The Adenovirus E4 17-Kilodalton Protein Complexes with the Cellular Transcription Factor E2F, Altering Its DNA-Binding Properties and Stimulating E1A-Independent Accumulation of E2 mRNA

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E2F is a cellular DNA-binding factor. Its binding activity is changed within adenovirus-infected cells so that it binds cooperatively to pairs of properly spaced and oriented E2F recognition sites. In the work described in this report, the conversion to cooperative binding was shown to require the adenovirus E4 17-kilodalton (kDa) polypeptide. Mutant viruses carrying alterations within the E4 17-kDa coding region failed to generate the infection-specific, cooperatively binding form of E2F. It was possible to alter E2F from uninfected cells so that it bound cooperatively by incubation with a partially purified fraction obtained from infected cells. The E4 17-kDa protein copurified with this activity and was also found to be present in a complex containing E2F. Consistent with its ability to alter the binding of E2F to its recognition sites within the E2 promoter, the E4 17-kDa polypeptide contributed to maximal expression of E2 mRNAs in some cell types. Its ability to enhance E2 transcription did not require expression of the E1A transactivator protein. These results are consistent with a model which proposes that the E4 17-kDa polypeptide binds to the cellular E2F factor, altering its binding behavior and thereby enhancing its ability to stimulate transcription.

Three different early adenovirus gene products have been shown to activate the expression of other viral genes *trans*. The best-studied activator is the E1A 289R protein. This E1A product is required for efficient activation of all remaining early viral genes (4, 28), and it functions to enhance the rate of transcription (36). The other activating products are the E1B 21-kilodalton (kDa) polypeptide (23, 51, 54), which has not been shown to act at the level of transcription, and an E4 gene product (13, 49).

The mechanism of transcriptional activation by the E1A protein is not yet clear (for reviews, see references 3, 11, and 40). It has been proposed that E1A-mediated activation of the adenovirus E2 early promoter involves the induction or "activation" of a cellular factor termed E2F (37, 38, 40). E2F is a sequence-specific DNA-binding activity that recognizes the sequence 5'-TTTC(G/C)CGC-3' (30). There are many such sites on the adenovirus chromosome, but four sites are located in regions that have been previously mapped as transcriptional control regions: two upstream of the E1A gene and two upstream of the E2 early start site. Kovesdi et al. (30) showed, by using an *in vitro* assay, that the ability of E2F to bind to the two E2F sites upstream of the E2 coding region increased dramatically in adenovirus-infected cells. This activation is not simply due to an increase in the amount of E2F activity that can bind to a single E2F site, but, rather, results from the production of a new form of E2F, called infection-specific E2F, which binds cooperatively to the two sites on the E2 early promoter in a manner that is dependent on the relative position and orientation of the two recognition sites (18). Since this factor binds to transcriptional control regions, one would predict

that it functions to facilitate transcription. Indeed, E2F purified from adenovirus-infected cells can stimulate transcription from a synthetic promoter containing E2F-binding sites in cell extracts (52).

Recently, it has been shown that the activation of E2F to cooperative binding requires an adenovirus gene product encoded by early region 4 (E4) (1, 16, 45) and can occur in the absence of E1A function (16). The E4 region is located at the right end of the adenovirus genome and is transcribed in the leftward direction, giving rise to a complicated set of differentially spliced mRNAs (12, 48). *In vitro* translation of E4-specific mRNAs (33) has shown that they encode at least six polypeptides from the region's seven open reading frames (ORFs [Fig. 1]). The study of viral mutants has given some insight into the function of these gene products (see, e.g., references 5, 15, 22, and 25). Large deletions of multiple E4 ORFs result in pleiotropic effects with defects in the expression of late viral genes, viral DNA accumulation, and shutoff of host cell macromolecular synthesis, whereas mutations affecting individual ORFs have relatively minor effects on viral growth. ORF3, which encodes a 14-kDa nuclear protein (8), and ORF6, which encodes a 34-kDa nuclear protein (6), appear to encode redundant functions involved in host cell shutoff and accumulation of late viral mRNAs (5, 25). Hemström et al. (22) have described a virus that contains only the portion of E4 encoding ORF6 and ORF6/7. This mutant grows normally on HeLa cells, displaying only minor differences from wild-type virus in the onset of DNA replication and in the accumulation of the E2A-encoded 72-kDa DNA-binding protein.

In this report, we present genetic and biochemical evidence that the adenovirus E4 ORF6/7 17-kDa protein is sufficient to produce the infection-specific form of E2F. A column fraction which contains an activity that can activate

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FIG. 1. Physical map of the E4 mRNAs and coding regions and the location of the mutation in *dl356*. The top of the figure positions the map in terms of map units and nucleotide sequence relative to the right end of the genome. Representative mRNAs that have been identified by cDNA cloning (12) are indicated by lines and arrows; introns are indicated by spaces, and polypeptide-coding regions are indicated by open rectangles. No AUG initiation codon or mRNA has been described for ORF5. Molecular masses of the predicted polypeptides encoded by each ORF and by fusion of different ORFs based on cDNA analysis are shown in parentheses. *dl356* contains a 2-base-pair deletion that disrupts ORF7 (15). The first and last nucleotide of each ORF, as well as the nucleotides which are present in *dl356*, are designated by nucleotide sequence numbers. Abbreviations: bp, base pairs; K, kilodaltons.

E2F from uninfected cells in vitro includes the E4 17-kDa protein. The ability to activate E2F in vitro copurified with the 17-kDa protein over a second, high-resolution high-performance liquid chromatography column. Furthermore, the 17-kDa protein is present in a complex with E2F, since monoclonal antibodies directed against the 17-kDa protein can specifically retard complexes that represent the infection-specific form of E2F and can deplete E2F activity from nuclear extracts. Consistent with its ability to interact with E2F, the 17-kDa protein can specifically stimulate the accumulation of E2 early mRNAs in certain cell types, since an adenovirus mutant unable to produce the E4 17-kDa protein exhibited reduced accumulation of these mRNAs compared with wild-type virus. Finally, the E4 17-kDa protein can act as a viral transactivator in the absence of E1A proteins, since a double mutant carrying small deletions in both the E1A and E4 17-kDa coding regions accumulated lower levels of E2 early RNA than did a mutant virus lacking only E1A function.

MATERIALS AND METHODS

Viruses, cells, and infection protocols. H5wt300 (wild-type adenovirus type 5 [Ad5]) (27), H5dl343 (21), H5dl356 (15), and H5dl1002-H5dl1014 (a gift from E. Bridge and G. Ketner [5]) have all been described previously. H5dl343/356 was constructed by ligation of the 0- to 76-map-unit *EcoRI*-generated fragment of *dl343* DNA to the 76- to 100-map-unit *EcoRI*-generated fragment of H5dl356. The presence in the resulting double mutant of the 2-base-pair deletion within the E1A coding region derived from H5dl343 was confirmed by the loss of an *AvaII* cleavage site, and the 2-base-pair deletion within E4 ORF6/7 derived from H5dl356 was confirmed by the loss of a *SmaI* cleavage site.

HeLa (American Type Culture Collection) and S49 (24) cells were grown in suspension culture in medium containing 10% horse serum. 293 cells, which contain and express the Ad5 E1A and E1B genes (14), were grown as monolayers in medium containing 10% calf serum. W162 cells, which express Ad2 E4 gene products (50), were grown as monolayers in 10% calf serum.

Purified wt300 and *dl356* (15) virion stocks were prepared from infected 293 cells by subjecting virions to two rounds of equilibrium density centrifugation. Purified virions were used to infect HeLa cells at a multiplicity of 1,000 particles per cell for analysis of their phenotypes unless otherwise noted. Stocks of the other viruses were generated from infected 293 or W162 cells by three cycles of freeze-thawing followed by low-speed centrifugation to remove debris.

For the time course experiments, the infections were carried out as follows. HeLa spinner cells were concentrated to 5×10^6 cells per ml by centrifugation at $1,500 \times g$ for 5 min at 25°C and suspended in medium containing 2% horse serum before being infected with purified wt300 or *dl356* at 1,000 particles per cell. Virus was allowed to absorb for 1 h, after which the cells were diluted to 5×10^5 cells per ml with medium containing 10% horse serum. At each time point, 5×10^7 cells were removed, pelleted, and washed once with phosphate-buffered saline (PBS) before being frozen in liquid nitrogen.

Oligonucleotides and peptides. Synthetic DNA used for this work was prepared, purified, and labeled as described previously (18). The sequences of one strand of the oligonucleotides containing either one or two E2F sites (underlined) were 5'-CCATTTTCGCGGAAACTGAATAAG-3' and 5'-GATGACGTACTTTTCGCGCTTAAATTTGAGAAAGGCGCGAAACTAGTCCTT-3', respectively. The P2 and P4 peptides were described previously (6). The P2 sequence is N-SRDRLPPFETETGGY-C, and the P4 sequence is N-YGEGGGRLNTRVL-C.

DNA band shift and off-rate analysis. DNA band shift assays were performed essentially as described previously (17). Each reaction contained less than 1 μ l of nuclear extract, 10 fmol of 32 P-labeled probe DNA, and 1 or 5 μ g of sonicated salmon sperm DNA for assays using a probe with one or two E2F binding sites, respectively. After separation of DNA-protein complexes by electrophoresis in a 4% polyacrylamide gel (bisacrylamide/acrylamide ratio, 1:20) in a buffer containing 10 mM Tris hydrochloride (pH 8) and 1 mM EDTA at 4°C, gels were transferred to dampened DE81 paper, dried, and exposed to X-ray film.

Off-rate analysis was performed exactly as previously described (16).

Preparation of extracts. Nuclear extracts were prepared by the method of Dignam et al. (7) as modified by Hardy and Shenk (18). Infected-cell extracts used for the purification of the induction-to-cooperative-binding activity were prepared 24 h after infection with Ad5 at a multiplicity of 20 PFU per cell.

For the time course experiments, all extracts were prepared at the same time as follows. After cells had thawed on ice, 2.5 packed-cell volumes of Triton lysis buffer (20 mM Tris [pH 8], 150 mM NaCl, 1 mM MgCl₂, 0.075% Triton X-100) were added, and the cells were allowed to incubate at 4°C for 10 min. The cells were disrupted by pipetting before being centrifuged in a Brinkman model 5415 Eppendorf centrifuge at setting 7. The supernatant was used to prepare cytoplasmic RNA as described below. The pellet was disrupted with one packed-cell volume of buffer C (7), and the extract was allowed to shake on ice for 45 min before centrifugation at 14,000 × g. This supernatant was considered the nuclear salt wash and was frozen in liquid nitrogen in small aliquots.

Chromatography of and assay for the induction-to-cooperative-binding activity. Chromatographic procedures were carried out at 4°C. To partially purify the induction-to-cooperative-binding activity, nuclear extracts from adenovirus-infected cells were dialyzed against 100 mM NaCl in buffer A (25 mM [HEPES; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.9], 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% [vol/vol] glycerol), clarified by centrifugation at 39,000 × g for 15 min, and loaded onto a cation-exchange column (Glass-Pak SP-5PW, 0.8 by 7.5 cm; Pharmacia LKB Biotechnology, Inc.) at a flow rate of 0.5 ml/min on a high-performance liquid chromatography system (Pharmacia LKB). Proteins were eluted with a 100 to 400 mM NaCl gradient (30 ml), 400 mM NaCl (5 ml), and a 400 to 1,000 mM NaCl gradient (5 ml), all in buffer A. The flowthrough (FT) (20 ml) from this column was then loaded onto an anion-exchange column (Glass-Pak DEAE-5PW, 0.8 by 7.5 cm; Pharmacia LKB), again at a flow rate of 0.5 ml/min. The column was washed with 100 mM NaCl in buffer A, and proteins were eluted by using a 100 to 320 mM NaCl gradient (30 ml), 320 mM NaCl (5 ml), and a 320 to 1,000 mM NaCl gradient (5 ml), all in buffer A.

The purification procedure and properties of E2F purified from uninfected or infected cells will be described in detail elsewhere (M. Marton and T. Shenk, manuscript in preparation). In brief, a nuclear extract was prepared as described above and subjected to DNA affinity chromatography (29) by using a matrix containing multiple E2F-binding sites.

To assay the ability of a column fraction containing an activity from infected cells to alter the DNA-binding properties of E2F from uninfected cells, we used the following reaction to monitor the induction of cooperative binding. A 5-μl portion of the column fraction was mixed with 5 μl of E2F that had been purified as described above. The mix was allowed to incubate for between 1 and 30 min at 22°C before being used in a DNA-binding assay. Each binding assay contained 10 fmol of ³²P-labeled probe DNA, 5 μg of sonicated salmon sperm DNA, and 3 μl of the above activation mix in 1× assay buffer (10 mM Tris [pH 8], 120 mM NaCl, 1 mM MgCl₂). Electrophoresis was carried out as above for band shift assays.

Preparation of antibodies and their use in DNA band shift assays. Monoclonal antibody (MAb) 1 was prepared against

the P2 peptide conjugated to keyhole limpet hemocyanin, and MAb 3 was raised against a β-galactosidase-E4 17-kDa fusion protein and generously supplied by Patricia Robinson of our laboratory.

DNA band shift assays which involved the addition of MABs against the E4 17-kDa protein were carried out as follows. After binding reactions were allowed to reach equilibrium (25 min at room temperature), increasing amounts of MAB tissue culture supernatants were added. The final salt concentration in each binding reaction was kept constant and at optimal binding conditions for E2F binding (120 mM NaCl for the double-site probe; 60 mM NaCl for the single-site probe).

Immunodepletion of E2F-binding activity from infected-cell extracts was performed as follows. A nuclear salt wash or affinity-purified E2F from infected cells was incubated for 2 h at 4°C with an equal volume of either MAB 1 or MAB 3 or with PBS and 4 mg of ovalbumin per ml to keep the ionic strength and protein concentration constant. After 1 μl of rabbit anti-mouse immunoglobulin G was added and the mixture was allowed to incubate for an additional 1 h at 4°C, 0.1 volume of protein A-positive *Staphylococcus aureus* cells (BMB) was added, and the mixture was allowed to incubate for 12 h at 4°C with shaking. The resulting precipitate was removed by centrifugation in a microcentrifuge for 10 min. The supernatant was assayed for E2F activity on both the single-site and double-site E2F DNA probes as described above.

Western immunoblot analysis. Fractions from the DEAE-5PW column (20 μl for E1A, 175 μl for E4 17-kDa protein) were subjected to electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide for E1A proteins, 15% acrylamide, bisacrylamide/acrylamide ratio, 1:108, for the E4 17-kDa proteins) and electrophoretically transferred to nitrocellulose at 1.5 mA for 1 h. Filters were blocked with 3% nonfat dry milk–1% bovine serum albumin in PBS overnight. M73 MAB (19) was used to probe for E1A protein, and MAB 3 was used to probe for the E4 17-kDa protein. After incubations with the first antibody at room temperature for 1 to 4 h, filters were washed three times with PBS–0.05% Tween 20 (10 min each) before a 1- to 2-h room temperature incubation with rabbit anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase (Sigma Chemical Co.). Filters were washed as above and developed in 0.4 mM 5-bromo-4-chloro-3-indolylphosphate and 0.4 mM Nitro Blue Tetrazolium (Sigma).

RNA isolation and analysis and RNase protection probes. RNA was isolated by the procedure of Farrell et al. (10) as modified by Engel et al. (9). Briefly, sodium dodecyl sulfate and proteinase K were added to the cytoplasmic fraction to 0.5% and 400 μg/ml, respectively. After incubation at 37°C for 35 min, the solution was extracted twice with phenol-chloroform (1:1) and once with chloroform. The nucleic acid was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. After centrifugation, the pellet was washed with 70 or 80% ethanol and quantified by spectrophotometry. The RNAs were analyzed by RNase protection by the procedure of Melton et al. (34). The relative amounts of RNA were quantified by densitometry of autoradiographs with a Bio-Rad model 620 densitometer. The E1A/E1B-specific probe was transcribed by T7 RNA polymerase from an *Eco*RI-linearized plasmid containing the first 1,833 bases of the adenovirus left end cloned into pGEM2. The E2 early probe was made by using T7 RNA polymerase and an *Eco*RI-linearized plasmid containing a *Sma*I-*Hind*III fragment (map units 73.2 to 76.6) cloned into pGEM4. The

FIG. 2. DNA band shift analysis of E2F activities that accumulate after infection with *wt300* and *dl356*, a mutant adenovirus that fails to produce the E4 17-kDa protein. Nuclear extracts were prepared from either mock-infected (lanes M) cells or at various times after infection (lanes 0.5 through 18) with either *wt300* (A and C) or *dl356* (B and D). Band shift analysis used a ^{32}P -labeled probe DNA that contained a single E2F-binding site (A and B) or two E2F-binding sites (C and D) arranged as they are found upstream of the E2 early start site. E2F-specific complexes formed on the single-site probe are labeled (E2F-a, E2F-b, E2F-c, E2F-d, and E2F-e); the cooperative complex formed on the double-site probe is designated E2F-Ad. As previously reported (16, 18), the amount of E2F-a activity varies from extract to extract and, in this experiment, is visible only after a long exposure of the gels shown in panels A and B. Unlabeled band in panels A and B is not E2F specific. Abbreviation: hpi, hours postinfection.

probe that specifically detects the E2 late start site was transcribed by SP6 RNA polymerase by using a plasmid linearized with *Hind*III that contains a *Kpn*I-*Hind*III fragment (map units 71.8 to 73.2) cloned into pGEM4. The E3-specific probe was transcribed by T7 RNA polymerase from an *Eco*RI-linearized plasmid that contains an *Eco*RI-*Bgl*II fragment (map units 75.9 to 77.9) cloned into pGEM2.

RESULTS

During a wild-type adenovirus infection, a new form of E2F activity can be detected which is distinct from the activity present in uninfected HeLa cells (16, 18). The infection-specific form of E2F and the E2F present in uninfected cells generate different band shift patterns by using double-stranded DNA oligonucleotides that contain either one or two E2F-binding sites as ^{32}P -labeled probes. Four E2F-specific bands were detected by using a single-site probe DNA and an uninfected-cell extract (Fig. 2A, E2F-a to E2F-d; lane M). These complexes represent sequence-spe-

cific interactions of cellular DNA-binding activities with the probe DNA, since their formation can be prevented by adding an excess of unlabeled single-site oligonucleotide (18). The pattern of shifted bands changed when an Ad5-infected cell extract was used in the assay. A new band of altered mobility was evident, (Fig. 2A, E2F-e). When an oligonucleotide probe (the double-site probe) containing the two E2F sites of the E2 early promoter was used in the assay, a slowly migrating complex (E2F-Ad) was formed when infected but not uninfected extracts were used (Fig. 2C and D). The E2F-e and E2F-Ad complexes represent sequence-specific interactions, since their formation can be prevented by adding an excess of unlabeled homologous oligonucleotide (18). Footprint analysis indicated that both E2F-binding sites are occupied in the E2F-Ad complex, and this complex involves the same cellular factors that bind to the single-site oligonucleotide, since the E2F-e and E2F-Ad activities copurify and since an excess of unlabeled single-site DNA can inhibit binding to ^{32}P -labeled double-site DNA (18).

TABLE 1. Summary of E4 mutants and their ability to induce the infection-specific form of E2F

Virus ^a	Mutated E4 ORF(s) ^b	Infection-specific E2F
<i>wr300</i>	None	+
<i>dl1002</i>	6, 6/7	—
<i>dl1003</i>	3/4, 4, 6, 6/7	—
<i>dl1004</i>	2, 3, 3/4, 4, 6, 6/7	—
<i>dl1005</i>	1, 2	+
<i>dl1006</i>	1, 2, 3, 3/4	+
<i>dl1007</i>	1, 2, 3, 3/4, 4, 6, 6/7	—
<i>dl1014</i>	1, 2, 3, 3/4, 6, 6/7	—
<i>dl355</i>	6	+
<i>in1012</i>	6/7	—
<i>dl356</i>	6/7	—

^a 300-series viruses are from Halbert et al. (15), and 1000-series viruses are from Bridge and Ketner (5).

^b The putative protein product for ORF5 (overlaps the 5' end of ORF6 and ORF6/7 [15]) has never been identified. This ORF is not mutated in either *dl356* or *in1012*; therefore, if an ORF5 product is made, it is not sufficient to produce the infection-specific form of E2F.

Additional assays (18) can distinguish the infection-specific form of E2F from the activity present in uninfected HeLa cells, and these assays demonstrate that the DNA-binding properties of E2F are altered qualitatively during an adenovirus infection. For example, infection-specific E2F, but not uninfected E2F, binds in an orientation-dependent and spacing-dependent manner, forms a complex with a bound half-life 10 times longer than that of uninfected E2F, and has a nonlinear dependence on protein concentration (18). All these properties are consistent with the conclusion that infection-specific E2F binds to the two E2F sites in the adenovirus E2 early promoter in a highly cooperative manner and that uninfected E2F binds to these sites independently or with very much less cooperativity.

Viruses carrying mutations within E4 ORF6/7 do not induce infection-specific E2F. A group of Ad5 deletion mutants defective in early region 4 (a map of the E4 region is shown in Fig. 1) were initially screened for the production of infection-specific E2F by band shift assay. The ORFs deleted in these individual viruses, which were isolated by Bridge and Ketner (5) and Cutt et al. (6), are shown in Table 1. Results obtained with single-site or double-site probe DNAs were consistent. Wild-type Ad5 (*wr300*), as well as *dl1005* and *dl1006*, generated infection-specific E2F when assayed on either the single-site or double-site DNA probes (data not shown; results summarized in Table 1). This demonstrated that the products of E4 ORFs deleted in the two mutants, ORF1, ORF2, ORF3, and ORF3/4, do not play a role in the production of the infection-specific forms of E2F activity. Mutants *dl1003*, *dl1004*, *dl1007*, and *dl1014* all failed to induce infection-specific E2F. These viruses all lack ORF6 and ORF6/7, suggesting that a product of one or both of these E4 coding regions is needed for the production of the infection-specific form of E2F.

Next, *dl356* (15) (Fig. 1), a mutant that contains a 2-base-pair deletion within ORF6/7 but grows to wild-type titers on HeLa cells, was analyzed. HeLa cells were infected at a multiplicity of 1,000 particles per cell (approximately 20 PFU per cell) with either *wr300* or *dl356* and harvested at various time intervals. Nuclear extracts prepared from HeLa cells as early as 3 h after infection with *wr300* generated complexes diagnostic of the infection-specific E2F-e complex on a single-site probe DNA (Fig. 2A) and E2F-Ad complex on a double-site probe DNA (Fig. 2C). Extracts prepared as late as 18 h (Fig. 2B and D) or 35 h (data not

shown) after infection with *dl356* failed to produce either infection-specific complex. A second, independently isolated E4 ORF6/7 mutant, *in1012*, also failed to produce the infection-specific complexes, and an E4 ORF6 mutant, *dl355*, behaved like wild-type virus in this assay (S. Bhattacharya, S. Hardy, M. Marton, and T. Shenk, unpublished data).

We conclude that an E4 ORF6/7 gene product either directly or indirectly alters the binding behavior of E2F in Ad5-infected cells.

In vitro activation of E2F. In addition to identifying the genetic component of adenovirus which was responsible for the production of the infection-specific form of E2F, we were interested in determining the nature of the alteration in E2F. It has been reported that E2F can be activated in vitro in a phosphorylation-dependent manner (2). We sought to reproduce and extend those observations. It was reported that extracts from adenovirus-infected cells, but not uninfected cells, contained an activity that could activate E2F, with activation being monitored by using either an exonuclease III assay or a band shift assay (2). The activity eluted in the FT of a cation-exchange column, while E2F bound to the column. We prepared nuclear salt wash extracts from uninfected and adenovirus-infected HeLa cells, subjected each to chromatography on an SP-5PW cation-exchange column, and assayed the fractions for E2F activity. Both uninfected-cell E2F (data not shown) (18) and infection-specific E2F (Fig. 3A) were retained on the column. The levels of E2F activity were highest in fractions 9 through 13. On a double-site probe DNA, infection-specific E2F generated both single-occupancy complexes in which only one E2F-binding site is occupied (Fig. 3A, arrowhead) and double-occupancy complexes which we have previously termed E2F-Ad (Fig. 3A, equals sign). As discussed above, the identities of these complexes were established by Hardy and Shenk (18). The FT from either uninfected (Fig. 4A, lane 3, and data not shown) or infected (Fig. 3A, lane FT, and Fig. 4A, lane 2) cell extracts contained no detectable E2F activity. Both FT fractions did contain an activity we have termed η factor (S. Hardy, M. Marton, and T. Shenk, unpublished), which is a distinct DNA-binding activity. η does not bind to the E2F recognition site and generates a complex that migrates slightly faster than the double-occupancy E2F complex.

To assay the ability of the material in the FT from uninfected- and infected-cell extracts to activate E2F, FT fractions were mixed with affinity-purified E2F from uninfected cells, ³²P-end-labeled oligonucleotide containing two E2F sites as on the adenovirus E2 early promoter was added, and the resulting complexes were subjected to electrophoresis. It has been shown previously that such E2F from uninfected cells preferentially forms a single-occupancy complex (18). Figure 4A, lane 1, shows a binding reaction containing only uninfected-cell E2F. As expected, only the single-occupancy complex was observed under these assay conditions. Lanes 2 and 3 confirm that the cation-exchange FT fractions from uninfected and infected cells did not contain detectable E2F-binding activity. When E2F was mixed with uninfected-cell FT, little difference was observed (Fig. 4A, lane 5); however, when infected-cell FT was added to uninfected E2F, a new band that comigrated with E2F-Ad, the infection-specific form of E2F, was observed (Fig. 4A, lane 4). This activated E2F has properties of authentic infection-specific E2F since it is competed by an oligonucleotide containing a single E2F site (data not shown) and has a bound half-life of more than 60 min (Fig. 4B).

FIG. 3. DNA band shift analysis of E2F activities and Western blot analysis of E4-specific polypeptides in fractionated nuclear extracts prepared from *w*3300-infected HeLa cells. Extracts were prepared 24 h after infection and resolved on an SP-5PW matrix. Material applied to the matrix (INPUT) and the FT are analyzed at the left side of the column's eluate profile. (A) Fractions were assayed by band shift assay with a ^{32}P -labeled probe DNA that contained two E2F sites arranged as they are found upstream of the E2 early start site. Symbols: =, E2F-Ad double-occupancy complexes; ▶, major single-occupancy complex. (B) Fractions were assayed by protein blot analysis with the E4-specific MAb 3. The positions of molecular mass markers are indicated.

Furthermore, since no induction of binding activity was observed on a single-site probe (data not shown), we conclude that the activation is not simply an induction of DNA-binding activity per se but rather an induction of the ability to E2F to bind cooperatively to the two E2F sites of the adenovirus E2 early promoter, as is the case during an adenovirus infection. In other words, the *in vitro*-activated E2F studied here has properties similar to those of the activated form that is generated *in vivo*.

The form of E2F activity that accumulates within adenovirus-infected cells was previously termed infection-specific E2F, and the complex it forms with a DNA containing two E2F-binding sites was named E2F-Ad (18). Until we have rigorously proved that the *in vitro*-generated activity is identical in all respects to the *in vivo*-generated, infection-specific E2F, we will refer to the *in vitro*-produced material as activated E2F.

The data presented thus far confirm the observation of Bagchi et al. (2) that a nuclear salt wash from infected cells can be fractionated on a cation-exchange column to produce a fraction containing an activity that can dramatically affect how E2F binds to DNA. However, in contrast to Bagchi et al. (2), who concluded that the fraction contained a kinase

activity, we were unable to inhibit the activation by addition of a kinase inhibitor (up to 1 mM adenosine-5'-O-3-thiotriphosphate [ATP- γ -S]; Fig. 4C), and, again in contrast to the earlier report, we were unable to activate E2F with the catalytic subunit of cyclic AMP-dependent protein kinase or inhibit the formation of the complex specific to the activated E2F with any alkaline phosphatase tested (data not shown). In addition, another observation made us question whether an enzymatic process was involved. A linear titration of the infected FT fraction in the activation assay resulted in a linear decrease in the amount of activated E2F complex, even in the presence of ATP (Fig. 4A). This result is consistent with the notion that some component or cofactor is limiting in the FT fraction (besides ATP) or consistent with a stoichiometric, i.e., noncatalytic alteration of E2F-binding activity. Finally, the activity was stable to boiling (Fig. 5B, lanes 1 and 2), and we are unaware of any examples of a protein kinase that is stable to extreme heat.

It seems quite clear that we are studying a different activity from that described by Bagchi et al. (2). The activity we have identified does not display the properties of a kinase and may function stoichiometrically. Since our assay detects a change in the DNA-binding behavior of E2F that is indicative of the cooperatively binding infection-specific form, we have named this activity the induction-to-cooperative-binding activity.

The activity copurifies with the E4 17-kDa protein. To further characterize the induction-to-cooperative-binding activity and to begin to address whether the E4 17-kDa protein was involved either directly or indirectly in the process, we further purified the activity over a high-performance liquid chromatography DEAE-5PW anion-exchange column. Each fraction was assayed for DNA-binding activity on the double-site probe DNA, and only the η activity (discussed above) was detected, eluting in fractions 11 and 12 (Fig. 5A). Fractions were further tested for their ability to alter the binding properties of E2F from uninfected cells. This activity eluted in an unusually tight peak at approximately 120 mM NaCl (Fig. 5B, fraction 3), far from the major protein peak, which was determined to be in fractions 10 to 12 (data not shown). We then assayed these column fractions for the presence of the E4 17-kDa protein by using a MAb (MAb 3) raised against a β -galactosidase-E4 17-kDa fusion protein. The Western blot revealed that the 17-kDa protein exactly copurified with the induction-to-cooperative-binding activity (Fig. 5C). It is also apparent from this Western blot that MAb 3 also recognizes the related protein encoded by ORF6 of early region 4. The protein, which has a predicted molecular mass of 34 kDa but which migrates in sodium dodecyl sulfate-gels with an apparent molecular mass of 25 kDa (6, 46), clearly separated away from the E4 17-kDa protein on the anion-exchange matrix. As a control, the column fractions were assayed for the presence of E1A protein (Fig. 5D), which demonstrated that the vast majority of the E1A protein separated from the 17-kDa protein in fraction 3. These results raised the possibility that the 17-kDa protein directly interacts with E2F to alter its binding properties, a possibility that was reinforced by performing a Western blot analysis to localize the E4 17-kDa protein in the elution profile of the infected-cell extract on the SP-5PW column (Fig. 3B). Although some E4 17-kDa protein was present in the FT, as expected (Fig. 5C), the bulk of it cofractionated with infection-specific E2F.

MAbs against the E4 17-kDa protein specifically recognize the infection-specific form of E2F. Since the 17-kDa protein copurified with the induction-to-cooperative-binding activity

FIG. 4. Analysis of the activity from wild-type virus-infected cells that can activate E2F from uninfected cells. DNA band shift assays of E2F activity were performed with a ^{32}P -labeled probe DNA that contained two E2F-binding sites arranged as they are found upstream of the E2 early start sites. Symbols: =, double-occupancy complexes; ▲, major single-occupancy complex. (A) Activation of E2F from uninfected HeLa cells. The SP-5PW FT fractions from uninfected (uninf) and infected (inf) HeLa cells were assayed for their ability to activate E2F, and the activity from infected cells was titrated (1, 2, 3, and 4 μl per reaction) in the presence of 1 mM ATP. (B) Off-rate analysis of E2F complexes formed by infection-specific E2F (lanes wild-type infected) or by activation of E2F from uninfected cells by the SP-5PW FT fraction of infected cell extract (lanes *in vitro* "activated"). Complexes on the ^{32}P -labeled probe DNA were formed, a 500-fold molar excess of unlabeled DNA identical to the probe was added, and samples were taken for electrophoretic analysis at the time after addition of competitor indicated above the lines. Each sample was loaded onto a running gel as soon as it was taken. As a result, samples were subjected to electrophoresis for somewhat different periods, leading to small differences between samples in the mobility of complexes and free DNA. (C) Comigration of the double-occupancy complexes generated by infected-cell nuclear salt wash extract (lane inf NSW) and by mixing the SP-5PW FT fraction from infected cells with E2F from uninfected cells, and the lack of a requirement for hydrolyzable ATP for formation of the activated E2F complex (the complex is formed in the presence of the ATP analog ATP- γ -S).

through two chromatographic steps, we decided to directly test the possibility that the viral protein was present in a complex with E2F and DNA. Previous studies have shown that addition of an antibody directed against DNA-binding proteins (32, 35, 42, 43) to a DNA band shift assay can either disrupt the protein-DNA complex or create a larger complex that migrates more slowly in a native band shift gel. We therefore tested a series of monoclonal antibodies raised against either a β -galactosidase-E4 17-kDa fusion protein or a peptide corresponding to a region within the N terminus of the 17-kDa protein. When either of two antibodies (MAb 1 [Fig. 6A] or MAb 3 [data not shown]) was added to a DNA-binding assay containing purified E2F and the E2 double-site probe, formation of the normal E2F-Ad complex was inhibited and a complex of slower electrophoretic mobility was observed (Fig. 6A, lanes 1 to 5, complex with antibody denoted by the star). A complex representing the major late transcription factor and its recognition sequence was not affected by the same concentration of antibody in the binding reaction (Fig. 6A, lanes 6 to 10). In addition, the "supershifted" complex could be formed by using affinity-purified E2F from infected cells (Fig. 6A, lane 11), further indicating that the antibody was specifically interacting with E2F-Ad in the binding reaction. Finally, the supershifted complex could be specifically competed with by using the P2 peptide (Fig. 6A, lanes 12 to 15) that was used to generate MAb 1, but was not inhibited by the P4 peptide (Fig. 6A, lanes 16 to 20), which represents a different portion of the E4 17-kDa protein. These data strongly argue that the E4 17-kDa protein is part of the E2F-Ad complex.

Previously, we recognized that the infection-specific form of E2F generated a distinct pattern of specific bands when

one used a DNA probe that contained only one E2F recognition site (16). On the single-site probe, a new band, designated E2F-e, becomes apparent during an adenovirus infection (16). We tested whether the E2F-e complex contained the 17-kDa protein by using the same MAbs that specifically supershifted the E2F-Ad complex. The addition of the anti-peptide MAb 1 could specifically shift the E2F-e complex and give rise to a more slowly migrating complex from an infected nuclear extract (Fig. 6B, lanes 1 to 5, complex with antibody marked by the star) or from affinity-purified E2F from infected cells (Fig. 6B, lanes 6 to 10). In this experiment, the other E2F-specific forms (especially E2F-c and E2F-d [18]) serve as convincing negative controls, since only the E2F-e complex is shifted. Finally, as in the case of the supershifted E2F-Ad complex, the supershifted E2F-e complex can be competed by the P2 peptide (Fig. 6B, lanes 11 to 15) but not the P4 peptide (Fig. 6B, lanes 16 to 20).

One of the E4 17-kDa-specific antibodies was also able to deplete E2F activity from preparations of infection-specific E2F. Antibodies to the E4 17-kDa protein were added either to infected nuclear extracts or to preparations of purified E2F from infected cells and allowed to incubate for 12 h at 4°C in the presence of rabbit anti-mouse immunoglobulin G and protein A-positive *S. aureus* cells. After the resulting precipitate was removed by centrifugation, the supernatant was tested for residual infection-specific E2F activity in band shift assays. MAb 3, but not MAb 1, depleted essentially all of the activity giving rise to the infection-specific E2F-Ad bands on the double-site probe and the E2F-e band on the single-site probe (Fig. 6C). The E2F-c and E2F-d complexes, which can be formed by E2F from uninfected

FIG. 5. DNA band shift analysis of E2F activities and Western blot analysis of E1A- and E4-specific polypeptides in fractionated nuclear extracts prepared from *wt300*-infected HeLa cells. The FT fractions from the SP-5PW column whose profile is displayed in Fig. 2 was applied to a DEAE-5PW matrix and eluted with a salt gradient. Material applied to the matrix (lane INPUT) and the FT were analyzed at the left side of the column's eluate profile. (A) Fractions were assayed by band shift assay with a 32 P-labeled probe DNA that contained two E2F sites arranged as they are found upstream of the E2 early start site. Symbols: =, position at which the E2F-specific double-occupancy complexes would migrate; ◆, η -specific complex. (B) Assay as in panel A, except that E2F from uninfected HeLa cells was added to each sample. Also, boiled (lane + BOIL) activity from fraction 3 was tested for its ability to activate E2F. (C) Fractions were assayed by protein blot analysis with E4-specific MAb 3. The positions of molecular mass markers are indicated. (D) Fractions were assayed by protein blot analysis with the E1A-specific MAb M73 (19). The positions of molecular mass markers are indicated.

cells (Fig. 2A), were not depleted (Fig. 6C), consistent with a requirement for the presence of the E4 17-kDa protein for depletion to occur.

In summary, the results of both supershift (Fig. 6A and B) and immunodepletion (Fig. 6C) experiments argue that the E4 17-kDa protein is present in complexes with infection-specific E2F. Furthermore, the E4 17-kDa protein and infection-specific E2F cofractionate on the cation-exchange matrix (Fig. 3). These results argue strongly that the E4 17-kDa protein is directly responsible for the infection-induced change in E2F-binding behavior.

The E4 17-kDa protein has little effect on accumulation of Ad5 early mRNA in HeLa cells. To determine whether the E4 17-kDa protein influenced mRNA accumulation, we assayed the steady-state levels of Ad5 early mRNAs in the cytoplasmic mRNA preparations from the *wt300* and *dl356* (E4 17-kDa protein mutant) infections described in Fig. 2. We examined E1A levels for two reasons. First, two E2F sites are present in a region which has previously been shown to be important in E1A expression (20). Second, even a small defect in the accumulation of E1A mRNA and their corresponding proteins might have a dramatic impact on the expression of other transcription units. E1A and E1B cytoplasmic mRNA accumulation assayed by RNase protection (34) appeared identical in *dl356*- and *wt300*-infected cells (Fig. 7). E1A 12S and 13S mRNAs were first detected at 2 h

and reached a plateau by 4 h after infection. E1B mRNA was first evident at 3 h after infection. Thus, the absence of infection-specific E2F did not detectably alter E1A mRNA accumulation in HeLa cells, even though two E2F-binding sites are present in the E1A transcriptional control region. It is important to note, however, that infection-specific E2F does not bind cooperatively to this pair of sites (18).

Since the alteration of E2F that we call the infection-specific form is based on an *in vitro* DNA-binding assay that requires properly oriented and spaced E2F sites as seen in the E2 early promoter, it seemed that the transcription unit most likely to be affected in a virus unable to produce this form of E2F would be the E2 early promoter. E2 early mRNA was first detected at 3 h after infection with *wt300*; its level increased until 10 h and then gradually declined through 18 h after infection (Fig. 8A). In cells infected with *dl356*, the kinetics of E2 early mRNA accumulation were identical to those observed for *wt300*. However, mRNA levels in the cytoplasm of *dl356*-infected cells were slightly (less than twofold) but consistently lower than in *wt300*-infected cells (Fig. 8B). Although this is consistent with the notion that the accumulation of the infection-specific form of E2F correlates with maximal expression of E2 early mRNA, it is not consistent with the hypothesis that the infection-specific form of E2F is essential for activation of the E2 early promoter.

FIG. 6. Immunological analysis of infection-specific E2F. (A) DNA band shift analysis was performed by using either a nuclear salt wash extract from infected HeLa cells (lanes NSW) or affinity-purified E2F from infected cells (lanes E2F). The nuclear salt wash extract was mixed with either 32 P-labeled E2F-specific double-site probe DNA or a 32 P-labeled DNA containing a binding site for the major late transcription factor (MLTF). After E2F was mixed with a 32 P-labeled probe DNA, E4-specific MAB 1 was added to the reaction mixtures as indicated. Some mixtures also received P2 peptide, which contains the epitope for MAB 1, and others received the nonspecific P4 peptide. Symbols: =, E2F-Ad double-occupancy complexes; ◀, major single-occupancy complex; ★, supershifted complex that includes MAB 1. (B) As in panel A, except that a 32 P-labeled probe DNA containing a single E2F site was used. E2F-c, E2F-d, and E2F-e complexes are labeled, and the supershifted complex that includes MAB 1 is indicated (★). (C) Immunodepletion of E2F activities giving rise to the E2F-Ad complex on a double-site probe and the E2F-e complex on a single-site probe using an E4-specific MAB. Either MAB 1 or MAB 3 was added to a nuclear salt wash or affinity-purified E2F from infected cells, and the resulting immunoprecipitate was removed. Band shift assays were then performed by using the immunodepleted sources of E2F. As a control, some E2F preparations received only PBS plus 4 mg of bovine serum albumin per ml, in which the antibodies were diluted.

At later times postinfection, a second E2 start site, located 1,137 base pairs downstream of the early start site, is utilized. These E2 late mRNAs were first detected at 7 h after infection, and no difference in the kinetics or level of mRNA expression was observed for *dl356*- and *wt300*-infected cells (Fig. 9).

Zajchowski et al. (55) have reported that promoter elements within the E2 early transcriptional control region influence E3 transcription. E3 is transcribed in the direction opposite to E2, with about 450 base pairs separating the two start sites. Accumulation of E3 mRNAs was examined, and no difference was evident for *dl356*- and *wt300*-infected cells (data not shown).

To summarize, we observed very little difference in the accumulation of early mRNAs in HeLa cells in the presence (*wt300*-infected HeLa cells) and absence (*dl356*-infected HeLa cells) of the infection-specific form of E2F. The only difference noted was for E2 early mRNAs, and this difference was modest. The level of E2 early mRNA was reduced by less than a factor of 2 in the absence of infection-specific E2F.

E4 17-kDa protein is required for normal E2 early RNA accumulation in S49 cells. Although we observed essentially no effect on E2 early expression in the absence of the infection-specific form of E2F in HeLa spinner cells, we wondered whether this was a cell-type-specific observation.

FIG. 7. RNase protection analysis of E1A and E1B RNAs that accumulate after infection of HeLa cells with *wt300* (A) or *dl356* (B). Cytoplasmic RNA was prepared from the harvested cells described in the legend to Fig. 2 and analyzed with a ^{32}P -labeled RNA probe specific for E1A and E1B RNAs. RNase-resistant products were analyzed by electrophoresis through a 5% polyacrylamide gel containing 7 M urea and visualized by autoradiography. Bands corresponding to E1A and E1B mRNA exons are indicated. Abbreviations: M, mock-infected cells; hpi, hours postinfection.

We chose to examine S49 cells, a mouse lymphoma cell line, because this line contains low endogenous levels of AP-1, a cellular factor which also binds the E2 early promoter and which has been implicated in E1A transactivation (35). S49 cells are nonpermissive for growth of adenovirus, but they do complete the early phase of adenovirus infection, including viral DNA replication, with delayed kinetics compared with HeLa cells (9; D. Engel and T. Shenk, unpublished observations). Thus, infected S49 cells were harvested after longer time intervals. The infection-specific form of E2F accumulated as expected in wild-type virus-infected cells but not in *dl356*-infected cells (Fig. 10A). Also, the levels of E1A cytoplasmic RNA were identical in *wt300*- and *dl356*-infected S49 cells (data not shown). However, *dl356*-infected S49 cells accumulated threefold-lower levels of E2 early RNA than *wt300*-infected cells did (Fig. 10B). Thus, the E4 17-kDa protein is necessary in S49 cells for maximal accumulation of E2 early mRNA. Presumably, the E4 17-kDa protein is exerting its effects by generating the infection-specific form of E2F.

E4 17-kDa protein can stimulate E2 early mRNA accumulation in S49 cells in the absence of E1A proteins. Once it had been established that the E4 17-kDa protein (and therefore presumably the infection-specific form of E2F) was required for maximal accumulation of E2 early mRNA, we chose to determine whether the E4 17-kDa protein-mediated activation was a part of the E1A transactivation process or whether it constituted a separate component of the activation of the E2 early promoter. To address this question, we constructed a virus defective in the production of both the E1A and the E4 ORF6/7 gene products so that we could access the ability of the E4 17-kDa protein to stimulate E2

early mRNA accumulation in the absence of E1A proteins. This virus, termed *dl343/356*, contained the E1A mutation from *dl343* (2-base-pair deletion near the N terminus of the E1A coding region [20]) as well as the E4 17-kDa mutation from *dl356*. HeLa or S49 cells were infected at a multiplicity of 100 PFU per cell, and cytoplasmic RNA and nuclear extracts were prepared as before. A high multiplicity of infection was used in this experiment since it has been shown previously that early mRNAs, such as the E4 species, are expressed independently of E1A products under these conditions (47). DNA band shift analysis (Fig. 11A; data not shown) of extracts prepared from both cell types confirmed that E1A proteins were not required for the formation of the infection-specific form of E2F, as previously reported (18). Likewise, analysis of E1A cytoplasmic RNA levels by RNase protection showed no difference between *dl343*- and *dl343/356*-infected cells (data not shown). The different cell lines did, however, yield different results when E2 early RNA was assayed. Whereas the HeLa cells exhibited little or no difference in E2 early mRNA accumulation (data not shown), S49 cells infected with the double mutant contained fourfold less E2 early RNA than cells infected with the E1A-negative virus *dl343* (Fig. 11B). There was no difference in the accumulation of E3 or E2 late mRNA (data not shown).

Thus, the E4 17-kDa protein is necessary for maximal accumulation of E2 early mRNAs in some cell types, and it can enhance E2 mRNA accumulation in the absence of E1A proteins. This suggests that the E4 17-kDa protein-mediated activation of the E2 early promoter, presumably through the activation of cellular E2F, can function as a separate com-

FIG. 8. RNase protection analysis of E2 early RNAs that accumulate after infection of HeLa with *wt300* (A) or *dl356* (B). Cytoplasmic RNA was prepared from the harvested cells described in the legend to Fig. 2 and analyzed as described in the legend to Fig. 7 by using a ^{32}P -labeled RNA probe specific for E2 early RNAs. Bands corresponding to two closely spaced start sites are designated +1, and those corresponding to a minor upstream start site are designated -26. Abbreviations: M, mock-infected cells; hpi, hours post-infection.

ponent of E2 early activation distinct from E1A transactivation.

DISCUSSION

Our results lead to several conclusions. First, the adenovirus E4 17-kDa protein is required to produce the infection-specific form of E2F (Fig. 2; Table 1). A requirement for an E4 gene product in the induction of infection-specific E2F activity is in agreement with results of recent work (1, 16, 45); this study serves to extend those results by identifying the specific E4 protein involved. Second, the E4 17-kDa protein acts directly to alter the DNA-binding properties of E2F by entering a complex with E2F (Fig. 3 to 6). Third, the 17-kDa protein is required for maximal accumulation of E2 early RNA during an adenovirus infection in certain cell types (Fig. 10; data not shown), suggesting that its interaction with E2F is a productive one that can lead to a higher level of transcription from a promoter that contains E2F sites. Finally, the 17-kDa protein can act as a viral transac-

FIG. 9. RNase protection analysis of E2 late RNAs that accumulate after infection of HeLa cells with *wt300* (A) or *dl356* (B). Cytoplasmic RNA was prepared from the harvested cells described in the legend to Fig. 2 and analyzed as described in the legend to Fig. 7 by using a ^{32}P -labeled RNA probe specific for E2 late RNAs. Bands corresponding to the major start sites are designated +1. Abbreviations: M, mock-infected cells; hpi, hours postinfection.

tivator even in the absence of E1A proteins (Fig. 11), suggesting that infection-specific E2F can constitute an E1A-independent mechanism by which adenovirus can activate its E2 early promoter.

Our conclusion that E1A function is not required for induction of infection-specific E2F activity is at odds with a series of earlier reports (2, 30, 31, 44, 52, 53) which suggested that the induction was E1A dependent. In general, these earlier studies could not detect E2F in uninfected cells because of the assay conditions used and could not detect infection-specific E2F in cells infected with mutant viruses lacking E1A functions since it appears only after a delay (16). Our results with the viral double mutant defective in both E1A and E4 17-kDa proteins (Fig. 11) lend further support to our earlier conclusion (16) that an E4 protein is the only viral gene product absolutely required for the production of the infection-specific form of E2F. The E4 17-kDa protein can act in an E1A-independent manner to stimulate cytoplasmic mRNA accumulation.

Given the strong evidence that E1A is unnecessary for the production of infection-specific E2F, we cannot account for

FIG. 10. DNA band shift analysis of infection-specific E2F activity and RNase protection analysis of E2 early RNAs that accumulate after infection of S49 cells with *wt300* or *dl356*. Infected cells were harvested at the indicated times (hours) postinfection (hpi). Nuclear extracts were prepared and assayed by DNA band shift analysis (A) for infection-specific E2F (E2F-Ad) by using a ^{32}P -labeled double-site probe DNA. Cytoplasmic RNA was prepared and assayed by RNase protection (B) for E2 early mRNAs (+1).

the recent results reported by Babiss (1), who studied E2F in rat CREF cells transformed with a virus carrying a defect in the E1A gene that leads to a cold-sensitive phenotype for transformation. At the nonpermissive temperature (32°C), the cells reverted to an apparently untransformed phenotype, while E4 expression was inferred to be unaffected. Curiously, the infection-specific form of E2F was not detected at 32°C , leading to the conclusion that E1A protein was necessary in addition to an E4 gene product to generate the infection-specific form of E2F. Perhaps the key E4 17-kDa protein was no longer produced at 32°C , even though some E4 mRNAs are still synthesized. It would decay rapidly relative to the time span of the experiment, since the protein has a half-life of about 4 h (6). Whatever the explanation for the temperature shift experiment, we believe that the role of E1A in the production of infection-specific E2F during lytic infection must be to a great extent, if not entirely, indirect. That is, E1A products serve to activate the expression of the E4 17-kDa protein, which, in turn, acts directly to create the infection-specific form of E2F.

Even though E1A products are not essential for induction of infection-specific E2F (16), a small proportion of the total amount of E1A protein was found in the same column fraction as the activating E4 17-kDa protein (Fig. 5D). The cofractionation may, of course, be entirely coincidental, but it raises the possibility that the E1A proteins interact with the E4 17-kDa protein and thereby play a role in the function of infection-specific E2F activity once induced. We have consistently observed a modest increase in the total level of E2F activity extracted from wild-type virus-infected cells (Fig. 2A) (16) and, to a lesser extent, from cells infected with mutant viruses unable to express the E4 17-kDa protein-coding region (Fig. 2B) (16), but not from cells infected with

FIG. 11. DNA band shift analysis of infection-specific E2F activity and RNase protection analysis of E2 early RNAs that accumulate after infection of S49 cells with *dl343* (E1A mutant) or *dl343/356* (E1A and E4 17-kDa double mutant). Infected cells were harvested at the indicated times (hours) postinfection (hpi). Nuclear extracts were prepared and assayed by DNA band shift analysis (A) for infection-specific E2F (E2F-Ad) by using a ^{32}P -labeled double-site probe DNA. Cytoplasmic RNA was prepared and assayed by RNase protection (B) for E2 early mRNAs (+1).

mutants unable to produce E1A proteins (16). These differences could simply reflect changes in the efficiency with which E2F is extracted from nuclei as parameters of the infection are altered. Nevertheless, we cannot rule out the possibility that E1A proteins influence E2F activity levels (transcriptional induction of the E2F gene?) independently of the E4 17-kDa protein-induced conversion of E2F to the infection-specific form.

We cannot explain the discrepancy in the nature of the *in vitro* activation of E2F between our results and those reported by Bagchi et al. (2). The activation that we observed did not possess characteristics predicted for the reaction described by Bagchi et al. (2), i.e., an E1A-dependent phosphorylation of E2F. We observed no ATP dependence and no requirement for the hydrolysis of ATP (Fig. 4C). We were also unable to reproduce the alkaline phosphatase-dependent inactivation of E2F-binding activity (data not shown) reported by Bagchi et al. (2). Further, in the *in vitro* activation process studied here, the amount of activated E2F observed could be linearly reduced by titrating down the amount of active fraction, suggesting that the activity might function stoichiometrically. Since the 17-kDa protein copurified with the ability to activate E2F through a second high-performance liquid chromatography column (Fig. 5B and C) and the 17-kDa polypeptide was present in the E2F-Ad complex (Fig. 6), it seems very likely that the E4 gene product is responsible for the induction of cooperative DNA binding by E2F.

Do the multiple E2F-specific complexes observed on a single-site probe DNA (E2F-a through E2F-e; Fig. 2A and B) contain functionally different forms of E2F? The band shift analyses with the MAbs against the E4 17-kDa protein have provided some insight into this question. Only the E2F-e form of E2F is recognized by the antibody. Although at the times tested in Fig. 2 both bands E2F-c and E2F-e are visible, at later times postinfection the E2F-c band is no longer detectable (16, 18). One possibility is that E2F-c is converted into E2F-e by the addition of the E4 17-kDa protein. However, this has not been demonstrated directly by adding pure E4 17-kDa protein to E2F from uninfected cells. It is apparent, however, that under the assay conditions used, only a subset of cellular E2F interacts with the E4 17-kDa protein in adenovirus-infected cells. In fact, we believe that the E4 17-kDa protein is produced in excess of the E2F that is able to bind it. Even though the bulk of the E4 17-kDa protein copurifies with the infection-specific form of E2F over the first cation-exchange column (Fig. 3), a small amount is present in the FT of this column. Our data support the interpretation that it is this E4 17-kDa protein in the FT that activates E2F to cooperative binding. The physical modifications that give rise to the different E2F species (E2F-a through E2F-d) are not clear. We do not think that the complexes result from different phosphorylation states of the factor, since treatment with phosphatases or protein kinases does not affect the relative proportions of the forms (Hardy et al., unpublished). Other obvious possibilities include a posttranslational modification other than phosphorylation, or the binding of one or more cellular components. Alternatively, the forms may represent products of different genes or possibly the product of a differentially spliced gene, one form of which has the ability to interact with the E4 17-kDa protein.

The stimulation of E2 early RNA accumulation by the E4 17-kDa protein (Figs. 10 and 11) is consistent with the observation that adenovirus E4 expression can be detected before E2 transcription in adenovirus-infected cells (39) and

the finding that the entire E4 gene could transactivate the E2 early promoter in a transient-expression assay (13). At present we do not understand the apparent cell type specificity of the ability of the E4 17-kDa protein to stimulate the accumulation of E2 early RNA. It is possible that HeLa cells, in contrast to S49 cells, have relatively high endogenous levels of cellular components (factors) that allow E1A products to transactivate at high efficiency, obviating the need for an E4 contribution to the activation. Moreover, because HeLa cells, but not S49 cells, contain endogenous human papillomavirus E7 protein which is able to transactivate the adenovirus E2 early promoter (41), the basal-level of E2 early expression may be higher in HeLa cells, making the contribution of E4 17-kDa protein more difficult to detect. Also, we do not fully understand the differences between our results and those of Reichel et al. (45), who observed a 10-fold reduction in E2 RNA levels in HeLa cells infected with a virus lacking the entire E4 region. The differences may result from variability between HeLa cell lines, or may be a result of different growth conditions. Further, Reichel et al. (45) used a mutant virus that lacked the entire E4 region, whereas we studied a mutant lacking only a portion of the E4 17-kDa coding region. Conceivably, another E4 product, present in our experiments and missing in the analysis performed by Reichel et al. (45), can influence E2 expression. Although we are confident that the E4 17-kDa protein is the only product necessary for induction of infection-specific E2F, we cannot rule out the possibility that another E4-product is able to activate E2 early expression by some other pathway.

Why does the presence of the E4 17-kDa protein in a complex with E2F stimulate the transcription of the E2 early gene? Perhaps it is supplying an activating domain to E2F or is able to bind part of the transcriptional apparatus and facilitate the formation of a stable transcription complex. Certainly, infection-specific E2F forms a much more stable complex on a synthetic oligonucleotide containing E2 early promoter sequences in an *in vitro* binding assay compared with E2F isolated from uninfected cells.

The ability of the E4 ORF6/7 gene product to stimulate the accumulation of E2 early mRNA in the absence of E1A products (i.e., *dl343*-infected S49 cells accumulated more E2 early RNA than did *dl343/356*-infected cells [Fig. 11]) indicates that adenovirus has evolved multiple, distinct pathways by which it can activate its E2 early promoter. So far, we have evidence for two such pathways: one is E1A dependent, and the other is E4 dependent. Both pathways are operative in wild-type virus-infected cells; *dl343*-infected cells contain only the E4-dependent pathway, and *dl343/356*-infected cells contain neither pathway. Perhaps the residual accumulation of E2 early RNA in *dl343/356*-infected cells (Fig. 11) results from an as yet undiscovered activation pathway. Alternatively, it could represent basal-level transcription due to cellular factors that include unmodified E2F activity.

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ADDENDUM

While this paper was under review, Huang and Hearing (26) reported that a peptide-specific antibody directed against the E4 17-kDa protein reduced the electrophoretic mobility of the complex formed between infection-specific E2F and DNA. The same antibody was able to clear infection-specific E2F DNA-binding activity from a nuclear extract. These results are in complete agreement with the experiments we report in Fig. 6 in which E4 17-kDa protein-specific monoclonal antibodies were used.

LITERATURE CITED

- Babiss, L. E. 1989. 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. *J. Virol.* **63**:2709-2717.
- Bagchi, S., P. Raychaudhuri, and J. R. Nevins. 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. *Proc. Natl. Acad. Sci. USA* **86**:4352-4356.
- Berk, A. J. 1986. Adenovirus promoters and E1A transactivation. *Annu. Rev. Genet.* **20**:45-79.
- Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp. 1979. A pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. *Cell* **17**:935-944.
- Bridge, E., and G. Ketner. 1989. Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* **63**:631-638.
- Cutt, J. R., T. Shenk, and P. Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **62**:543-552.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
- Downey, J. F., D. T. Rowe, S. Bacchetti, F. L. Graham, and S. T. Bayley. 1983. Mapping of a 14,000-dalton antigen to early region 4 of the human adenovirus 5 genome. *J. Virol.* **45**:514-523.
- Engel, D. A., S. Hardy, and T. Shenk. 1988. cAMP acts in synergy with E1A protein to activate transcription of the adenovirus early genes E4 and E1A. *Genes Dev.* **2**:1517-1528.
- Farrell, P. J., R. J. Broeze, and P. Lengyel. 1979. Accumulations of an mRNA and protein in interferon-treated Ehrlich ascites tumor cells. *Nature (London)* **279**:523-525.
- Flint, J., and T. Shenk. 1989. Adenovirus E1A protein: paradigm viral transactivator. *Annu. Rev. Genet.* **23**:141-161.
- Freyer, G. A., Y. Katoh, and R. J. Roberts. 1984. Characterization of the major mRNAs from adenovirus 2 early region 4 by cDNA cloning and sequencing. *Nucleic Acids Res.* **12**:3503-3519.
- Goding, C., P. Jalinot, D. Zajchowski, H. Boeuf, and C. Kedinger. 1985. Sequence-specific *trans*-activation of the adenovirus E1A early promoter by the viral E1V transcription unit. *EMBO J.* **4**:1523-1528.
- Graham, F. L., J. Smiley, W. C. Russell, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59-72.
- Halbert, D. N., J. R. Cutt, and T. Shenk. 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* **56**:250-257.
- Hardy, S., D. A. Engel, and T. Shenk. 1989. An adenovirus early region 4 gene product is required for induction of the infection specific form of cellular E2F activity. *Genes Dev.* **3**:1062-1074.
- Hardy, S., and T. Shenk. 1988. Adenoviral control regions activated by E1A and the cAMP response element bind the same factor. *Proc. Natl. Acad. Sci. USA* **85**:4171-4175.
- Hardy, S., and T. Shenk. 1989. E2F from adenovirus-infected cells binds cooperatively to DNA containing two properly oriented and spaced recognition sites. *Mol. Cell. Biol.* **9**:4495-4506.
- Harlow, E., B. R. Franza, and C. Schley. 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early regional 1A products. *J. Virol.* **55**:533-546.
- Hearing, P., and T. Shenk. 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. *Cell* **33**:695-703.
- Hearing, P., and T. Shenk. 1985. Sequence-independent auto-regulation of the adenovirus type 5 E1A transcription unit. *Mol. Cell. Biol.* **5**:3214-3221.
- Hemström, C., K. Nordqvist, U. Pettersson, and A. Virtanen. 1988. Gene product of regional E4 of adenovirus type 5 modulates accumulation of certain viral polypeptides. *J. Virol.* **62**:3258-3264.
- Herrmann, C. H., C. V. Dery, and M. B. Mathews. 1987. Transactivation of host and viral genes by the adenovirus E1B 19K tumor antigen. *Oncogene* **2**:25-35.
- Horibata, K., and A. W. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell Res.* **60**:61-77.
- Huang, M., and P. Hearing. 1989. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J. Virol.* **63**:2605-2615.
- Huang, M.-M., and P. Hearing. 1989. The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA-binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev.* **3**:1699-1710.
- Jones, N., and T. Shenk. 1978. Isolation of deletion and substitution mutants of adenovirus type 5. *Cell* **13**:181-188.
- Jones, N., and T. Shenk. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* **76**:3665-3669.
- Kadonaga, J. T., and R. Tjian. 1986. Affinity purification of sequence-specific DNA-binding proteins. *Proc. Natl. Acad. Sci. USA* **83**:5889-5893.
- Kovesdi, I., R. Reichel, and J. R. Nevins. 1986. Identification of a cellular transcription factor involved in E1A trans-activation. *Cell* **45**:219-228.
- Kovesdi, I., R. Reichel, and J. R. Nevins. 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proc. Natl. Acad. Sci. USA* **84**:2180-2184.
- Kristie, T. M., and B. Roizman. 1986. DNA-binding site of major regulatory protein $\alpha 4$ specifically associated with promoter-regulatory domains of α genes of herpes simplex virus type 1. *Proc. Natl. Acad. Sci. USA* **83**:4700-4704.
- Matsuo, T., S. Hashimoto, W. S. M. Wold, J. Symington, A. Rankin, and M. Green. 1982. Identification of adenovirus 2 early region 4 polypeptides by *in vitro* translation and tryptic peptide map analysis. *J. Virol.* **41**:334-339.
- Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035-7056.
- Müller, U., M. P. Roberts, D. A. Engel, W. Doerfler, and T. Shenk. 1989. Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. *Genes Dev.* **3**:1991-2002.
- Nevins, J. R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* **26**:213-220.
- Nevins, J. R. 1986. Control of cellular and viral transcription during adenovirus infection. *Crit. Rev. Biochem.* **19**:307-322.
- Nevins, J. R. 1987. Regulation of early adenovirus gene expression. *Microbiol. Rev.* **51**:419-430.
- Nevins, J. R., H. S. Ginsberg, J. M. Blanchard, M. C. Wilson, and J. E. Darnell. 1979. Regulation of the primary expression of

- the early adenovirus transcription units. *J. Virol.* **32**:727–733.
40. Nevins, J. R., P. Raychaudhuri, A. S. Yee, R. J. Rooney, I. Kovesdi, and R. Reichel. 1988. Transactivation by the adenovirus E1A gene. *Biochem. Cell Biol.* **66**:578–583.
 41. Phelps, W. C., C. L. Yee, K. Münger, and P. M. Howley. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* **53**:539–547.
 42. Rauscher, F. J., III, L. C. Sambucetti, T. Curran, R. J. Distel, and B. M. Spiegelman. 1988. Common DNA binding site for *Fos* protein complexes and transcription factor AP-1. *Cell* **52**:471–480.
 43. Rauscher, F. J., III, P. J. Voulalas, B. R. Franza, Jr., and T. Curran. 1988. *Fos* and *Jun* bind cooperatively to the AP-1 site: reconstitution *in vitro*. *Genes Dev.* **2**:1687–1699.
 44. Reichel, R., I. Kovesdi, and J. R. Nevins. 1988. Activation of a preexisting cellular factor as a basis for adenovirus E1A-mediated transcription control. *Proc. Natl. Acad. Sci. USA* **85**:387–390.
 45. Reichel, R., S. D. Neill, I. Kovesdi, M. C. Simon, P. Raychaudhuri, and J. R. Nevins. 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for *trans*-activation of E2 transcription and for E2F activation. *J. Virol.* **63**:3643–3650.
 46. Sarnow, P., P. Hearing, C. W. Anderson, D. N. Halbert, T. Shenk, and A. J. Levine. 1984. Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J. Virol.* **49**:692–700.
 47. Shenk, T., N. Jones, W. Colby, and D. Fowlkes. 1979. Functional analysis of adenovirus-5 host-range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harbor Symp. Quant. Biol. **44**:367–375.
 48. Tigges, M. A., and H. J. Raskas. 1984. Splice junctions in adenovirus 2 early region 4 mRNAs: multiple splice sites produce 18 to 24 RNAs. *J. Virol.* **50**:106–117.
 49. Venkatesh, L. K., and G. Chinnadurai. 1987. Activation of the adenovirus 2 protein IX promoter by DNA replication in a transient expression assay. *Nucleic Acids Res.* **15**:2235–2250.
 50. Weinberg, D. H., and G. Ketner. 1983. A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. *Proc. Natl. Acad. Sci. USA* **80**:5383–5386.
 51. White, E., B. Faha, and B. Stillman. 1986. Regulation of adenovirus gene expression in human WI38 cells by an E1B-encoded tumor antigen. *Mol. Cell. Biol.* **6**:3763–3773.
 52. Yee, A. S., P. Raychaudhuri, L. Jakoi, and J. R. Nevins. 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Mol. Cell. Biol.* **9**:578–585.
 53. Yee, A. S., R. Reichel, I. Kovesdi, and J. R. Nevins. 1987. Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. *EMBO J.* **6**:2061–2068.
 54. Yoshida, K., L. Venkatesh, M. Kuppuswamy, and G. Chinnadurai. 1987. Adenovirus transforming 19kd T antigen has an enhancer-dependent trans-activation function and relieves enhancer repression mediated by viral and cellular genes. *Genes Dev.* **1**:645–658.
 55. Zajchowski, D. A., P. Jalinot, and C. Kedinger. 1988. E1A-mediated stimulation of the adenovirus EIII promoter involves an enhancer element within the nearby EIIa promoter. *J. Virol.* **62**:1762–1767.