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Mutually Exclusive Interaction of the Adenovirus E4-6/7 Protein and the Retinoblastoma Gene Product with Internal Domains of E2F-1 and DP-1

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The binding of E2F to the adenovirus (Ad) E2a promoter is stimulated by the Ad E4-6/7 protein. E2F DNA binding activity is composed of a heterodimer of related but distinct proteins of the E2F-1 and DP-1 families. The E4-6/7 protein induces the cooperative and stable binding of E2F to an inverted repeat binding site in the E2a promoter apparently by providing a dimerization interface to two adjacent E2F heterodimers. The product of the retinoblastoma gene product (Rb) represses the transcriptional activity of E2F by direct protein-protein interaction. In this report, we have examined the regions of E2F-1 and DP-1 that are required for the induction of cooperative E2F binding to the E2a promoter by the E4-6/7 protein. Our results demonstrate that an internal segment of E2F-1, that is conserved among members of the E2F family, is required for functional interaction with the E4-6/7 product. Consistent with this observation, other members of the E2F family (E2F-2 and E2F-3) productively interact with E4-6/7. DP-1 also is necessary for stable interaction with E4-6/7 and an internal segment of DP-1 is required that is positioned in a location similar to that of the conserved E2F-1 domain. Interestingly, the binding of E4-6/7 and the binding of Rb to E2F are mutually exclusive, and our results show that the same internal segments of E2F-1 and DP-1 that are required for E4-6/7 binding are also required for stable interaction with Rb. These results suggest that the Ad E4-6/7 protein mimics Rb in part for the protein interaction requirements for E2F binding, although with different functional consequences. While Rb binding represses E2F activity, the E4-6/7 protein stimulates transactivation of the Ad E2a promoter.

The mammalian transcription factor E2F was first described as a nuclear activity that bound to two inverted recognition sites in the adenovirus (Ad) E2a promoter (38, 39). The binding activity of E2F to these sites is stimulated upon Ad infection by the Ad E4-6/7 protein, which forms a stable complex with E2F and induces the cooperative binding of E2F to the inverted repeat (termed E2F induction [33, 46, 49]). The induction of E2F binding to the Ad E2a promoter in vitro is directly correlated with transcriptional activation of the E2a promoter in vivo (46, 49, 53, 54). E2F transcriptional activity is also positively modulated by the Ad E1A gene products (38, 39, 45, 60, 66) at least in part through the dissociation of E2F from proteins that repress E2F activity (1, 5, 59) (discussed below). Binding sites for E2F have been identified in the promoters of a number of cellular genes involved in DNA synthesis and regulation of the cell cycle. Functionally important E2F binding sites are present in the c-*myc*, dihydrofolate reductase gene, *cdc2*, and b-*myb* promoters (6, 15, 31, 41, 48, 68). It appears that by recruiting E2F activity to the E2a promoter for the expression of viral replication genes, Ad has concomitantly created an optimal environment for DNA synthesis by inducing the expression of cellular activities important for facilitating progression through and entry into the S phase of the cell cycle (reviewed in references 51 and 52).

Modulation of transcription factor activity by protein-protein interactions has emerged as an important mode of transcriptional regulation. Recent studies have demonstrated that E2F is regulated by interactions with multiple cellular proteins and that the association of E2F with these proteins varies with specific stages of the cell cycle. In G_0 and early G_1 fibroblasts, E2F is predominantly found in a complex with p130, which additionally mediates association with either cyclin A/cdk2 or cyclin E/cdk2 kinase complexes (12, 13, 26, 44). A similar protein complex containing E2F, p107, cyclin E, and cdk2 is detected in late G_1 phase, while p107, cyclin A, and cdk2 are components of an S-phase E2F complex (8, 16, 19–21, 42, 47, 56, 65). The p130 and p107 proteins are related to a third direct binding partner of E2F, the product of the retinoblastoma susceptibility gene (Rb) (2, 3, 10, 11, 55). Rb-E2F complexes are found in both G_1 and S phase (63, 65). Inhibition of cell proliferation, a hallmark of normal Rb function, is relieved when the Rb-E2F interaction is disrupted by mutation of Rb or dissociated by the DNA tumor virus transforming protein large T antigen, E1A, or E7 (1, 5, 9, 30, 58; reviewed in references 18 and 70). Recent evidence suggests that Rb may function, in part, to negatively regulate E2F function by inhibiting transactivation by E2F and acting as a repressor of E2F-responsive promoters (22, 25, 27, 29, 30, 71). The p130 and p107 complexes, also targets for DNA tumor virus proteins, may also be inhibitory, but their exact function has not been elucidated (63, 72, 73). This evidence implicates E2F as a target in the cell cycle signalling pathways governed by p130, p107, and Rb and transcriptional regulation linked to E2F as one of the molecular mechanisms by which these proteins function.

The regulation of E2F binding activity has achieved an added level of complexity with the recent definition of multiple components involved in E2F binding activity. Initially, a cDNA encoding a protein with the characteristics of E2F was cloned (28, 37). This protein, referred to as E2F-1, is a member of a family of proteins (E2F-1, -2, and -3 [35, 43]) capable of forming heterodimers with DP-1, a second protein with E2F binding properties (4, 29, 40, 54). Heterodimers between E2F

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family members and DP-1 demonstrate enhanced binding to E2F sites in vitro and increased transactivation of a promoter containing E2F sites in vivo (4, 29, 40, 54). In addition, this heterodimerization is required for stable interaction with Rb (29, 40) and is necessary to form the Ad infection-specific complex on the E2a promoter with the Ad E4-6/7 protein (discussed above) (54). Sequence comparison of E2F family members has revealed a conserved, potential basic-helix-loop-helix region with an adjacent leucine zipper domain and shows significant similarity to DP-1 in helix II and the zipper region. Homology between E2F members also is found in the Rb interaction domain and a region located between amino acid residues 251 and 317 of E2F-1 (the marked box [43]). Thus, E2F activity consists of a family of related proteins with the ability to dimerize and form multiple protein complexes. The differential regulation of target genes may depend on the binding affinities of each member of the family to a given promoter, on the regulation of this affinity by protein contacts within the family and/or with other proteins not contacting DNA, and on other factors binding to different sites in the upstream regulatory region.

It is not known how the physical interaction of the Ad E4-6/7 protein with the E2F/DP-1 heterodimer results in the formation of a stable, ternary protein-DNA complex on the E2a promoter (double-site complex). Analyses using deletion mutants in E4-6/7 defined the C-terminal 70 amino acids as necessary and sufficient for induction of cooperative binding and for transactivation (50, 53). Additionally, point mutagenesis throughout this region delineated a bipartite E2F interaction domain and a potential dimerization/induction domain centered around amino acid 125 (50, 54). Here we describe an analysis of the sequences in E2F-1 and DP-1 that are required for the induction of E2F binding to the E2a promoter. We have localized the region of E2F-1 required for E4-6/7 interaction to the functionally uncharacterized, conserved domain between amino acid residues 245 and 313 and to a similarly localized region of DP-1 (amino acids 243 to 328). Furthermore, a five-amino-acid substitution within this region of E2F-1 abolishes formation of the double-site complex. These results suggest that E4-6/7 makes independent protein contacts with both E2F-1 and DP-1 and that interaction occurs via a conserved region not required for heterodimerization between E2F-1 and DP-1. Additionally, we have shown that the binding of E4-6/7 and the binding of Rb to a E2F-1/DP-1 heterodimer are mutually exclusive and that this exclusivity can be explained by overlapping structural requirements of the E2F-1/DP-1 heterodimer for binding by E4-6/7 and Rb.

MATERIALS AND METHODS

Construction of wild-type and mutant E2F-1, DP-1, and E4-6/7 plasmids. The human E2F-1 (hE2F) (28, 37) and human DP-1 (hDP-1) cDNAs (29) were kindly provided by K. Helin and E. Harlow. The bacterial expression plasmid pET-3a-E2F-1 was constructed using the pET-3 vector system described by Rosenberg et al. (61). The E2F-1 cDNA in pBS-SK was adapted for insertion into the pET-3a *Bam*HI site by ligation of two annealed oligonucleotides, 5′-GATCC CATATGGC-3′ and 5′-CAAGGCCATATGG-3′, to the *Sty*I site at amino acid 2 of E2F-1 and subsequent digestion with *Bam*HI to release the cDNA. The majority of carboxy-terminal deletion mutants were generated by full or partial digestion of pET-3a-E2F-1 with a restriction enzyme that contains a site within the E2F-1 coding sequence. This was followed by digestion with *Esp*I, which digests pET-3a just 5′ to the T7 terminator, repair with Klenow polymerase, and religation of the plasmid. This provided stop codons in all three reading frames immediately 3′ to the fusion. C-terminal deletion mutant ΔC 1-313 was generated by sequential digestion with exonuclease III and mung bean nuclease to create a blunt-ended deletion. The deletion fragment was released by digestion with *Xba*I, which has a site in the vector, and subcloned into a *Xba*I-blunt *Esp*I pET-3a vector. Internal deletion mutants were generated by standard subcloning procedures using appropriate restriction sites (62). The five-amino-acid substitution mutant was generated by digesting with *Afl*II and *Bgl*II restriction enzymes, removing the overhangs by digestion with mung bean nuclease, and inserting a 12-bp *Eco*RI linker. pET-3a-E2F-1Δ81-313 was constructed by digestion with *Bst*UI, ligation of a *Bam*HI linker, and excision of the insert with a *Bam*HI and *Afl*II digestion. The insert was then ligated into pET-3a-E2F-1Δ1-313 vector digested with the same enzymes. E2F-2 and E2F-3 coding sequences were subcloned from plasmids containing the respective cDNAs (kindly provided by J. Lees and E. Harlow) by PCR and subsequent ligation into the pET vector. The pET-E2F-2 clone contains E2F-2 amino acids 24 to 247; the pET-E2F-3 clone contains E2F-3 amino acids 1 to 21 fused to amino acids 127 to 425.

The bacterial expression plasmid pET-3a-hDP-1 was constructed by inserting a *Bst*YI fragment from pBS-SK-DP-1 (29) containing the DP-1 coding sequence into the *Bam*HI site of pET-3a. Carboxy-terminal deletion mutants of pET-3a-hDP-1 were generated as described above for pET-3a-E2F-1. The murine DP-1 (mDP-1) cDNA (23) was kindly provided by R. Girling and N. La Thangue in vector pGC (69). pET-3a-mDP-1Δ61-341 was constructed from pGEX-DP-1ΔN61 (54a) by digestion with *Bam*HI to release the fragment containing amino acids 61 to 341 and ligation into pET-3a. The resulting construct has the sequence 5′-CCGGAATTCCGGGAG-3′ between the pET-3a *Bam*HI site and amino acid 61.

The bacterial expression plasmid pGEX-ORF 7, containing open reading frame (ORF) 7 of the E4-6/7 gene fused in frame with glutathione *S*-transferase (GST), was constructed by digesting pBS-E4-6/7 (53) with *Mlu*I, repair with Klenow polymerase, release of the insert with *Hin*dIII digestion, and insertion of the fragment into *Sma*I-*Hin*dIII-digested pGEX-KG (24). pET-E4-6/7, containing amino acids 2 to 150 of E4-6/7, was previously described (54). All mutants generated were confirmed by dideoxy sequencing.

The eukaryotic expression vector pCMV-HA-DP-1 was kindly provided by K. Helin and E. Harlow and contains a hemagglutinin epitope-tagged hDP-1 cDNA (29). A frameshift mutation was introduced at amino acid 273 by digestion with *Bsa*AI and the subsequent insertion of an 8-bp *Spe*I linker. A frameshift mutation was introduced at amino acid 333 by digestion with *Pst*I and repair with T4 DNA polymerase. The eukaryotic expression vector pCMX-45-E2F-1 contains the E2F-1 cDNA in a cytomegalovirus vector with a 13-amino-acid epitope tag for monoclonal antibody (MAb) M45 (54) inserted at the *Sty*I site between amino acids 2 and 3. Frameshift mutations were introduced at amino acids 292 and 313 by insertion of homologous DNA fragments derived from the E2F-1 mutant bacterial expression plasmids described above.

Bacterial and in vitro expression of proteins. A 10-ml culture of *Escherichia coli* BL21(DE3) lysogen (67) transformed with pET-3a-hDP-1 constructs or *E. coli* BL21(DE3) lysS lysogen (67) transformed with pET-3a-E2F-1 constructs was grown in LB medium to saturation overnight at 37°C. The following day, the 10-ml culture was used to inoculate a 100-ml culture of LB medium and allowed to grow for 1 h. Isopropylthiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM, and incubation was continued for 4

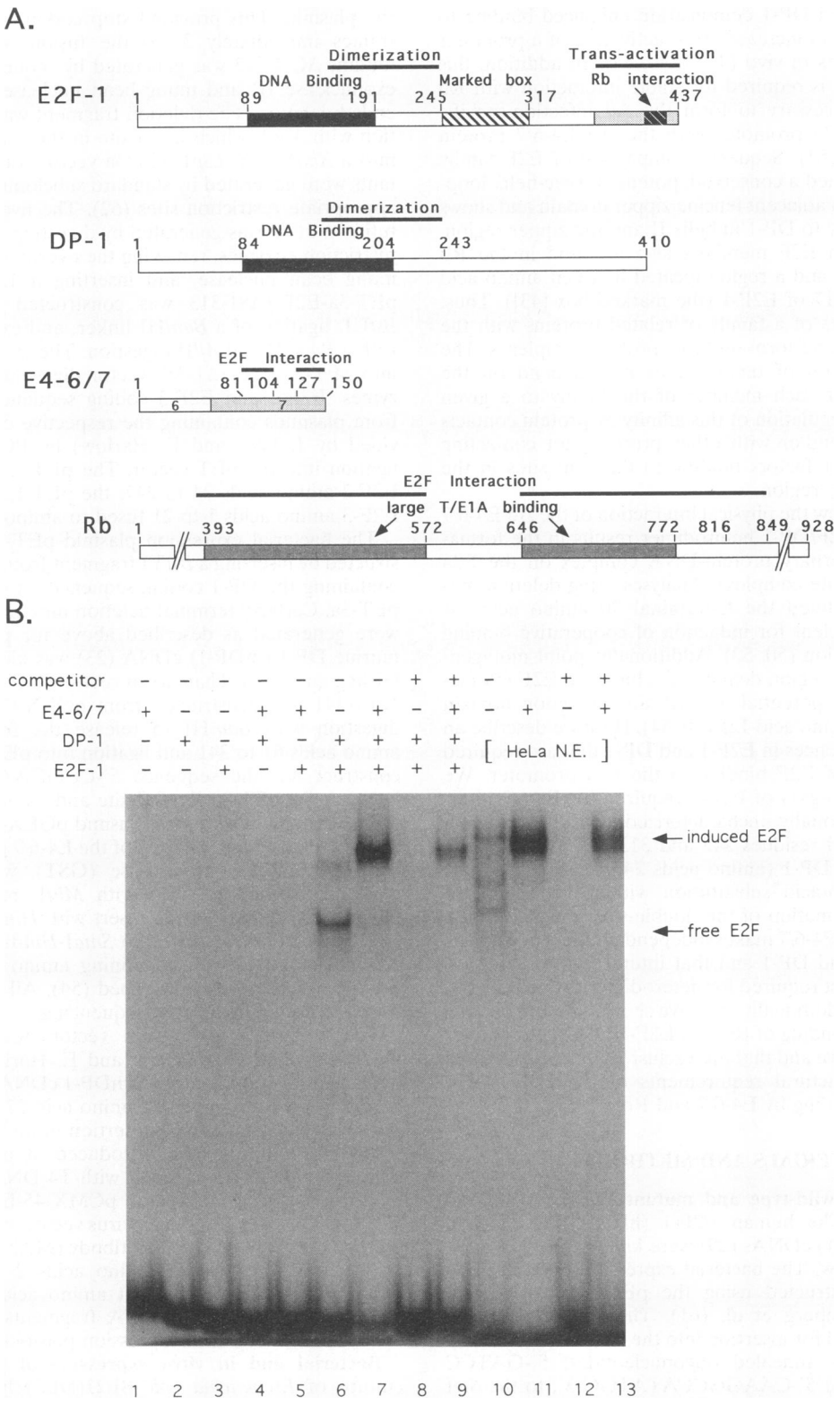


FIG. 1. (A) Schematic representation of the E2F-1, DP-1, E4-6/7, and Rb coding sequences. The numbers above each coding sequence refer to the amino acid residues delineating the structural regions required for the biochemical function(s) of each protein. Each functional domain is correlated with its defined structural region by shading of that region or by a heavy black bar overlying the region. The function for a particular domain is listed above the ascribed structural region. (B) Reconstitution of the double-site complex with hE2F-1, hDP-1, and E4-6/7 in a gel mobility shift assay. A double E2F site fragment from the E2aE promoter (E2aE sequences −30 to −73) was used in binding reactions containing each of the bacterially expressed proteins either individually (lanes 1 to 3), in pairwise combinations (lanes 4 to 6 and 8), and with all three together (lanes 7 and 9). HeLa cell nuclear extract (N.E.) was assayed in place of hE2F-1 and hDP-1 in lanes 10 to 13. Bacterially expressed GST–E4-6/7

hs. Bacterial pellets were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C, washed once with phosphate-buffered saline (PBS) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and resuspended in 1/50 original volume of TNE (50 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA) and 1 mM PMSF. Bacteria were lysed by incubation with lysozyme (DE3) or by one freeze-thaw cycle [DE3(lysS)], incubation at 37°C with 0.1% deoxycholic acid, and subsequent digestion with DNase I. Inclusion bodies containing the overexpressed DP-1 and E2F-1 proteins were washed three times with TNE-PMSF and resuspended with 8 M urea in TNE containing 10 mM dithiothreitol (DTT). Remaining particulate material was removed by centrifugation. E2F-1 and DP-1 proteins were visualized on 12.5% Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. A 50-ml culture of bacteria (*E. coli* DH5) transformed with pGEX-ORF 7 was grown overnight to saturation. The following day, a 500-ml culture was inoculated and allowed to grow for 1 h. IPTG was then added to 0.1 mM, and incubation continued for another 4 h. Bacterial pellets were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C, washed once with PBS and 1 mM PMSF, and resuspended in 25 ml of TNE with 1 mM PMSF, 10 mM DTT, and 1% Triton X-100. Aliquots of 5 ml were sonicated on ice for one 20-s burst, and soluble protein was recovered by centrifugation at 12,000 × *g* for 20 min. The soluble protein was pooled and loaded onto a 10-ml glutathione-agarose column (Sigma), washed with buffer DB (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.2 mM EDTA, 5 mM DTT, 1 mM PMSF), and eluted with buffer DB and 20 mM reduced glutathione (Sigma). Fractions were tested for activity in a gel mobility shift assay with uninfected nuclear extract from HeLa cells (see below), and active fractions were pooled.

Plasmids were linearized and transcribed in vitro with T7 RNA polymerase. Wheat germ extracts were programmed with 1 to 2 μg of RNA in the presence of ^{35}S-Translabel (ICN) as described by the manufacturer (Promega). In vitro translation products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Extract preparation and gel mobility shift assays. Nuclear and cytoplasmic extracts were prepared by the method by Dignam et al. (17). The supernatant obtained after isolation of the nuclei was adjusted to 100 mM KCl, spun at 100,000 × *g* for 1 h, and saved as the cytoplasmic fraction. Cytoplasmic and nuclear fractions were dialyzed against DB, and the dialysate was cleared by centrifugation at 25,000 × *g*.

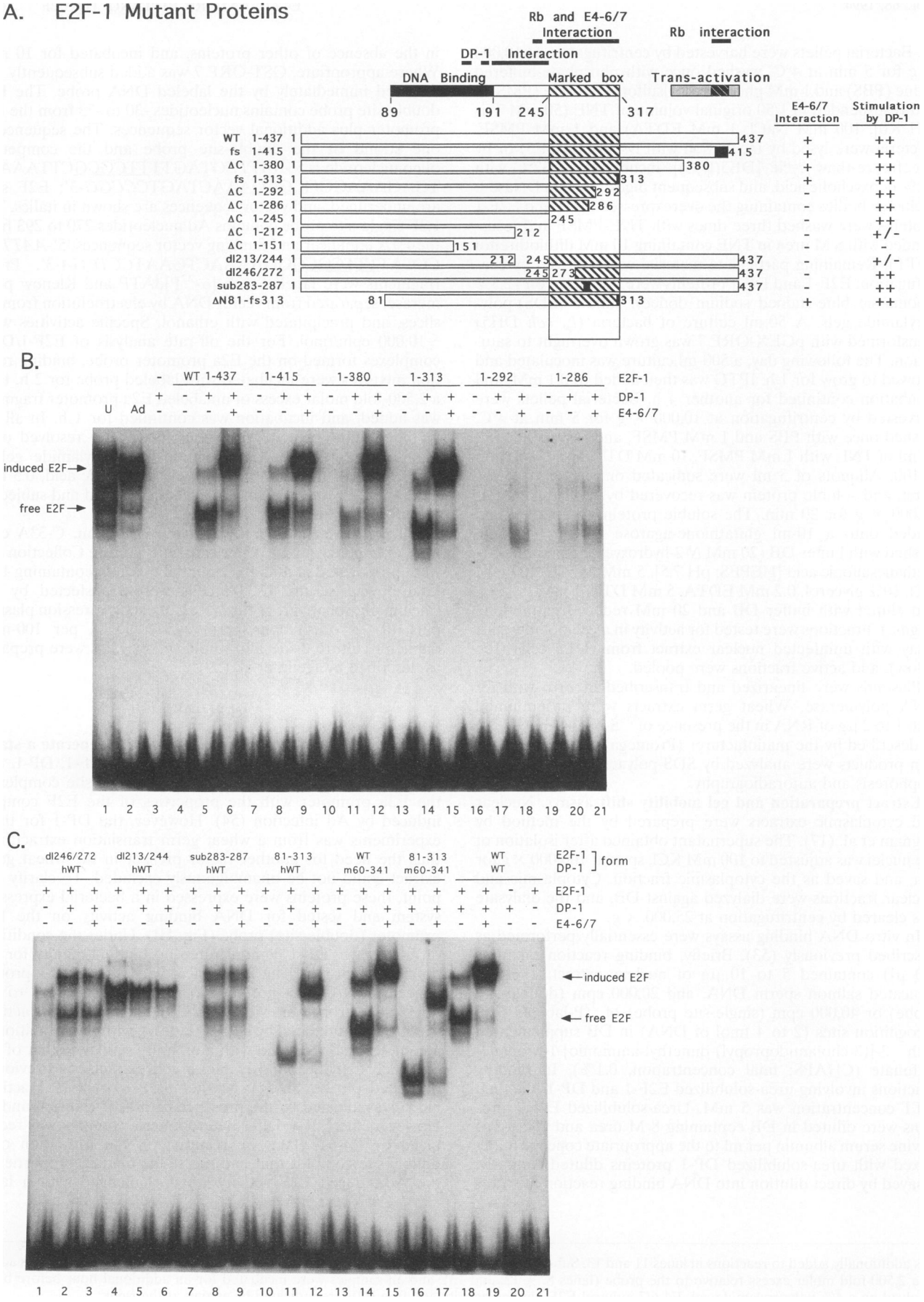
In vitro DNA binding assays were essentially performed as described previously (53). Briefly, binding reaction mixtures (20 μl) contained 5 to 10 μg of nuclear extract, 1 μg of sonicated salmon sperm DNA, and 20,000 cpm (double-site probe) or 40,000 cpm (single-site probe) of ^{32}P-labeled E2F recognition sites (2 to 4 fmol of DNA) in DB supplemented with 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; final concentration, 0.1%). In binding reactions involving urea-solubilized E2F-1 and DP-1, the final DTT concentration was 5 mM. Urea-solubilized E2F-1 proteins were diluted in DB containing 8 M urea and 100 μg of bovine serum albumin per ml to the appropriate concentration, mixed with urea-solubilized DP-1 proteins diluted similarly, assayed by direct dilution into DNA binding reaction mixtures in the absence of other proteins, and incubated for 10 min. Where appropriate, GST-ORF 7 was added subsequently and followed immediately by the labeled DNA probe. The E2F double-site probe contains nucleotides -30 to -73 from the E2a promoter plus additional vector sequences. The sequence of one strand of the double-site probe and the competitor oligonucleotide is 5′-*AATT*CGTAGTTTCGCGCTTAAATT TGAGAAAGGGCGCGAAACTAGTC*CCGG*-3′; E2F sites are underlined, and vector sequences are shown in italics. The E2F single-site probe contains Ad nucleotides 270 to 293 from the E1A enhancer and flanking vector sequences: 5′-*AATTCC CCCAT*TTTCGCGGGAAAACTGAAT*CCTCGA*-3′. Probe fragments were labeled with [α-^{32}P]dATP and Klenow polymerase, separated from vector DNA by electroelution from gel slices, and precipitated with ethanol. Specific activities were ~10,000 cpm/fmol. For the off-rate analysis of E2F-1/DP-1 complexes formed on the E2a promoter probe, binding reaction mixtures were incubated with labeled probe for 2 h, then a 2,500-fold molar excess of unlabeled E2a promoter fragment was added, and incubation was continued for 1 h. In all gel mobility shifts, protein-DNA complexes were resolved on a 4%, 30:1 (acrylamide/bisacrylamide) polyacrylamide gel in 0.5× TBE (25 mM Tris [pH 8.3], 25 mM boric acid, 0.5 mM EDTA) at 4°C at 10 V/cm. The gels were dried and subjected to autoradiography.

Cells, transfections, and extract preparation. C-33A cells were obtained from the American Type Culture Collection and were propagated in minimal essential medium containing 10% fetal bovine serum. C-33A cells were transfected by the calcium phosphate procedure (5 μg of each expression plasmid plus 10 μg of salmon sperm carrier DNA per 100-mm-diameter culture dish), and whole cell extracts were prepared as described by Helin et al. (29).

RESULTS

E2F-1, DP-1, and E4-6/7 are sufficient to generate a stable double-site complex on the E2a promoter. E2F-1, DP-1, and E4-6/7 are sufficient to reconstitute a double-site complex at the E2a promoter with the properties of the E2F complex induced by Ad infection (54). However, the DP-1 for these experiments was from a wheat germ translation extract, and thus the need for another factor present in the wheat germ extract could not be unambiguously excluded. To clarify this point, these proteins were expressed in a bacterial expression system and tested for DNA binding activity on the E2a promoter (double-site) probe (Fig. 1B). Under the conditions of our assay, hDP-1 demonstrated no binding affinity for the double-site probe (lane 2), even at very high hDP-1 protein concentrations (data not shown). Binding of hE2F-1 to the double-site probe was evident (lane 3), and this binding activity was greatly stimulated by hDP-1 (lane 6), with the formation of a complex that migrated with a mobility similar to that of the free E2F activity found in HeLa cell extracts, as previously described (4, 29, 40, 54). Neither hDP-1 or hE2F-1 activity alone was affected by the presence of E4-6/7 (lanes 4 and 5). However, the E2F-1/DP-1 heterodimeric complex was recognized by E4-6/7 (lane 7), resulting in the formation of a double-site complex that had the stable binding properties of the Ad-induced E2F activity when challenged with a large

was additionally added to reactions in lanes 11 and 13. Subsequent to the binding reaction, unlabeled, double E2F site competitor DNA was added to a 2,500-fold molar excess relative to the probe (lanes 8, 9, 12, and 13), and all samples were incubated for an additional hour before being resolved on a 4% polyacrylamide gel. E4-6/7-induced E2F binding activity and free E2F are indicated by arrows at the right.


A. E2F-1 Mutant Proteins
Rb and E4-6/7 Interaction
Rb interaction
DP-1 Interaction
DNA Binding
Marked box
Trans-activation
89
191
245
317
E4-6/7 Interaction
Stimulation by DP-1
WT 1-437 1 437 + ++
fs 1-415 1 415 + ++
ΔC 1-380 1 380 + ++
fs 1-313 1 313 + ++
ΔC 1-292 1 292 - ++
ΔC 1-286 1 286 - ++
ΔC 1-245 1 245 - ++
ΔC 1-212 1 212 - +/-
ΔC 1-151 1 151 - -
dl213/244 1 212 245 437 - +/-
dl246/272 1 245 273 437 - ++
sub283-287 1 437 - ++
ΔN81-fs313 81 313 + ++
B.
WT 1-437
1-415
1-380
1-313
1-292
1-286
E2F-1
DP-1
E4-6/7
U
Ad
induced E2F
free E2F
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
C.
dl246/272 hWT
dl213/244 hWT
sub283-287 hWT
81-313 hWT
WT m60-341
81-313 m60-341
WT WT
E2F-1 DP-1] form
E2F-1
DP-1
E4-6/7
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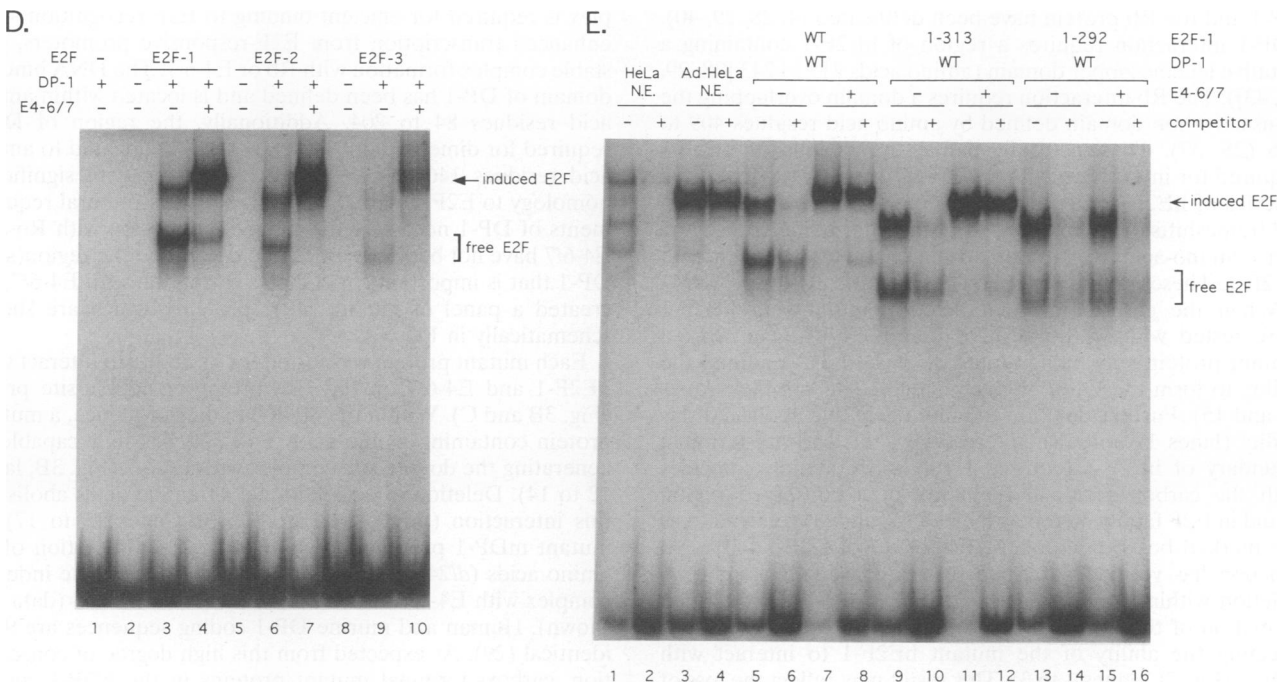

FIG. 2. (A) Schematic diagram of hE2F-1 wild-type (WT) mutant proteins and their activities in vitro. The top part depicts the structural regions of the hE2F-1 coding sequence and the functions assigned to them, as shown in Fig. 1A. In the bottom part, the rectangles represent the coding region of hE2F-1 and the sequences remaining with each mutant protein. The number located at the deletion endpoint of each mutant protein refers to the last remaining amino acid present in that protein. The region defined as the marked box has been highlighted with diagonal stripes in each protein. The darkly shaded box at the carboxy terminus of mutant fs (frameshift) 1-415 reflects 13 additional amino acids present as a result of a frameshift after amino acid 415 of E2F-1. Similarly, two additional amino acids are present at the carboxy terminus of fs 1-313. The fs constructs, carboxy-terminal truncations (ΔC), and ΔN81-fs313 are named according to the amino acids remaining in the mutant protein. The two internal deletion mutant proteins are named according to the amino acids removed by the deletion, and the five-amino-acid substitution (sub283-287) is named for the amino acids changed by the mutation. A summary of the results from experiments presented in Fig. 2B and C is shown in the columns on the right. (B) Interaction of E4-6/7 and DP-1 with carboxy-terminal mutant E2F-1 proteins. A DNA probe containing the double E2F binding site from the E2aE promoter was mixed with individual E2F-1 mutant proteins and assayed either alone (lanes 4, 7, 10, 13, 16, and 19), together with hDP-1 (lanes 5, 8, 11, 14, 17, and 20), or in combination with hDP-1 and GST–E4-6/7 (lanes 6, 9, 12, 15, 18, and 21), and DNA-protein complexes were resolved by gel mobility shift assay. hDP-1 assayed alone is shown in lane 3. Nuclear extract from either uninfected (U) or Ad-infected (Ad) HeLa cells was also assayed and is shown in lane 1 or 2, respectively. The specific E2F-1 mutations assayed are indicated above the horizontal lines at the top. The presence of hDP-1 or GST–E4-6/7 in a specific reaction is denoted by a + or − over the lane for that reaction. E4-6/7-induced E2F binding activity and free E2F are indicated by arrows on the left. WT, wild type. (C) Identification of minimal domains of E2F-1 and DP-1 required for interaction with E4-6/7. The double E2F binding site probe was mixed with individual E2F-1 mutant proteins and assayed either alone (lanes 1, 4, 7, and 10), together with hDP-1 (lanes 2, 5, 8, and 11), or together with hDP-1 and GST–E4-6/7 (lanes 3, 6, 9, and 12), and DNA-protein complexes were resolved by gel mobility shift assay. Similarly, mutant DP-1 (m60-341) was tested alone (lane 13) and with wild-type (WT) hE2F-1 in the absence (lane 14) and presence (lane 15) of GST–E4-6/7. Minimal E2F-1 and minimal DP-1 were tested together without (lane 16) and with (lane 17) GST–E4-6/7. Wild-type E2F-1 and DP-1 were assayed individually (lanes 20 and 21) or together without (lane 18) or with (lane 19) GST–E4-6/7. The presence of E2F-1, DP-1, or GST-E4-6/7 in a specific reaction is denoted by a + or − over the lane for that reaction. The specific form of E2F-1 or DP-1 in the reaction is indicated above the horizontal lines at the top. E4-6/7-induced E2F binding activity and free E2F are indicated for the wild-type proteins by arrows on the right. (D) E2F-2 and E2F-3 form the induced E2F double-site complex. hE2F-1, -2, or -3 was incubated with the E2F double-site probe either individually (lanes 2, 5, and 8), with hDP-1 (lanes 3, 6, and 9), or with hDP-1 and GST–E4-6/7 (lanes 4, 7, and 10), and DNA-protein complexes were resolved by gel mobility shift assay. The presence of DP-1 or GST–E4-6/7 in a specific reaction is denoted by a + or − over the lane for that reaction. The presence of E2F-1, -2, or -3 in the reaction is indicated above the horizontal lines at the top. E4-6/7-induced E2F binding activity (arrow) and free E2F (bracket) are indicated on the right. (E) Off-rate analysis of E2F activity generated with carboxy-terminal mutant hE2F-1 proteins. The E2F double-site probe was mixed with uninfected HeLa nuclear extract (N.E.; lanes 1 and 2), Ad-infected HeLa nuclear extract (lanes 3 and 4), wild-type (WT) hE2F-1 and wild-type hDP-1 (lanes 5 to 8), E2F-1 fs 1-313 and wild-type hDP-1 (lanes 9 to 12), and E2F-1Δ1-292 and wild-type hDP-1 (lanes 13 to 16), and DNA-protein complexes were resolved by gel mobility shift assay. Lanes 7, 8, 11, 12, 15, and 16 contained GST–E4-6/7 protein. Binding reaction mixtures contained excess quantities of hE2F-1 and hDP-1 proteins to allow formation of the slower-migrating complex in the absence of GST–E4-6/7 (lanes 5, 9, and 13), which represents the occupancy of both E2F binding sites on the probe. Reaction mixtures were incubated for 2 h, at which time reactions in even-numbered lanes were challenged with a 2,500-fold molar excess of unlabeled double-site probe DNA, and all reaction mixtures were incubated for an additional hour. E4-6/7-induced E2F binding activity (arrow) and free E2F (bracket) are indicated at the right.

excess of specific competitor DNA (lanes 7 to 13). These data demonstrate that bacterially expressed hE2F-1, hDP-1, and E4-6/7 are sufficient to form the induced E2F double-site complex in vitro.

A conserved region of hE2F-1 is required for induction of the double-site complex by E4-6/7 in vitro. Formation of the hE2F-1/hDP-1 heterodimer is necessary for stable interaction with Rb, and domains of hE2F-1 required for interaction with

DP-1 and the Rb protein have been delineated (4, 28, 29, 40). hDP-1 interaction requires a region of hE2F-1 containing a putative leucine zipper domain (amino acids 215 to 243 [28, 29, 40, 43]). The Rb interaction requires a domain overlapping the transactivation domain defined by amino acid residues 409 to 426 (28, 37). To structurally define the regions of hE2F-1 required for interaction with the E4-6/7 protein, we generated a series of hE2F-1 mutant proteins containing carboxy-terminal frameshifts or truncations, in-frame internal deletions, and a five-amino-acid substitution within the coding sequence of hE2F-1. These mutants are shown schematically in Fig. 2A.

When the carboxy-terminal deletion mutants in hE2F-1 were tested with wild-type hDP-1 and E4-6/7 (Fig. 2B), a mutant protein containing amino acids 1 to 313 retained the ability to form the E4-6/7-induced double-site complex (lanes 14 and 15). Further deletion to amino acid 292 abolished this ability (lanes 17 and 18). Interestingly, this carboxy-terminal boundary of hE2F-1 required for E4-6/7 function coincides with the carboxy-terminal boundary of a conserved region found in E2F family members (E2F-1, -2, and -3) referred to as the marked box (amino acids 251 to 317 of E2F-1 [43]). No function has yet been ascribed to this conserved domain. A deletion within the marked box (hE2F-1Δ246-272) abolished formation of the E4-6/7-induced double-site complex without affecting the ability of the mutant hE2F-1 to interact with hDP-1 (Fig. 2C, lanes 1 to 3). This result may reflect the loss of a region for direct interaction with E4-6/7 or a change in the position (spacing) of an interaction domain relative to other regions of the hE2F-1 or hDP-1 protein. Evidence that this conserved region indeed constitutes a direct interaction domain for E4-6/7 was provided by the inability of a mutant hE2F-1 protein containing a five-amino-acid substitution within this region to form the E4-6/7-induced double-site complex (hE2F-1-sub283-287; Fig. 2C, lanes 7 to 9), although the association of this mutant hE2F-1 with hDP-1 was not affected. Deletion of the putative leucine zipper domain of hE2F-1 (hE2F-1Δ213-244) abrogated interaction with both DP-1 and E4-6/7 (Fig. 2C, lanes 4 to 6). If the conservation of the marked box in fact reflects the conservation of a protein-protein interaction domain that has been exploited by Ad, we would expect that the other E2F family members would also be capable of forming the double-site complex with E4-6/7. Bacterially expressed hE2F-2 and hE2F-3 formed the double-site complex as readily as hE2F-1 in the presence of hDP-1 and E4-6/7 (Fig. 2D). These results implicate the marked box as a domain of hE2F-1, -2, and -3 that is required for interaction with E4-6/7 and the formation of the Ad-induced double-site complex on the E2a promoter.

When high concentrations of wild-type or mutant hE2F-1 proteins were assayed with hDP-1 on the double-site probe, occupancy of both E2F binding sites could be achieved, and a slower-migrating complex was seen along with the faster-migrating single-site complex typically referred to as free E2F (Fig. 2C and E, lanes 5, 9, and 13). This slower-migrating complex was not seen when similar protein concentrations on a probe containing a single E2F binding site were used (data not shown). However, the double-site complex formed in the absence of E4-6/7 was not stable to competitor challenge, in contrast to the induced complex formed with E4-6/7 (Fig. 2E, lanes 5 to 16). From these results, we conclude that disruption of a protein-protein interaction between E4-6/7 and hE2F-1 prevents the formation of a stable DNA binding complex on the E2a promoter.

A region of DP-1, separate from the DNA binding and E2F-1 heterodimerization domain, is required for E4-6/7 interaction in vitro. As mentioned, an hE2F-1/hDP-1 heterodimeric complex is required for efficient binding to E2F recognition sites, enhanced transcription from E2F-responsive promoters, and stable complex formation with Rb or E4-6/7. The DNA binding domain of DP-1 has been defined and is located within amino acid residues 84 to 204. Additionally, the region of DP-1 required for dimerization with E2F-1 was delineated to amino acid residues 146 to 238 of DP-1, a region with significant homology to E2F-1 (4, 23). In contrast, the structural requirements of DP-1 necessary for complex formation with Rb and E4-6/7 have not been described. To determine the region(s) of DP-1 that is important for complex formation with E4-6/7, we created a panel of mutant DP-1 proteins, which are shown schematically in Fig. 3A.

Each mutant protein was tested for its ability to interact with hE2F-1 and E4-6/7 on the E2a promoter double-site probe (Fig. 3B and C). Within the mDP-1 coding sequence, a mutant protein containing amino acids 1 to 329 was fully capable of generating the double-site complex with E4-6/7 (Fig. 3B, lanes 12 to 14). Deletion of an additional 41 amino acids abolished this interaction (mDP-1Δ1-288; Fig. 3B, lanes 15 to 17). A mutant mDP-1 protein containing an internal deletion of 32 amino acids (*dl*244-275) also was unable to form the induced complex with E4-6/7 but could interact with hE2F-1 (data not shown). Human and murine DP-1 coding sequences are 95% identical (29). As expected from this high degree of conservation, carboxy-terminal mutant proteins in the hDP-1 coding sequence provided results equivalent to results for their murine counterparts (Fig. 3C). When a mutant hE2F-1 protein containing amino acids 81 to 313 was mixed with a mutant DP-1 protein containing amino acids 60 to 341, E4-6/7 was fully capable of generating the induced double-site complex (Fig. 2C, lanes 10 to 19). From these results, we conclude that a region of DP-1, independent of the DNA binding and hE2F-1 interaction domains and analogous in position to the marked box of hE2F-1, is required for stable association with E4-6/7 and formation of the induced double-site complex on the Ad E2a promoter. Our data suggest that a structural unit comprising the DNA binding domains, heterodimerization domains, and sequences just C terminal to these domains in both E2F-1 and DP-1 is necessary and sufficient to form a stable complex on the E2a promoter with the E4-6/7 protein. E4-6/7 apparently makes protein-protein contacts with the marked box of E2F-1 and an equivalent region of DP-1.

Mutant E2F-1 and DP-1 proteins, expressed in vivo, demonstrate binding properties similar to those of proteins expressed in vitro. To determine if the results for bacterially expressed proteins could be confirmed following expression of hE2F-1 and hDP-1 in vivo, extracts from C-33A cells transfected with expression vectors carrying wild-type or mutant hE2F-1 and hDP-1 coding regions were assayed for interaction with E4-6/7 on the E2a promoter double-site probe (Fig. 4). The hE2F-1 protein was tagged with a 13-amino-acid epitope for MAb M45 (54), and the hDP-1 protein was tagged with the influenza hemagglutinin epitope recognized by MAb 12CA5 (29). As previously described (29), coexpression of hE2F-1 and hDP-1 resulted in the formation of an E2F complex readily detected above the level of endogenous C-33A E2F activity (lane 3 versus lane 7). Both the endogenous C-33A E2F activity and the E2F activity observed following transfection were induced for binding to the double-site probe by E4-6/7 (lanes 4 and 8), but only the E2F activity in transfected cell extracts was recognized by MAbs M45 and 12CA5 (lanes 5 and 6 versus lanes 9 and 10), as expected. The wild-type and mutant hE2F-1 and hDP-1 proteins behaved similarly when synthesized in vivo as when produced in vitro, with one exception. An E2F-1 protein containing amino acids 1 to 313 retained full

ability for E4-6/7 induction, while this interaction was lost with a deletion to amino acid 286 (lanes 11, 12, 15, and 16). Similarly, a mutant hDP-1 protein containing amino acids 1 to 333 was induced by E4-6/7 (lanes 19 and 20). Unfortunately, we have not been able to visualize expression of a hDP-1 protein containing amino acids 1 to 273 above background levels (lanes 23 and 24), possibly because of protein instability in vivo. We conclude from this experiment that the requirements for E4-6/7 binding to bacterially expressed hE2F-1 and hDP-1 proteins determined in vitro are recapitulated when these proteins are expressed in vivo.

Mutually exclusive E2F binding by Rb and E4-6/7. Previous experiments using extracts from cells containing little or no free E2F activity have suggested that the ability of the E4-6/7 protein to interact with E2F is blocked by the binding of cellular proteins, such as Rb and p107, to E2F (1). E2F can be made available by the action of the Ad E1A 12S and 13S proteins, which form a complex with these cellular proteins, thereby removing them from E2F (1, 59). To understand the relationship of the E4-6/7 protein's ability to interact with E2F to that of cellular proteins such as Rb and explain the apparent exclusivity of binding to E2F, we investigated the effect of the E4-6/7 protein on the endogenous cellular protein-E2F complexes present in HeLa cell nuclear extracts. Several E2F-specific complexes were resolved by using a single E2F binding site probe (Fig. 5A, lane 1). One of these complexes contained E2F complexed with Rb, since it was recognized by the Rb MAb C36 (lane 3); a significant amount of free E2F was present in the extract. Titration of bacterially expressed E4-6/7 protein into these binding reaction mixtures had no apparent effect on the preexisting complexes of E2F and cellular proteins, while E4-6/7 was readily able to bind the free E2F protein (lanes 5 to 10).

Cytoplasmic extracts from HeLa cells are an abundant source of free E2F activity separated from the majority of E2F that is complexed with cellular proteins (Fig. 5B, lane 1). Titration of Rb into binding reactions containing cytoplasmic E2F activity demonstrated that Rb was capable of interacting with only a portion of the free E2F activity present in this extract (Fig. 5B, lane 3, demonstrates the result obtained with a fourfold excess of Rb over that required to generate the maximum amount of complex possible [data not shown]). In contrast, the E4-6/7 protein was able to complex with all of the free E2F activity available (Fig. 5B, lane 2). To investigate the apparently exclusive nature of Rb-E2F and E4-6/7–E2F complexes, we performed an order-of-addition experiment. First, an excess of Rb was allowed to incubate with cytoplasmic E2F activity for 1 h. Individual binding reactions were then challenged with increasing amounts of E4-6/7 protein, followed immediately by the addition of single-site probe and incubation for another 30 min (Fig. 5B, lanes 3 to 9). As expected for an exclusive interaction, the secondarily added E4-6/7 did not affect the performed Rb-E2F complexes and was relegated to binding to the remaining E2F activity not accessible to Rb. In the converse experiment, increasing quantities of E4-6/7 were incubated with cytoplasmic E2F for 1 h, challenged with an excess of Rb, and incubated for an additional 30 min with single-site probe (Fig. 5B, lanes 11 to 17). Under these conditions, quantities of E4-6/7 capable of occupying all of the free E2F completely excluded Rb from E2F interaction. These results demonstrate that the interactions of Rb and E4-6/7 with E2F are mutually exclusive and that Rb and E4-6/7 compete for similar E2F species. The observation that Rb can interact with only a fraction of the free E2F in the extract while E4-6/7 can interact with the "Rb-competent" E2F as well as the remaining E2F activity suggests that E4-6/7 may be a pleiotropic E2F-binding protein.

Stable Rb-E2F-1/hDP-1 complex formation has structural requirements of hE2F-1 and hDP-1 similar to those for binding to the E4-6/7 protein. E4-6/7 and Rb can each form a stable trimolecular complex with the hE2F-1/hDP-1 heterodimer. Since amino acid residues 409 to 426 are necessary for interaction of Rb with E2F-1 (28) and amino acids 251 to 317 are necessary for E4-6/7–E2F interaction, it is not clear why Rb binding and E4-6/7 binding are mutually exclusive, although steric considerations are warranted in the context of the folded E2F heterodimer. The results described above prompted us to analyze the structural domains of hE2F-1 and hDP-1 that are required for stable Rb binding. Neither hE2F-1 nor hDP-1 has been analyzed genetically with respect to formation of a trimolecular DNA binding complex with Rb.

The addition of Rb to a binding reaction mixture containing bacterially expressed hE2F-1 and hDP-1 generated the trimolecular Rb-E2F-1/DP-1 complex (Fig. 5C, lanes 2 and 3). As expected, a frameshift mutation within the Rb interaction domain (E2F-1Δ1-415) abolished this interaction (Fig. 5C, lanes 5 and 6). Interestingly, a deletion mutation and a five-amino-acid substitution mutation within the marked box region also abolished the ability of Rb to interact with the hE2F-1/hDP-1 heterodimer (Fig. 5C, lanes 11, 12, 17, and 18). Mutations in hDP-1 were also tested in this assay. A carboxy-terminal deletion retaining amino acids 1 to 333 readily formed the complex with Rb (Fig. 5C, lanes 20 and 21). However, a deletion to amino acid 272 (hDP-1Δ1-273) abolished interaction with Rb but not hE2F-1 (Fig. 5C, lanes 23 and 24). These results strongly suggest that Rb interaction with the hE2F-1/hDP-1 heterodimer requires the carboxy-terminal domain (amino acids 409 to 426) of E2F-1 in conjunction with the conserved marked box region of E2F-1 and the analogous region of DP-1, both of which are necessary for E4-6/7 binding. In fact, with the exception of a requirement for the carboxy-terminal interaction domain, Rb exhibited structural requirements for binding to hE2F-1 similar to those for binding to E4-6/7.

DISCUSSION

Total cellular E2F activity appears to consist of several individual E2F activities whose components are composed of two distinct families of proteins with E2F properties. Members of one family, referred to as E2F-1, -2, and -3 (28, 35, 37, 43), preferentially forms heterodimers with a member of the second family, called DP-1 (23, 29). Formation of a E2F-1/DP-1 heterodimer has been correlated with efficient binding to E2F recognition sites, enhanced transcription from E2F-responsive promoters, and generation of a stable protein complex with E4-6/7 or Rb (4, 29, 40, 54). We have analyzed the structural requirements of the E2F-1/DP-1 heterodimer that are necessary for stable complex formation with E4-6/7 and Rb. Mutagenesis of the human E2F-1 coding sequence implicated the conserved marked box domain (amino acids 251 to 317) as necessary for interaction with E4-6/7. Carboxy-terminal deletions to amino acid 313 of E2F-1, which maps to the C-terminal border of the marked box, retained ability to form the double-site complex (Fig. 2B). Further C-terminal deletions abolished this ability (Fig. 2B). Internal deletions and a five-amino-acid substitution within the marked box, leaving the C terminus intact, also abolished E4-6/7 interaction (Fig. 2C). Mutations generated amino terminal to the marked box also impaired DNA binding or dimerization with DP-1 (Fig. 2C), and therefore the N-terminal boundary for E4-6/7 binding has

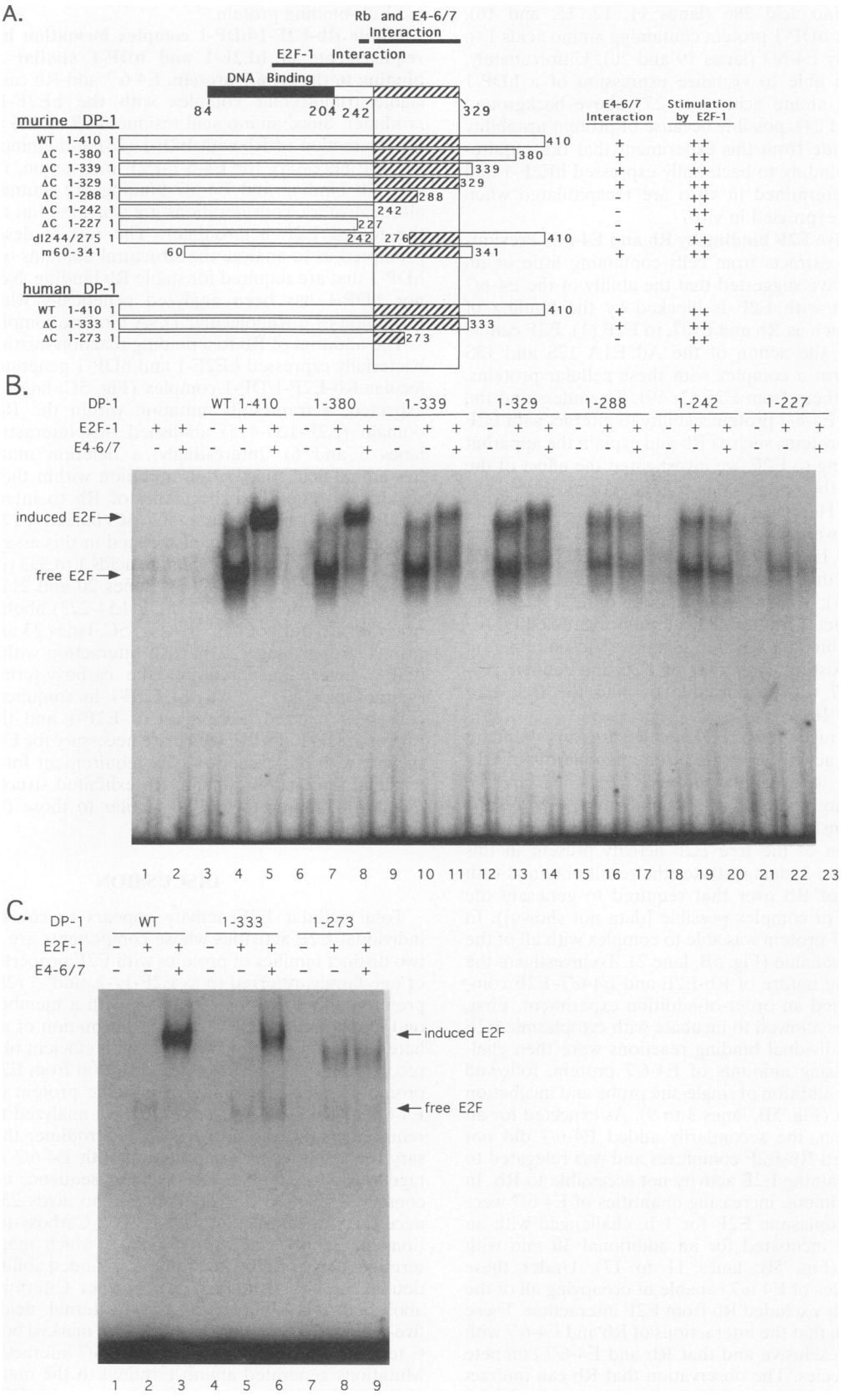
A.
Rb and E4-6/7 Interaction
E2F-1 Interaction
DNA Binding
84
204
242
329
E4-6/7 Interaction
Stimulation by E2F-1
murine DP-1
WT 1-410 1 410 + ++
ΔC 1-380 1 380 + ++
ΔC 1-339 1 339 + ++
ΔC 1-329 1 329 + ++
ΔC 1-288 1 288 - ++
ΔC 1-242 1 242 - ++
ΔC 1-227 1 227 - +
dl244/275 1 242 276 410 - ++
m60-341 60 341 + ++
human DP-1
WT 1-410 1 410 + ++
ΔC 1-333 1 333 + ++
ΔC 1-273 1 273 - ++
B.
DP-1 WT 1-410 1-380 1-339 1-329 1-288 1-242 1-227
E2F-1 + + - + + - + + - + + - + + - + + - + + - + +
E4-6/7 - + - - + - - + - - + - - + - - + - - + - - +
induced E2F
free E2F
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
C.
DP-1 WT 1-333 1-273
E2F-1 - + + - + + - + +
E4-6/7 - - + - - + - - +
induced E2F
free E2F
1 2 3 4 5 6 7 8 9

FIG. 3. (A) Schematic diagram of murine and human DP-1 proteins and their activities in vitro. The top part shows a schematic representation of the structural regions of the DP-1 coding sequence and the functions assigned to them, as depicted in Fig. 1A. WT, wild type. In the middle part of the diagram, the rectangles represent the coding region of murine DP-1 and the sequences present in each of the mutant proteins. The bottom portion depicts a similar representation for the human DP-1 proteins. The number located at the deletion endpoint of each mutant protein refers to the last remaining amino acid present in that protein. The region delineated as analogous to the marked box of E2F-1 and defined functionally for E4-6/7 and Rb interaction is highlighted with diagonal stripes in each protein. The carboxy-terminal truncations (ΔC) and m60-341 are named according to the amino acid residues remaining in the mutant protein. The internal deletion mutation (*dl*244/275) was named for the amino acids removed by the deletion. A summary of results from experiments presented in Fig. 3B and C is shown in the columns on the right. (B) Interaction of E4-6/7 and hE2F-1 with carboxy-terminal mutant mDP-1 proteins. The E2F double-site DNA probe was incubated with individual mDP-1 mutant proteins alone (lanes 3, 6, 9, 12, 15, 18, and 21), together with hE2F-1 (lanes 4, 7, 10, 13, 16, 19, and 22), and in combination with both hE2F-1 and GST–E4-6/7 (lanes 5, 8, 11, 14, 17, 20, and 23), and DNA-protein complexes were resolved by gel mobility shift assay. hE2F-1 assayed alone and with GST–E4-6/7 is shown in lanes 1 and 2, respectively. The specific mDP-1 mutations assayed are indicated above the horizontal lines at the top. The presence of hE2F-1 or GST–E4-6/7 in a specific reaction is denoted by a + or − over the lane for that reaction. E4-6/7-induced E2F binding activity and free E2F are indicated by arrows on the left. (C) Interaction of E4-6/7 and hE2F-1 with hDP-1 is equivalent to that with mDP-1. The E2F double-site DNA probe was incubated with individual hDP-1 mutant proteins alone (lanes 1, 4, and 7), together with hE2F-1 (lanes 2, 5, and 8), and in combination with both hE2F-1 and GST-E4-6/7 (lanes 3, 6, and 9), and DNA-protein complexes were resolved by gel mobility shift assay. The specific hDP-1 mutations assayed are indicated above the horizontal lines at the top. The presence of hE2F-1 or GST–E4-6/7 in a specific reaction is denoted by a + or − over the lane for that reaction. E4-6/7-induced E2F binding activity and free E2F are indicated by arrows on the right.

not been unambiguously determined. Further mutagenesis will be required to resolve this question. Mutations were similarly generated in the coding sequence of the murine and human DP-1 proteins with similar results. Deletions from the carboxy terminus and internal deletions defined a region of DP-1 (amino acids 243 to 329) required for E4-6/7 binding (Fig. 3B and C). On the linear DP-1 coding sequence, relative to the DNA binding/dimerization domains, this region of DP-1 is

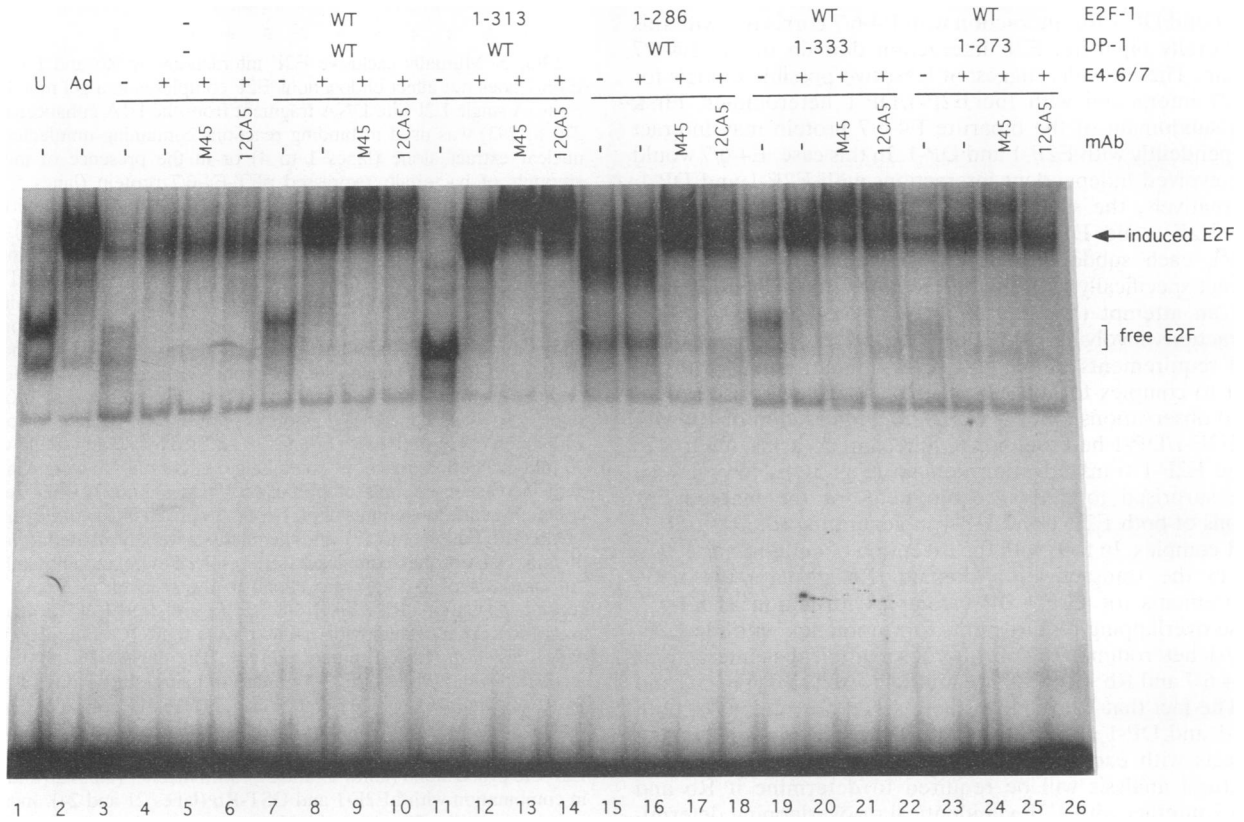

FIG. 4. hE2F-1 and hDP-1 mutant proteins expressed in vivo demonstrate binding properties similar to those of proteins expressed in vitro. C-33A cells were either mock transfected (lanes 3 to 6) or transfected with wild-type (WT) hDP-1 (lanes 7 to 18) in combination with wild-type hE2F-1 (lanes 7 to 10), hE2F-1–ΔC 1-313 (lanes 11 to 14), or hE2F-1–ΔC 1-286 (lanes 15 to 18). Similarly, cells were transfected with wild-type hE2F-1 (lanes 19 to 26) in combination with hDP-1–ΔC 1-333 (lanes 19 to 22) or hDP-1–ΔC 1-273 (lanes 23 to 26). Whole cell extracts were prepared and assayed on the E2F double-site probe in the absence (lanes 3, 7, 11, 15, 19, and 23) or presence (lanes 4 to 6, 8 to 10, 12 to 14, 16 to 18, and 20 to 22) of GST–E4-6/7. After incubation for 1 h, reaction mixtures were placed on ice, and incubation was continued for an additional hour in the absence (lanes 1 to 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, and 24) or presence of MAb M45 (lanes 5, 9, 13, 17, 21, and 25) or 12CA5 (lanes 6, 10, 14, 18, 22, and 26). Uninfected (U; lane 1) and Ad-infected (Ad; lane 2) HeLa nuclear extracts were assayed for comparison. DNA-protein complexes were resolved on a 4% polyacrylamide gel. The presence of GST–E4-6/7 is denoted by a + or − over the lane for that reaction. The form of hE2F-1 or hDP-1 present in the reaction is indicated above the horizontal lines at the top. E4-6/7-induced E2F binding activity (arrow) and free E2F (bracket) are indicated on the right.

localized similarly and has a function analogous to that of the marked box of E2F-1.

Cress et al. (14) examined a large panel of mutants in E2F-1 for transactivation by E4-6/7 in vivo and concluded that the leucine zipper region and C-terminal sequences were required for E4-6/7 function. In our own studies, deletion of the leucine zipper abolished E2F-1 interaction with DP-1 and E4-6/7, although loss of E2F-1/DP-1 heterodimerization would be expected to preclude E4-6/7 binding. These results are consistent with those of Cress et al. and suggest that the N-terminal boundary of E2F-1 required for E4-6/7 interaction extends into the leucine zipper region. By inference, we would predict that this may also be true for DP-1. We did not find a requirement for C-terminal sequences of E2F-1 for E4-6/7 binding in vitro. The transactivation studies of Cress et al. would measure both E4-6/7 binding and transactivation properties, suggesting the interesting possibility that E4-6/7 may influence the C-terminal transactivation domain of E2F-1 by a conformational change or direct interaction. This putative function of E4-6/7 would not be required to observe the induction of cooperative E2F-1/DP-1 binding in vitro. This point remains to be clarified.

Genetic and biochemical analysis of the E4-6/7 protein has defined the E2F interaction domain as structurally bipartite (50, 54). E4-6/7 amino acid residues 81 to 104 and 127 to 150 are both necessary and sufficient for interaction with E2F (54b). The requirement for distinct structural regions of both E2F-1 and DP-1 for interaction with E4-6/7 correlates with this structurally bipartite, E2F interaction domain of the E4-6/7 protein. These results suggest at least two possible models for E4-6/7 interaction with the E2F-1/DP-1 heterodimer. First, each subdomain of the bipartite E4-6/7 protein may interact independently with E2F-1 and DP-1. In this case, E4-6/7 would have evolved independent interactions with E2F-1 and DP-1. Alternatively, the subdomains of E4-6/7 could interact with both E2F-1 and DP-1 as a composite interface. In such a model, each subdomain of E4-6/7 would have evolved to interact specifically with the heterodimer as a structural unit.

In an attempt to understand the mutually exclusive E2F interactions involving E4-6/7 and Rb, we analyzed the structural requirements of the E2F-1/DP-1 heterodimer with respect to complex formation with Rb. As expected from published observations (14, 22, 27, 28, 37), interaction of Rb with the E2F-1/DP-1 heterodimer requires amino acids 409 to 426 of the E2F-1 transactivation domain (Fig. 5C). However, we were surprised to find a requirement for the marked box regions of both E2F-1 and DP-1 in generating the Rb–E2F-1/DP-1 complex. In fact, with the exception of amino acids 409 to 426 in the transactivation domain, Rb displays the same requirements for E2F-1/DP-1 complex formation as E4-6/7. These overlapping requirements for interaction with the E2F-1/DP-1 heterodimer explain the observation that interactions of E4-6/7 and Rb with E2F are mutually exclusive (Fig. 5A and B). The fact that Rb requires the marked box regions of both E2F-1 and DP-1 also suggests that Rb makes protein-protein contacts with each of these proteins. A detailed genetic or structural analysis will be required to determine if Rb and E4-6/7 interact with E2F via identical or overlapping determinants.

These results are entirely consistent with the results of Helin et al. (29), who demonstrated that both E2F-1 and DP-1 are required for stable interaction with Rb in vivo. Thus, in vivo binding when assayed by coimmunoprecipitation (29) or in vitro binding when assayed by a gel mobility shift assay (Fig. 5C) exhibits more stringent requirements for Rb binding to E2F activity compared with the ability of Rb to bind independently to C-terminal sequences of E2F-1 when assayed in the context of a GST precipitation assay (22, 27, 28, 37). However, only the C-terminal segment of E2F-1 is required for repression of the transcriptional activity of E2F-1 in vivo when assayed in the context of a GAL4 fusion protein (22, 27). It

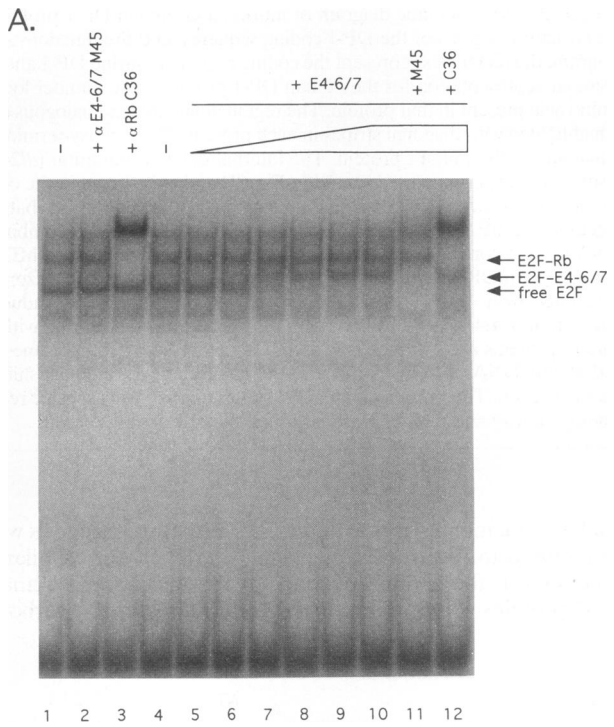

FIG. 5. Mutually exclusive E2F interactions by Rb and E4-6/7. (A) E4-6/7 does not affect endogenous E2F complexes in a gel mobility shift assay. A single E2F site DNA fragment from the E1A enhancer (Ad5 nt 270 to 293) was used in binding reactions containing uninfected HeLa nuclear extract alone (lanes 1 to 4) or in the presence of increasing amounts of bacterially expressed pET-E4-6/7 protein (lanes 5 to 12). Lanes 10 to 12 have equivalent amounts of pET-E4-6/7 protein. Following a 30-min incubation, MAb M45 (αE4-6/7 to M45; lanes 2, 11) or C36 (αRb C36; lanes 3 and 12) was added, and incubation was continued for an additional 30 min. DNA-protein complexes were resolved by gel mobility shift assay. Rb-E2F, E4-6/7–E2F, and free E2F protein-DNA complexes are indicated with arrows on the right. (B) An order-of-addition experiment demonstrates mutually exclusive E2F binding by E4-6/7 and Rb. The single E2F site probe was incubated with HeLa cell cytoplasmic extract either alone (lanes 1 and 2), with an excess of GST-Rb (lanes 3 to 9), with increasing amounts of pET-E4-6/7 (lanes 11 to 17), or with an excess of pET-E4-6/7 (lanes 10 and 18) for 30 min. Following the 30-min incubation, reactions containing excess GST-Rb were challenged with increasing amounts of pET-E4-6/7 (lanes 3 to 9) while reactions containing various amounts of pET-E4-6/7 were challenged with an excess of GST-Rb (lanes 11 to 17), and the mixtures were incubated for another 30 min. DNA-protein complexes were resolved by gel mobility shift assay. An excess of pET-E4-6/7 was added to the reaction in lane 2 for the second incubation. Rb-E2F, E4-6/7–E2F, and free E2F protein-DNA complexes are indicated with arrows on the right. (C) Interaction of Rb with hE2F-1 and hDP-1 mutant proteins. The single E2F site probe was incubated with individual E2F-1 mutant proteins either alone (lanes 1, 4, 7, 10, 13, and 16), with hDP-1 (lanes 2, 5, 8, 11, 14, and 17), or in combination with hDP-1 and GST-Rb (lanes 3, 6, 9, 12, 15, and 18). Similarly, individual mutant hDP-1 proteins were incubated with single E2F site probe alone (lanes 19 and 22), with hE2F-1 (lanes 20 and 23), or in combination with hE2F-1 and GST-Rb (lanes 21 and 24). Incubation was continued for 30 min, and DNA-protein complexes were resolved by gel mobility shift assay. The form of hE2F-1 or hDP-1 present in the reaction is indicated above the horizontal lines at the top. Rb-E2F and free E2F complexes are indicated with arrows on the right. WT, wild type.

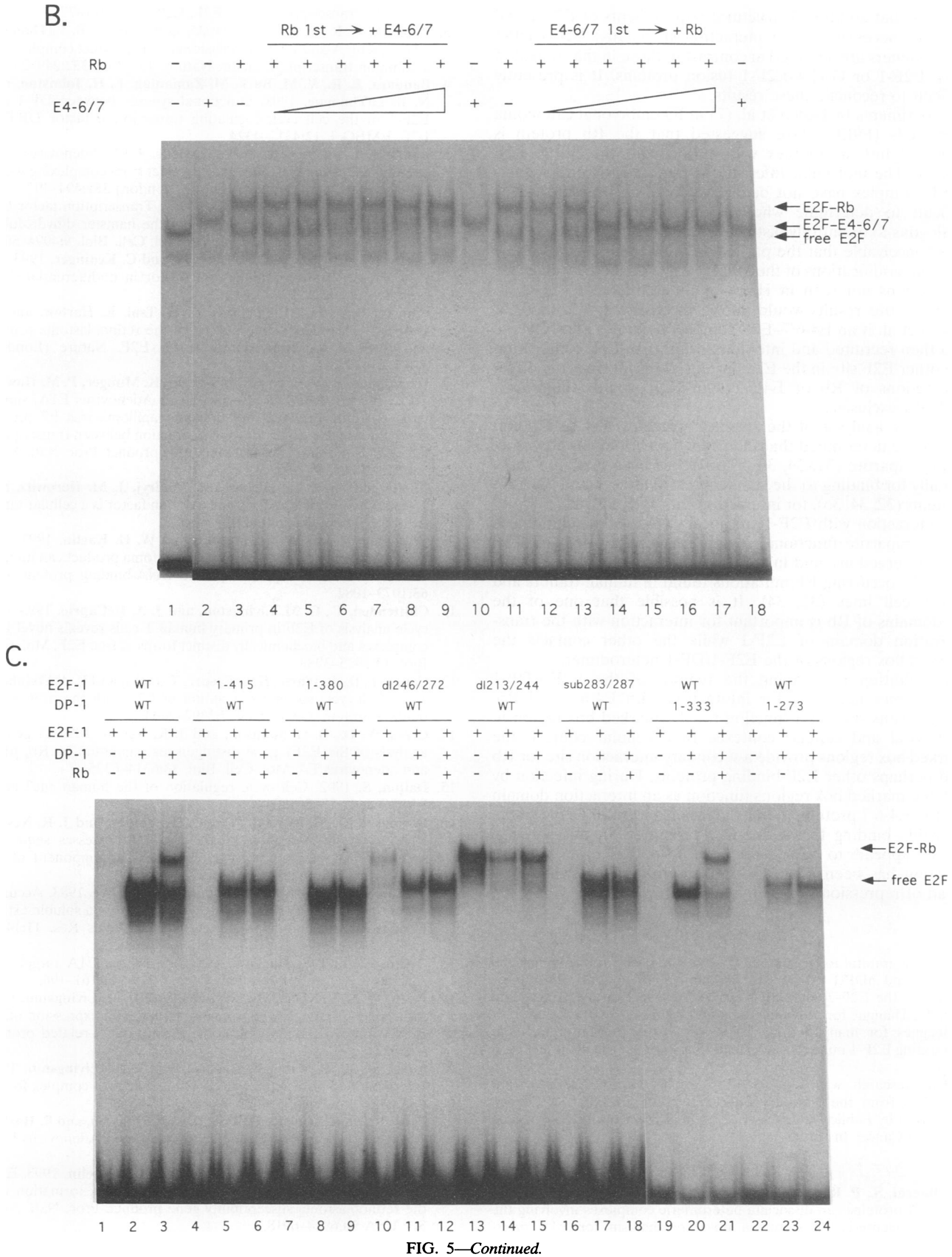

FIG. 5—*Continued.*

appears that additional structural requirements of E2F-1 and DP-1 are necessary for Rb interaction when native E2F-1/DP-1 heterodimers are analyzed in contrast to those in the context of GST–E2F-1 or GAL4–E2F-1 fusion proteins. It is presently difficult to reconcile these results.

Experiments by Bocco et al. (7) in F9 embryonal carcinoma stem cells (F9EC) have suggested that the Rb protein is recruited into a complex with E4-6/7 and the F9EC E2F activity. The molecular interactions required to generate this F9EC complex have not been described, and therefore it is difficult to determine whether this represents a situation analogous to or entirely distinct from that found in HeLa cells. It is conceivable that the particular components or posttranslational modifications of the F9EC E2F or Rb activity allow for interactions not seen in HeLa or differentiated F9 cells. In addition, our results would not be inconsistent with those of Bocco et al. if an E4-6/7–E2F complex bound to one E2F site and then recruited and interacted with a Rb-E2F complex on the other E2F site in the E2a promoter. In this case, the direct interactions of Rb or E4-6/7 with E2F would still remain mutually exclusive.

Genetic analyses of the "pocket" proteins such as Rb and p107 have determined that their functional domains are structurally bipartite (32, 34, 36, 57). Rb has been analyzed functionally for binding to the simian virus 40 large T and Ad E1A proteins (32, 34, 36), for interaction with E2F activity (57), and for interaction with E2F-1 specifically (64). Both subdomains of this bipartite functional region are important for each of these interactions, and in addition, they are common sites for naturally occurring Rb mutations found in human tumors and tumor cell lines (32, 34). It is possible that one of the subdomains of Rb is important for interaction with the transactivation domain of E2F-1 while the other contacts the marked box regions of the E2F-1/DP-1 heterodimer.

In addition to defining the regions of the E2F-1/DP-1 heterodimer necessary for interaction with E4-6/7 and Rb, these results suggest a function for the marked box region in both viral and cellular contexts. In a cellular context, the marked box regions provide a secondary interaction site for Rb and perhaps other E2F-binding proteins. During infection by Ad, the marked box regions function as an interaction domain for the E4-6/7 protein, thereby creating a form of E2F capable of stable binding to the Ad E2a promoter. In this manner, E4-6/7 appears to be a highly evolved pocket protein whose function has been adapted for transcriptional activation instead of repression.

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