suggests that, like E1A protein, E2f protein is not absolutely required for E2A gene expression at this late time following infection but could be functioning early to increase the rate of template recruitment or factor association in the generation of the E2A gene transcription complex. While I cannot rule out the possibility that *dl366* virus infection of the CR-G cell line results in E2A expression by the integrated viral genomes, the conclusion remains the same: the role of E2f in the process of E2A transcription is to increase the rate of transcription initiation.

DISCUSSION

In this report, I have demonstrated that the activation of the cellular transcription factor E2f, which in a cell-free assay leads to an increased affinity for binding to the E2A promoter, is brought about by an interaction with or modification by the viral E1A 289R protein and an undefined E4 gene product. While previous studies had clearly implicated E1A gene products in this activation process (26–28, 41), they did not distinguish whether the E1A proteins were playing a direct role in E2f activation or were simply stimulating expression of another direct-acting viral factor. By using a well-characterized panel of cloned In340 virus-transformed CREF cells that differentially express the viral E2A and E4 gene products, a correlation between E1A and E4 gene expression and E2f activation was established. Additional studies using an Ad5hr1-transformed CREF cell line which contains a cold-sensitive mutation in the E1A gene, affecting the expression of the 289R protein, demonstrated a direct role (or a role other than the transcriptional activation of the viral E4 gene) for this protein in the process of E2f activation.

One question that remains unresolved concerns the mechanism by which E1A and E4 gene products modify or interact with cellular E2f. This could occur by two distinct mechanisms: (i) the induction of E2F gene expression at the level of transcriptional initiation or (ii) the modification of a preexisting cellular protein. These two processes are distinguished by the need for cellular protein synthesis. The studies of Reichel et al. (38) demonstrate that after a 1-h virus infection of HeLa cells, protein synthesis is no longer necessary for E2f activation. However, since E1A and E4 gene products could be synthesized during the first hour of infection, it is possible that E2f protein is also newly expressed. The recent studies of Green et al. (12), however, support the idea that E2f activation does not require cellular gene expression, since a synthetic peptide containing a domain specific to the E1A 289R protein (this is the same protein that Reichel et al. [38] have shown to participate in E2f activation) can increase the rate of E2A gene expression *in vitro*. It is interesting to speculate on the type of protein-protein interaction(s) that must occur during the process of E2f activation. To date, only cold-sensitive mutations (suggesting conditional defects in protein-protein interactions) have been isolated in the E1A 289R protein. Since this protein is involved in E2f activation, and since we have shown a temperature dependence on E2f activation by using a cold-sensitive virus-transformed cell line, this may in fact

suggest a physical interaction between this protein and cellular E2f protein.

While E2f binds to both the E1A enhancer and the E2A promoter, only in the case of the E1A enhancer has this interaction been shown to lead to an increased rate of transcription (16, 27). In this article, I suggest that binding of the E2f factor to the *cis*-regulatory elements of the E2A gene leads to increased rates of E2A gene transcription (Fig. 6). However, there is a potential paradox, since it has previously been shown that E4 gene expression (which is required for E2f activation) is suppressed by the product of the E2A gene, the 72-kilodalton single-stranded DNA-binding protein (4, 36). It is possible that the activated E2f may persist for a period of time sufficient to maintain the maximal rate of E2A gene expression in the absence of continued E4 gene expression (prior to the onset of viral DNA replication). Of course, the half-life of the E4 protein(s) will also contribute to the levels of activated E2f. Furthermore, our observation that E2A gene expression is E2f independent at 22 h postinfection is reminiscent of the same observation in E1A mutant virus-infected cells (10, 35) for all the E1A-dependent early promoters and supports the role of E2f as being stimulatory but not absolutely necessary for expression.

It should be noted that the studies of Halbert et al. (13), describing the phenotypic properties of the *dl366* virus upon infection of HeLa cells, failed to detect a reduction in E2A mRNA accumulation compared with that in wild-type virus-infected cells. This may suggest that E2f plays a more significant role in establishing the active transcription complex in rodent cells. This could occur mechanistically by (i) E2f-DNA interaction leading to a template modification that facilitates factor binding or (ii) E2f interaction with specific cellular transcription factors, thereby increasing the rate of factor recruitment on the DNA template. Alternatively, the inability to detect a defect in E2A mRNA accumulation in *dl366* virus-infected HeLa cells may be discerned only when scoring the rate of transcription initiation.

The findings presented in this report have broad implications for the early stages of adenovirus transformation of rodent cells. As evident from the panel of the transformed cells that are presented in Table 1, one cellular phenotype is missing, namely, the transformed cell that expresses high levels of the viral early genes (including E2A) as a consequence of activated E2f levels. Several studies have suggested that the constitutive expression of the E2A gene product is lethal to human cells (25; L. Babiss and H. Ginsberg, unpublished observations). Since it is likely that this protein could adversely affect cellular DNA replication, such a cell line could not be isolated. Therefore, during the early stages of the viral DNA integration process or immediately preceding the first round of cellular DNA replication, at least one of three events may occur to decrease E2A mRNA levels. Because of the pattern of viral DNA integration, either the E2A or E4 genes could be deleted. Both of these situations would lead to a decrease or a lack of E2A gene expression. These hypotheses could explain why a virus containing a temperature-sensitive lesion in the E2A gene integrates complete viral genomes into the CREF cell chromosomes at the nonpermissive temperature of 39°C and integrates incomplete (E2A gene deleted) genomes at the permissive temperature (L. Babiss, P. Fisher, and H. Ginsberg, unpublished observation). Of course, it is possible that this reflects viral DNA replication prior to viral DNA integration and may not pertain to E2A gene regulation. Alternatively, and of more potential interest, there is a possibility that selective pressure allows the isolation of a transformed

cell which expresses an altered form of the E2f protein that can no longer be activated by E1A and E4 gene products. The CR-D cell line is a potential candidate, since E2f activation cannot occur in these cells after virus infection, therefore ruling out E1A and E4 protein defects. Of course, we cannot rule out the possibility that the CR-D cell line expresses an altered form of an E4 gene product that has a *trans*-dominant effect, thereby negating the function of the wild-type E4 gene product provided in *trans*.

These findings also have implications for our understanding of the biological characteristics of transformed cells. By using the panel of cell lines described in this article, a direct correlation between E2A and E2F levels in the transformed cell and the tumorigenicity of that cell can be determined. Such correlations will give a greater insight into the interrelationship between changes in viral and cellular gene expression which leads to cellular transformation and tumor induction. Furthermore, the observation that a viral oncogene product can modulate the activity of a preexisting cellular transcription factor suggests a possible mechanism that commonly occurs to initiate the transformed-cell phenotype.

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