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The C-terminal 70 amino acids of the adenovirus E4-ORF6/7 protein are essential and sufficient for E2F complex formation

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ABSTRACT

E2F is a cellular transcription factor that binds to the adenovirus (Ad) E1A enhancer and E2aE promoter regions, to the cellular c-myc P2 and dihydrofolate reductase promoters, and to other viral and cellular regulatory regions. The binding activity of E2F to the Ad E2aE promoter is dramatically increased during an adenovirus infection (termed E2F induction). E2F induction is dependent on the expression of the 150 amino acid E4-ORF6/7 protein which forms a direct, physical complex with E2F to mediate the cooperative and stable binding of E2F to inverted sites in the E2aE promoter. Using *in vitro* DNA binding assays to measure the formation of the infection-specific complexes, we have defined the minimal domain of the E4-ORF6/7 protein, the C-terminal 70 amino acids, required to complex with E2F and stabilize its binding at the E2aE promoter. The ability of mutant E4-ORF6/7 proteins to form the stable E2F-E2aE promoter complex *in vitro* correlated well with their ability to trans-activate E2 transcription *in vivo*. These observations support a model in which the E4-ORF6/7 protein binds to E2F to induce the cooperative binding of two E2F molecules to the E2aE promoter thereby activating E2 transcription.

INTRODUCTION

The temporal regulation of adenovirus early gene expression has been extensively studied and the Ad early class II promoters, in utilizing both cellular and viral specific transcription factors, provide an excellent system to investigate the regulation of transcription initiation by the cellular RNA pol II. The products of Ad early regions E1A and E4 regulate viral gene expression. The E1A proteins trans-activate the Ad early region promoters and a variety of other viral and cellular promoter regions apparently by multiple mechanisms (for review see refs. 1–3). Recent studies have demonstrated a role for an E4 gene product, the E4-ORF6/7 protein, that functions to physically mediate the cooperative binding of E2F to two sites at the E2aE promoter (4–6). The E2aE promoter is complex and contains two overlapping promoters directing the initiation of transcription from a major start site at +1 and a minor start site at −26 (7, 8). Two TATA box-like elements, one each about 30 base pairs upstream of their respective start sites, direct efficient transcription initiation (7, 9). Two other cellular transcription factors bind to the E2aE promoter region. An ATF binding site is located between −68 and −77 (10–12), and two E2F binding sites, inverted with respect to each other, are located between −35 and −68 (13, 14). Each of these elements contributes to basal E2aE expression and each has been implicated in the ability of E1A to trans-activate the E2aE promoter (8, 9, 15–19).

E2F is a 54 kDa cellular transcription factor that was initially identified in Ad-infected cells by its ability to bind to the E2aE promoter region (13, 14, 20). E2F also binds to two sites in the Ad E1A enhancer, to the cellular c-myc P2 and dihydrofolate reductase promoters, and to other viral and cellular regulatory regions. These binding sites are important for both basal and Ad-induced expression *in vivo* and a single E2F site has been shown to confer E1A inducibility to a heterologous promoter (21–25). The binding activity of E2F to the E2aE promoter is dramatically increased during the early phase of an adenovirus infection (4, 5, 12–14, 26, 27) and this induction of E2F binding activity correlates with increased E2aE transcription *in vivo* (5, 6, 18, 20, 21, 28). The Ad infection-specific form of E2F, but not E2F from uninfected cells, binds cooperatively to two sites in the E2aE promoter (27, 29, 30). The generation of the infection-specific form of E2F requires the expression of the Ad E4-ORF6/7 protein (4–6).

The Ad2 E4-ORF6/7 product is a 150 amino acid protein generated from the fusion of E4 ORFs 6 and 7 by mRNA splicing (Figure 1; 31, 32). The involvement of the E4-ORF6/7 protein in a direct physical complex with E2F at the E2aE promoter has been shown using E4 mutants in viral infections, utilizing peptide-specific antisera to the E4-ORF6/7 protein in gel mobility shift assays, and using *in vitro* E2F induction assays with *in vitro* produced E4-ORF6/7 protein (4–6). The E4-ORF6/7 protein also forms a complex with E2F in the absence of DNA binding (4, 5). The stoichiometry of the complex in solution versus that formed on the E2aE promoter has yet to be determined.

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The E4-ORF6/7 protein forms a complex with E2F in solution, with E2F bound to a single E2F site, and mediates the stable binding of E2F at the E2aE promoter double site. As a result, we have postulated that E4-ORF6/7 protein functions to bind to E2F and physically mediate its cooperative binding at the E2aE promoter. This model predicts that the E4-ORF6/7 protein may have domains for both direct interaction with E2F and for dimerization. Analysis of a viral frame-shift mutant has shown that the C-terminal region of the E4-ORF6/7 protein is important for E2F induction (4–6). To investigate the structural aspects of the E4-ORF6/7 protein involved in the induction of E2F binding activity, we have generated a series of mutants in the E4-ORF6/7 coding sequence and assayed the formation of the E4-ORF6/7-E2F complex *in vitro*. This analysis has allowed us to define the minimal domain, the C-terminal 70 amino acids, that is important for binding to E2F and inducing cooperative binding of E2F at the E2aE promoter *in vitro*. The same sequences are required for trans-activation of the E2 promoter *in vivo*.

MATERIALS AND METHODS

Wild type and mutant E4-ORF6/7 plasmids, probes, and oligonucleotides

The wild type plasmid, pBS-E4-6/7, was constructed using a cDNA corresponding to Ad2 E4-ORF6/7 (clone 101, ref. 31, kindly provided by M. Perricaudet). The cDNA was excised with Pst I and inserted into the Pst I site of pBS-KS (Stratagene) in an orientation allowing transcription with T7 RNA polymerase. Each of the mutant proteins was constructed according to standard recombinant DNA techniques. The N-terminally deleted proteins were fused at amino acid 2 using the Tth111-I site and were named according to the second restriction site used to create the deletion. The internally deleted constructs were named according to the two restriction sites used to generate the deletion endpoints. 3′ IN was an insertion of an 8 bp BglII linker into a repaired BspMI site. 3′ FS was an insertion of 3 BglII 8-mers into the same BspMI site. 3′ TN was a truncation made by run off transcription of a BglII digest of 3′ IN. Restriction site overhangs were made flush with either Klenow polymerase, or Mung bean nuclease where necessary, to maintain the correct E4-ORF6/7 reading frame. The plasmid, p*dl*356*, was constructed by digestion of pBS-E4-6/7 with XmaI and filling in the ends with Klenow polymerase. This 4 bp insertion generates the same frame-shift mutation as the viral deletion mutant *dl*356 (32, 33). All of the mutant constructs were confirmed by DNA sequencing except Mlu/Pvu and Pvu/Sma which were confirmed by protein expression.

The E2F single site probe is a 51 bp fragment that contains Ad nt 270 to 293 from the E1A enhancer and flanking sequences from a pUC9 vector. The E2aE promoter double site probe is a 54 bp fragment that contains E2 sequences from −30 to −73 and flanking pBS vector sequences. The sequence of the single site probe is: 5′-AATTCCCCCATTTCGCGGGAAAAC-TGAATCCTCGA-3′/5′-TCGAGGATTCAGTTTTCC CGCGAAAATGGGGGAATT-3′. The sequence of the double site probe is: 5′-AATTCGTAGTTTCGCGCTTAAATTTG-AGAAAGGGCGCGAAACT AGTCCCGG-3′/5′-CCGGGAC-TAGTTTCGCGCCCTTTCTCAAATTTAAGCGCGAAAAC-TACGAATT-3′. The E2F sites are underlined. ^{32}P-labeled probes were generated using ^{32}P-alpha-dATP and Klenow polymerase to yield probes with a specific activity of 5000–10,000 cpm/fmole. Probe DNAs were isolated by electroelution from a polyacrylamide gel and precipitated from ethanol three times.

In vitro transcription and translation, and antibodies

1–2 μg of linearized wild type and mutant E4-ORF6/7 plasmids were transcribed *in vitro* using T7 RNA polymerase according to the manufacturer's instructions (Promega). RNAs produced by this method were purified by phenol/chloroform extraction and quantitated visually on a 1.5% agarose gel run in 1X TAE buffer (40 mM Tris-Acetate, 2 mM EDTA) against a standard concentration of cellular rRNA. 1–2 μg of E4-ORF6/7 RNAs were then used to program either rabbit reticulocyte or wheat germ translation extracts in the presence of ^{35}S-methionine according to the manufacturer's instructions (Promega). Incorporation of ^{35}S-methionine was measured by TCA precipitation to quantitate the extent of translation relative to translation in the absence of exogenously added RNA. Equal cpms of the wild type and mutant products were subjected to SDS-PAGE, flourographed, and visualized by autoradiography. The E4-ORF6/7 peptide-specific antibodies R3 and R7 have been described (4, 32).

In vitro DNA binding assays

A binding assay (25 μl) contained 20 mM HEPES (pH7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 0.08 mM EDTA, 10% glycerol, 0.1% NP-40, 2 μg of sonicated salmon sperm DNA as non-specific competitor, and 5–10 μg of nuclear or cytoplasmic extract. This mixture was pre-incubated for 10 minutes at room temperature, followed by addition of ^{32}P-labeled single site or double site probe (10,000 cpm, 1–2 fmoles DNA). Incubation was continued for 1 hour at room temperature, and could be continued overnight at 4°C. The protein-DNA complexes were resolved on a 4% 30:1 (acryl:bis) polyacrylamide gel run in 0.5× TBE (25mM Tris 8.3, 25 mM boric acid, 0.5 mM EDTA) at 4°C and at 10V/cm. The gel was dried and subjected to autoradiography. The cytoplasmic extract of uninfected HeLa cells is a rich source of E2F separated from many DNA binding proteins; cytoplasmic extracts gave the cleanest mobility shift patterns and were used for most *in vitro* E2F binding reactions. Identical results were obtained using nuclear extracts from uninfected HeLa cells. When the E4-ORF6/7 mutants were assayed, equimolar amounts of mutant

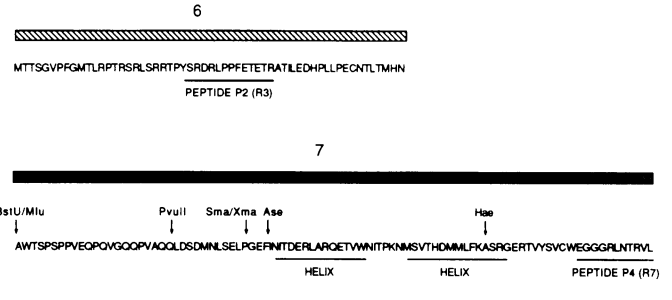

Figure 1. E4-ORF6/7 protein sequence. The entire E4-ORF6/7 protein coding sequence is shown represented by the single letter amino acid code. Open reading frames 6 and 7 are indicated by striped and stippled boxes, respectively, above the sequence. Two putative alpha-helical regions described in the text are indicated by bars below the amino acid sequence. Restriction nuclease cleavage sites used to construct certain important mutants are shown. Peptides P2 and P4 used to raise antisera R3 and R7, respectively, are indicated below the sequence by bars.

proteins (based on TCA precipitations) were added to each binding reaction. Total protein content was kept constant by supplementing reactions with unprogrammed translation extract.

Cells, transfections, nuclear and cytoplasmic extracts, and protein expression

Suspension cultures of HeLa cells were maintained in medium containing 7% calf serum (4). Nuclear extracts were prepared according to the procedure of Dignam et al (34). The cytoplasmic fraction was adjusted to 100 mM KCl and clarified by centrifugation at 100,000×g for 60 minutes. The nuclear and cytoplasmic fractions were dialysed against buffer D (20mM HEPES (pH 7.5), 100mM KCl, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After dialysis, the extracts were clarified by centrifugation at 25,000×g for 20 minutes. Ad-infected cell extracts were harvested 6 to 12 hours post-infection with Ad5 *wt*300 at an multiplicity of infection of 500 particles/cell, as described above.

Monolayer HepG2 cells (ATCC) and HeLa cells were grown in medium containing 10% fetal calf serum or 10% calf seurm, respectively. Cell monolayers were transfected by the calcium phosphate precipitation method (35). Briefly, the cells were split the day before transfection and transfected the following day with 1 μg of a target plasmid (pE2-CAT; 16), 1 μg of effector plasmid (pCMV-E4-6/7, discussed below), and 18 μg of salmon sperm carrier DNA per 100 mm dish. The cells were incubated overnight with the precipitate, and then washed with Tris buffered saline solution (TBS), TBS with 3 mM EGTA, and TBS. Fresh medium was added, and the cells were harvested 24 hours later and assayed for chloramphenicol acetyl transferase (CAT) activity as described previously (36). The results were quantitated by excising the acetylated and non-acetylated forms of ^{14}C-chloramphenicol from the thin layer chromatogram and counting by liquid scintillation spectroscopy. For the analysis of *in vivo* protein expression, transfected cells were incubated in methionine-free medium for 30 minutes, and then pulse-labelled with ^{35}S-methionine (250μCi/ml) for 4 hours from 18−22 hours after transfection. Cellular extracts were prepared and E4-ORF6/7 proteins immunoprecipitated as previously described (32). The products were analyzed by SDS-PAGE.

RESULTS

Reconstitution of the Ad infection-specific complex *in vitro*

E2F binds to pairs of sites in both the E1A enhancer and the E2aE promoter regions. At the E1A enhancer the E2F sites are direct with respect to each other and are separated by 63 bp (13, 14); E2F in Ad-infected cell extracts does not bind cooperatively to the E1A enhancer region (27). In contrast, the two E2F sites in the E2aE promoter are inverted with respect to each other and are separated by 23 bp (21); E2F in Ad-infected cell extracts binds cooperatively and stably to this region (E2F induction; 27, 29, 30). Using a probe from the E1A enhancer region that contains a single E2F binding site and uninfected HeLa cell nuclear extract, four E2F-specific complexes are detected using a gel mobility shift assay (Figure 2, lane 1; complexes a-d in refs. 5, 26, 27). Each of these complexes are competed using

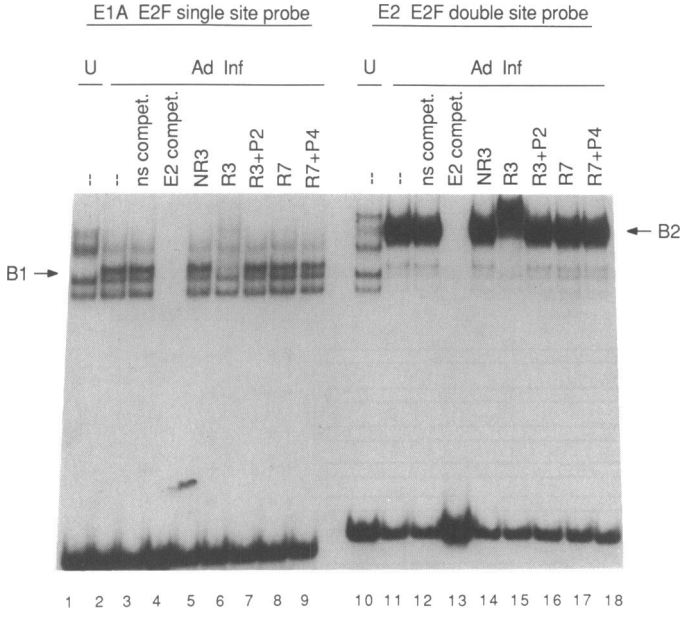

Figure 2. Binding of E2F-specific complexes to single and double E2F binding sites. A single E2F binding site fragment from the E1A enhancer (Ad5 nt 270−293, lanes 1−9) and a double E2F site fragment from the E2aE promoter (E2aE sequences from −30 to −73, lanes 10−18) were used in binding reactions containing uninfected (U) or adenovirus-infected (Ad Inf) HeLa cell nuclear extract. Competitions for E2F binding were performed using non-specific competitor DNA (lanes 3 and 12) or unlabeled E2aE promoter DNA (lanes 4 and 13) at a 100-fold molar excess relative to the probe. Subsequent to the binding reaction, the samples were incubated at 4°C for one hour with no antiserum (lanes 1−4, 10−13), pre-immune serum NR3 (lanes 5, 14), immune antiserum R3 without (lanes 6, 15) or with (lanes 7, 16) the cognate peptide P2, or immune antiserum R7 without (lanes 8, 17) or with (lanes 9, 18) the cognate peptide P4. The DNA-protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel and detected by autoradiography. The E2F-E4-ORF6/7-specific complexes are indicated by arrows (B1, single site; B2, double site).

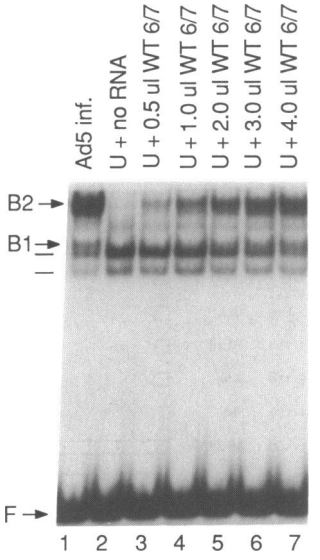

Figure 3. Reconstitution of the Ad infection-specific complexes *in vitro*. The E2F double site fragment from the E2aE promoter was used in binding reactions containing nuclear extract prepared from Ad-infected (Ad5 inf., lane 1) or uninfected (U, lanes 2−7) HeLa cells. Unprogrammed reticulocyte lysate (+ no RNA, lane 2) and increasing amounts of *in vitro* translated E4-ORF6/7 (+ WT 6/7, lanes 2−7) were added to the binding reactions. Binding reactions were performed for 1 hour at room temperature. The DNA-protein complexes were resolved on a 4% polyacrylamide gel and detected by autoradiography. The E2F-E4-ORF6/7-specific complexes are indicated by arrows (B1 and B2). E2F-specific complexes not containing the E4-ORF6/7 protein are indicated with dashes. The free, unbound probe is shown (F).

the E2aE promoter region probe (lane 4). The lower two complexes appear to contain only E2F; the doublet is possibly due to proteolysis (20). The upper two complexes likely represent E2F complexed with cellular proteins. Similar low-mobility complexes are observed in murine L cell extracts and are directly disrupted by the E1A proteins (37). With extracts prepared at early times after Ad infection, the upper complexes are reduced (as expected due to E1A expression) and a novel complex is detected (B1, lane 2; complex e in ref. 26). This complex contains the E4-ORF6/7 protein as shown by the altered mobility following the addition of a peptide-specific antiserum (R3, Figure 1) that is directed against the N-terminal region of the E4-ORF6/7 protein (lane 6); this effect is blocked by the cognate peptide (lane 7). A peptide-specific antibody directed against the C-terminal region of the E4-ORF6/7 protein (R7, Figure 1) did not recognize the E2F-E4-ORF6/7 complex (lane 8). This antibody also did not interfere with the formation of this complex if incubated with the extract prior to the binding reaction (data not shown). This result is consistent with the role of the C-terminal region of the E4-ORF6/7 protein in complex formation with E2F (see below). Similar complexes are detected using an E2F double site probe (the E2aE promoter) using uninfected HeLa cell extract (lane 10). Following Ad infection, the upper complexes are reduced and a new, major E2F-specific complex is observed (B2, lane 11), as previously described (5, 26, 27). A weak single site E4-ORF6/7-E2F complex is also detected with this probe (B1). The mobility of these new Ad-specific complexes are altered by the R3, but not R7, antiserum (lanes 15 and 17). These results recapitulate reported findings, as well as establish that the E4-ORF6/7 C-terminal antiserum (R7) does not recognize the E2F-E4-ORF6/7 complex at either a single or double binding site.

To study the involvement of the E4-ORF6/7 protein in the E2F-specific complexes, we utilized an *in vitro* system that reconstituted the infection-specific complexes using uninfected Hela cell extracts and E4-ORF6/7 protein synthesized by *in vitro* translation. The protein products produced in the translation reaction (Figure 5, lane 2) comigrated with E4-ORF6/7 proteins detected in immunoprecipitations from lysates of Ad-infected cells pulse-labelled for short periods of time, although the full-length E4-ORF6/7 protein is the major product that accumulates *in vivo* (32). While the smaller E4-ORF6/7 proteins appear to be unstable *in vivo*, these products accumulate *in vitro* (Figure 5). Figure 3 shows a gel mobility shift assay using the E2aE promoter double site probe and uninfected Hela nuclear extract incubated with E4-ORF6/7 protein synthesized *in vitro*. As increasing amounts of E4-ORF6/7 protein were titrated into the binding reaction (lanes 3–7), the infection-specific complexes (B2 and B1) were reconstituted and E2F binding activity was induced (B2). The *in vitro* reconstituted complexes were supershifted by the R3, but not R7, antiserum (data not shown). This result also demonstrates that the E4-ORF6/7 protein is the only viral protein required to produce the E2F infection-specific complex, as previously described (6).

The C-terminal 70 amino acids of the E4-ORF6/7 protein are essential and sufficient for E2F complex formation

Experiments using an adenovirus E4-ORF6/7 frame-shift mutant, *dl*356, implicated the C-terminus of the E4-ORF6/7 protein in the induction of E2F binding (4–6). To define the structural domains important for the ability of the E4-ORF6/7 protein to bind to E2F and induce cooperative binding at the E2aE promoter, we generated a series of in-frame deletion mutants within the E4-ORF6/7 coding sequence (Figures 1 and 4). These mutants were constructed by fusing the initiating ATG start codon to downstream sequences to create a set of N-terminally deleted proteins or by fusing an upstream restriction site to a downstream site to create internal deletion mutants. In addition, mutants with

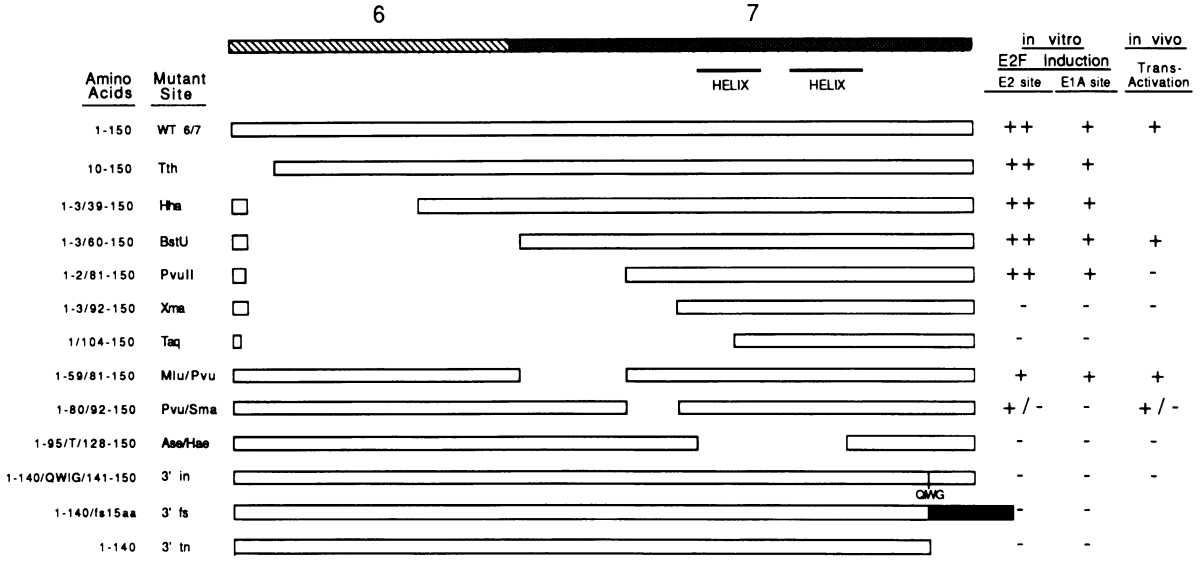

Figure 4. Schematic diagram of E4-ORF6/7 mutants and summary of their activities *in vitro* and *in vivo*. The top part of the figure shows a schematic representation of the wild-type E4-ORF6/7 coding sequence, as depicted in Figure 1. The open boxes represent the coding region remaining with each mutant protein. The amino acid numbers listed for each mutant refer to those remaining at the junction of each deletion. The names of the mutants refer to the restriction site(s) used to generate the mutant, except for the C-terminal mutations which are named according to the type of mutation constructed (insertion, frameshift, and truncation). A summary of the results from the experiments presented in Figures 6 (E2F induction, E2aE double site) and 7 (E2F binding, E1A single site), and Table 1 (E2aE trans-activation) is shown in the columns on the right.

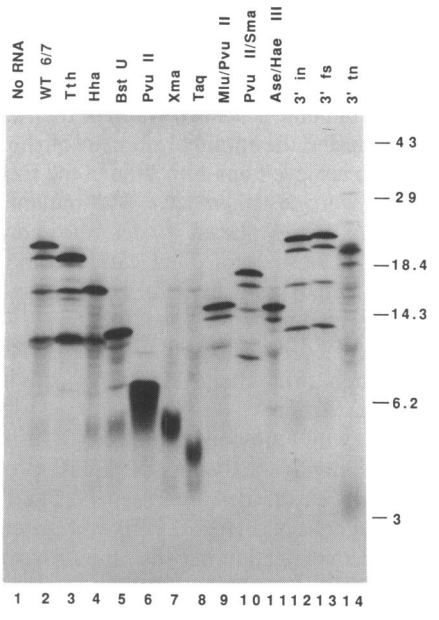

Figure 5. *In vitro* translated wild type and mutant E4-ORF6/7 proteins. Wild type and mutant E4-ORF6/7 genes were transcribed and translated *in vitro*. The ^{35}S-labelled proteins were resolved on a 20% SDS-polyacrylamide gel and the products were visualized by flourography. Equal molar TCA-precipitable counts were loaded for each sample.

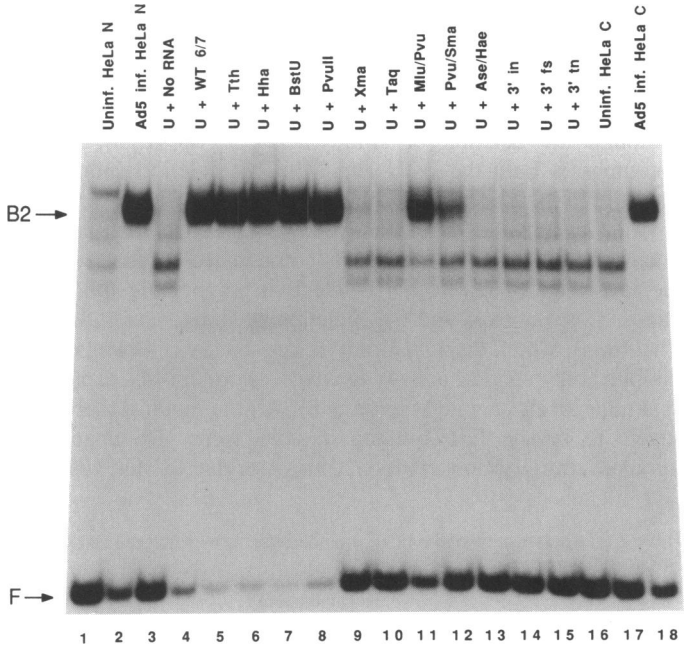

Figure 6. The C-terminal 70 amino acids of the E4-ORF6/7 protein are required for the induction of cooperative E2F binding to the E2aE promoter. The E2F double site fragment from the E2aE promoter was used in binding reactions containing nuclear (lane 1) or cytoplasmic (lanes 3–17) extract prepared from uninfected HeLa cells, or containing nuclear (lane 2) or cytoplasmic (lane 18) extract prepared from Ad-infected Hela cells. Equal molar amounts of the wild type and mutant E4-ORF6/7 proteins produced by *in vitro* translation were added to the binding reactions shown in lanes 3–16, as indicated. Binding reactions were performed overnight at 4°C. The DNA-protein complexes were resolved on a 4% polyacrylamide gel and detected by autoradiography. The induced E2F-E4-ORF6/7 complex is indicated by an arrow (B2). The free, unbound probe is shown (F).

alterations to the C-terminal ten amino acids of the E4-ORF6/7 protein, including an insertion, a frame-shift and a truncation, were generated.

The wild type and mutant E4-ORF6/7 genes were transcribed and translated *in vitro*. The ^{35}S-labelled mutant products were visualized by SDS-PAGE (Figure 5). Translation of the wild-type E4-ORF6/7 RNA produced several protein species (lane 2) which also have been detected in immunoprecipitations of Ad-infected cell lysates (32; data not shown). Immunoprecipitations of *in vitro* synthesized mutant proteins and of wild type proteins pulse-labelled *in vivo* for very short times or analyzed in pulse-chase experiments have allowed us to rule out post-translational modification of the E4-ORF6/7 protein and to assign the major species to internal initiation events (32; data not shown). The slowest migrating species in the wild type translation corresponds to the full-length 150 amino acid product. The second and fourth major species appear to correspond to initiation at the second and third in-frame methionines (Figure 1), respectively, based on size, immunoprecipitation pattern using the R3 and R7 antisera (data not shown), and the pattern of polypeptides observed with deletion mutants that remove internal ATG codons (Figures 4 and 5). The third major species appears to correspond to an initiation originating from an in-frame CTG codon based on the same criteria. The sizes of all the mutant proteins (lanes 3–14) correlated with that expected from the size of their mutations.

The ability of the E4-ORF6/7 protein to interact with E2F at both single and double sites suggested that we would be able to test the ability of the E4-ORF6/7 protein to complex with E2F (single site shift) separate from its ability to induce cooperative DNA binding (double site shift). The wild type and mutant E4-ORF6/7 proteins were first tested in the double site assay (Figure 6). The *in vitro* translated proteins were incubated with uninfected HeLa cell extract and the E2aE promoter double site probe and analyzed in a gel mobility shift assay. The wild type E4-ORF6/7 protein produced *in vitro* induced E2F binding activity to a similar extent as that seen with an extract from Ad-infected HeLa cells (Figure 6, compare lanes 2 and 4). Mutant E4-ORF6/7 proteins with N-terminal deletions extending to amino acid 80 also induced E2F binding to wild-type levels (lanes 5–8). In contrast, larger N-terminal deletions (lanes 9 and 10), or internal deletions or alterations within the C-terminal 70 amino acids (lanes 12–16) impaired or completely abolished the ability of E2F to stably bind the E2aE double site probe. The extent of formation of the B2 (induced) complex depends on the time of reaction incubation (Figure 3, 1 hour vs. Figure 6, overnight) and the amount of E4-ORF6/7 protein added (Figure 3). The results presented in Figure 6 display the reduced E2F induction by the Pvu/Sma mutant (lane 12) and complete lack of induction by the C-terminal mutants (lanes 12–17) even under conditions of extensive E2F induction by the wild type E4-ORF6/7 protein. Identical E2F induction results were obtained with the Pvu mutant and the other mutant proteins using binding reaction conditions where the extent of E2F induction was varied (data not shown). These results demonstrate that the C-terminal 70 amino acids of the E4-ORF6/7 protein are necessary and sufficient for induction of E2F binding activity to the E2aE promoter.

The ability of the wild type and mutant E4-ORF6/7 proteins to bind to E2F at a single site was next assayed (Figure 7). In this assay, the band corresponding to the E2F-E4-ORF6/7 complex appears diffuse in the reactions containing the wild type protein (lane 3) and the mutant proteins with smaller N-terminal deletions (lanes 4–15). We attribute this to the presence of

multiple species in the reaction originating from internal initiations during translation (see Figure 5). This interpretation is consistent with the fact that multiple species were not apparent using Ad-infected cell extracts (lane 1) where the predominant E4-ORF6/7 product is the full-length protein (32). Again, the wild type and the mutant forms with N-terminal deletions to amino acid 80 were capable of binding E2F and retarding the mobility of the E2F complex (lanes 3–7, 10). Any mutation in the C-terminal 70 amino acids abolished the E2F-E4-ORF6/7 interaction in the single site assay (lanes 8–9, 11–15). The Pvu/Sma mutant (lane 11), which deletes amino acid residues 81–91, had no activity in the single site assay but retained partial activity in the double site assay. This eleven amino acid deletion appears to define the N-terminal boundary of the E4-ORF6/7 polypeptide required for full activity. These experiments did not detect a mutant that could bind to E2F at a single site but not induce stable E2F binding at a double site. Thus, we are presently unable to separate the ability of the E4-ORF6/7 protein to complex with E2F from its function in inducing cooperative DNA binding. The C-terminal 70 amino acids are essential and sufficient for both of these functions.

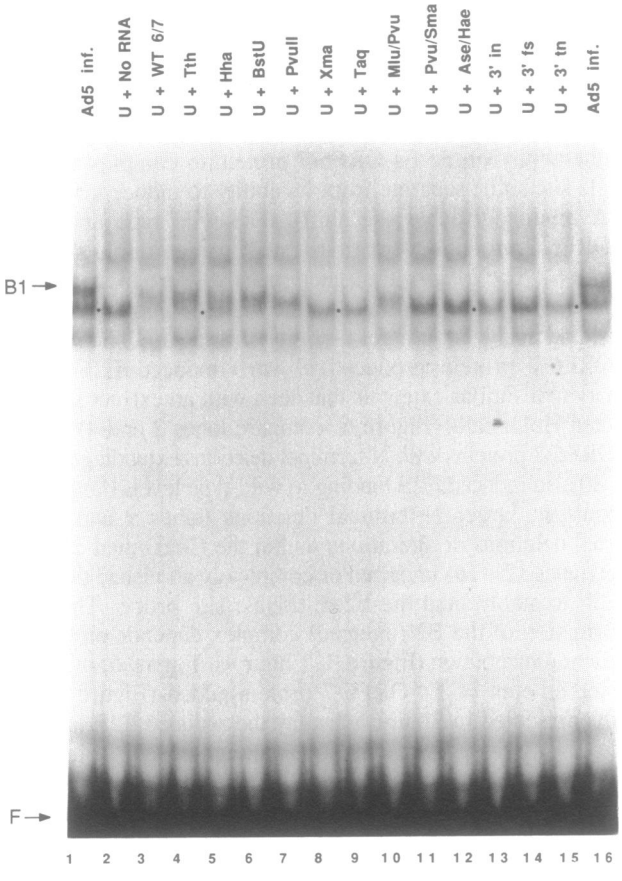

Figure 7. The C-terminal 70 amino acids of E4-ORF6/7 are required for binding to E2F at a single binding site. The E2F single site fragment from the E1A enhancer was used in binding reactions containing cytoplasmic extract prepared from Ad-infected (lane 1) or uninfected (lanes 2–16) HeLa cells. Equal molar amounts of the wild type and mutant E4-ORF6/7 proteins produced by *in vitro* translation were added to the binding reactions in lanes 2–15, as indicated. Binding reactions were incubated for 1 hour at room temperature. The DNA-protein complexes were resolved on a 4% polyacrylamide gel and detected by autoradiography. The E2F-E4-ORF6/7 complex is indicated by an arrow (B1). The free, unbound probe is shown (F). The dots between individual lanes align the E2F-DNA complexes not containing the E4-ORF6/7 protein for reference.

Trans-activation *in vivo* correlates with the ability to induce cooperative E2F binding activity *in vitro*

Previous experiments have demonstrated a role for the E4-ORF6/7 protein in the trans-activation of the E2aE promoter (6, 28, 38). To define the minimal domain of the E4-ORF6/7 protein required to achieve trans-activation of the E2aE promoter, mutant plasmids were tested *in vivo*. The mutant E4-ORF6/7 coding sequences were placed under the control of the Cytomegalovirus (CMV) immediate early promoter/enhancer (39) and cotransfected with the E2aE promoter fused to the chloramphenicol acetyltransferase gene (pE2-CAT; 16). The level of E2aE expression in the context of various E4-ORF6/7 mutants was assayed by monitoring the level of CAT activity in cellular extracts after transfection.

In agreement with previous studies (6), the wild-type E4-ORF6/7 protein trans-activated the E2aE promoter 8- to 30-fold, depending on the cell line used (Table 1). For these studies both HeLa cells and HepG2 cells were used. The HeLa cells allow a direct correlation between the *in vitro* and *in vivo* results because the *in vitro* studies were performed with HeLa cell extracts. The HepG2 cells were used because they yielded the most dramatic trans-activation. Trans-activation of the E2aE promoter by the wild type E4-ORF6/7 protein was dependent on the integrity of both E2F binding sites, as assayed using E2-CAT plasmids with linker scanning mutations in these sites (16; data not shown). In either cell line, the deletion of amino acids 4–59 (BstU) resulted in less than a two-fold reduction in trans-activation relative to the wild type, while the deletion of amino acids 60–80 (Mlu/Pvu) reduced trans-activation less than 2.5-fold (Table 1). These 2 to 3-fold reductions in trans-activation appear to reflect decreased levels of these proteins, relative to the wild type protein level, visualized following transfection of these mutant plasmids (Figure 8, compare lanes 2, 3, 7). Although the deletion of amino acids 3–80 (Pvu), which encompasses both the BstU and Mlu/Pvu deletion endpoints, allowed full induction of E2F binding activity *in vitro*, this mutant protein was not able to trans-activate the E2aE promoter *in vivo*. The interpretation of this result is complicated by the fact that we have been unable to detect the Pvu polypeptide following transfection (lanes 4 and 5). Deletion of amino acids 81–91 (Pvu/Sma), which displayed only residual activity *in vitro*, also exhibited only residual activity *in vivo*; this protein was expressed to similar levels as wild type (lane 8). All the mutants that were unable to induce E2F binding *in vitro* were also unable to stimulate transcription *in vivo* (Xma, Ase/Hae, 3′ IN, *dl*356*;

Table 1. Trans-activation of the E2aE promoter *in vivo* by wild-type and mutant E4-ORF6/7 proteins.

E4-ORF6/7 Plasmid	Fold-Induction* HeLa	HepG2
Mock	1.0	1.0
WT	8.6	30.4
BstU	6.9	16.4
Pvu	1.1	1.2
PvuΔ	1.3	1.4
Xma	1.1	1.1
Mlu/Pvu	6.6	12.1
Pvu/Sma	2.6	3.4
Ase/Hae	1.2	1.0
3′ in	1.0	1.2
dl356*	0.7	0.9

*Values are presented as fold-induction over the uninduced level obtained with pE2-CAT (16) alone and reflect the average of five independent experiments.

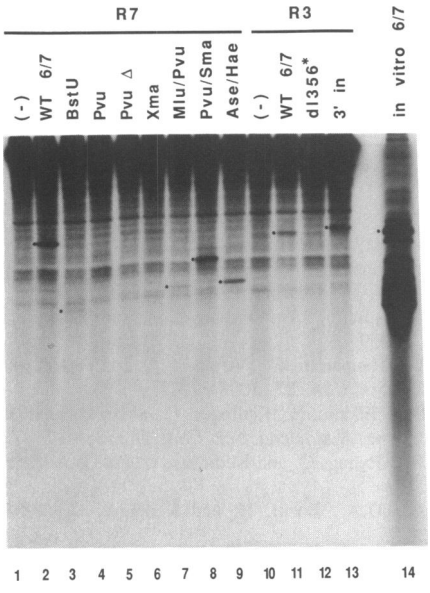

Figure 8. Wild type and mutant E4-ORF6/7 proteins produced *in vivo*. HepG2 cells were mock-transfected (−, lanes 1 and 10) or transfected with plasmids expressing the wild type and mutant E4-ORF6/7 proteins, as indicated (lanes 1−13). Pvu Δ (lane 5) represents a varient clone which lacks the GC-tail generated during preparation of the E4-ORF6/7 cDNA and contained in the leader sequences of the expressed mRNAs. This GC-tail reduces *in vitro* E4-ORF6/7 translation to a moderate extent. Cells were pulse-labelled with ^{35}S-methionine from 18−22 hrs. post-transfection. Cellular extracts were prepared and E4-ORF6/7 proteins immunoprecipitated using the immune antisera R7 (lanes 1−9) or R3 (lanes 10−13). The products were resolved on a 20% SDS polyacrylamide gel, flourographed, and visualized by autoradiography. The specific wild type and mutant proteins are indicated by dots adjacent in individual lanes. Lane 14 displays the wild type E4-ORF6/7 *in vitro* translation products.

Table 1). Of these defective mutants, only the Ase/Hae and 3'IN mutants could be detected at the protein level (lanes 9 and 13). Considering only those mutants that could be detected at the protein level, the ability of the E4-ORF6/7 protein to trans-activate the E2aE promoter *in vivo* strongly correlated with the ability of mutant proteins to induce stable E2F binding at this promoter *in vitro*.

DISCUSSION

Using an *in vitro* system to reconstitute the Ad infection-specific E2F complexes, we have begun to dissect the structure-function relationships of the E4-ORF6/7 protein with respect to its ability to interact with E2F and to induce the cooperative binding of E2F to the E2aE promoter region. The addition of *in vitro* translated E4-ORF6/7 protein to a HeLa cell extract is sufficient to induce E2F binding activity to the E2aE double site and to retard the migration of a single site DNA-E2F complex in mobility shift assays (Figures 3, 5, and 6). These results agree with previous observations using either *in vitro* translated or partially purified fractions of the E4-ORF6/7 protein (5, 6, 30). We have now extended these observations to demonstrate that reconstitution of these E2F infection-specific complexes, and thus the induction of stable and cooperative binding of E2F to its E2aE recognition sites, requires only the C-terminal 70 amino acids of the E4-ORF6/7 protein (summarized in Figure 4). This minimal domain is both essential and sufficient for full induction of E2F binding activity. All mutations to the N-terminal 80 amino acids of the E4-ORF6/7 protein had no effect on its ability to induce E2F binding activity to the E2aE double site DNA (Figure 5) or retard the single site DNA-E2F complex (Figure 6). In contrast, any mutation to the C-terminal 70 amino acids either drastically reduced or completely abolished both of these functions. Previous experiments have suggested that the C-terminus of the E4-ORF6/7 protein is important for the induction of E2F binding activity. A viral mutant, *dl*356, generates a frameshift in the E4-ORF6/7 coding sequence after amino acid 91. Extracts made from *dl*356-infected cells were unable to induce E2F binding activity (4−6).

Additionally, an antiserum specific to the C-terminal eleven amino acids of the E4-ORF6/7 protein (R7), was unable to clear E2F binding activity from an Ad-infected extract or supershift the single or double site infection-specific complexes in a mobility shift assay (4; Figure 2). An antiserum specific to the N-terminus of the E4-ORF6/7 protein (R3), however, was capable of both of these activities (Figure 2; 4, 5). As predicted from these results, our data demonstrate that an intact C-terminus of the E4-ORF6/7 protein is essential for both interaction with E2F and induction of E2F binding activity. Indeed, a deletion, a frameshift, or an insertion in the C-terminal 10 amino acids of the E4-ORF6/7 protein abolished all activity, as did a deletion of 33 amino acids from the C-terminal third of the protein (Ase/Hae).

The Pvu/Sma mutant contains an eleven amino acid internal deletion that appears to define the N-terminal border of the domain required for E2F interaction and induction. This mutant protein was not capable of retarding the single site DNA-E2F complex, but retained a residual ability to induce cooperative binding on the E2aE double site DNA. The interaction of this mutant protein with E2F appears to be destabilized such that only the cooperative interactions at the E2aE double site could weakly capture the complex. The Xma polypeptide, a deletion mutant lacking these same eleven amino acids in addition to the N-terminal 80 amino acids, had no activity in either assay. In contrast, the BstU mutant protein, which contains the C-terminal 80 amino acids of the E4-ORF6/7 protein, bound to E2F and induced cooperative DNA binding efficiently. These results confirm that this eleven amino acid region (amino acids 81 to 91) is required for efficient interaction with E2F. The presence of amino acids N-terminal to the deletion with the Pvu/Sma mutant may partially compensate for the loss of a portion of the E2F interaction domain. Alternatively, certain mutations (eg. Xma) may have a global effect on the conformation of the C-terminal domain which may be partially rescued by the presence of additional protein sequence N-terminal to this region (eg. Pvu/Sma). Both the Pvu/Sma and Xma mutant proteins have deletion endpoints just upstream of a region predicted by protein structure computer programs to contain two alpha helices separated by a spacer region (see Figures 1 and 4). This region was shown to be necessary for E4-ORF6/7 function (Figure 4) and preliminary results from site-directed mutagenesis suggest that alpha helical structures in these regions are functionally significant (S. Schmid, R. O'Connor and P. Hearing, unpublished results). By analogy to the helix-loop-helix family of transcription factors (40, 41), this region may provide a dimerization domain. These results are consistent with a model where the very C-terminal sequences of the E4-ORF6/7 protein, and possibly residues between amino acids 81 and 91, interact with E2F while the putative helix-loop-helix region provides a dimerization domain. Further studies will be required to test this model.

The ability of E4-ORF6/7 mutants to trans-activate the E2aE promoter *in vivo* (Table 1 and Figure 4) correlates directly with their ability to induce the formation of stable and cooperative binding of E2F to the same promoter *in vitro*, with one exception. The Pvu mutant protein functioned as well as wild type for E2F binding and induction *in vitro*, but had no trans-activation activity *in vivo*, implying that this mutant protein may have lost the ability to confer trans-activation to the E2aE promoter-bound E2F-E4-ORF6/7 complex. However, we were not able to detect this mutant protein following transfection and thus it is more likely that the loss of trans-activation *in vivo* reflects the lack of accumulated protein product. This possibility is supported by the observation that the BstU and Mlu/Pvu mutant proteins trans-activated the E2aE promoter *in vivo* nearly as well as the wild type product. Thus, sequences N-terminal to amino acid 80 do not appear to be essential for E2aE trans-activation. Mutants Ase/Hae and 3′ IN were inactive both *in vivo* and *in vitro* and the Pvu/Sma mutant displayed the same residual activity *in vivo* that it did *in vitro*. These three mutants were expressed *in vivo* to comparable levels as the wild type protein. From those mutants in which we are able to detect protein *in vivo*, there clearly is a strong correlation between the ability to trans-activate the E2aE promoter *in vivo* and the ability to form a stable complex with E2F *in vitro*. It remains to be determined whether the E4-ORF6/7 protein independently contributes to the transcriptional activation confered by the E2aE infection-specific complex.

From these data it seems probable that the major role for E4-ORF6/7 at the E2aE promoter is a structural one to stabilize the binding of two E2F molecules to binding sites in the E2aE promoter. How does E4-ORF6/7 accomplish the induction of stable and cooperative E2F binding? Our data are consistent with several models. The E4-ORF6/7 protein may possess one domain allowing stable binding to E2F in solution and a second domain allowing interaction with a second molecule of E2F at the E2aE promoter. This second domain would mediate cooperative E2F DNA binding. In this case, a heterotrimeric complex containing two E2F molecules and one E4-ORF6/7 protein would stably bind to the E2aE promoter. An alternative model suggests that a heterotetrameric E2F-E4-ORF6/7 complex forms at the E2aE promoter where two E2F-E4-ORF6/7 heterodimers interact in a stable manner. In this case, the E4-ORF6/7 protein binds to E2F and provides a dimerization domain for stable DNA binding. A third possibility that the E4-ORF6/7 protein binds to two molecules of E2F in solution has not been excluded but we think it less likely because of the small magnitude of the E4-ORF6/7 induced mobility shift on a single E2F site relative to the shift on the E2aE double site. A more pronounced change in the mobility shift pattern would be expected if two E2F proteins were bound at a single site. We have attempted mixing experiments using wild type and mutant E4-ORF6/7 products to test these models. The mixing of defective mutant E4-ORF6/7 proteins with the wild type protein did not interfere with (ie. squelch) E2F induction *in vitro* or E2aE trans-activation *in vivo* (data not shown). Thus we at present are unable to distinguish between these models. More direct biochemical approaches will be required to resolve the stoichiometry of the E2F-E4-ORF6/7-E2aE promoter bound complex.

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