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Interacting Domains of E2F1, DP1, and the Adenovirus E4 Protein

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Recent experiments demonstrate that a family of related proteins constitute the E2F transcription factor activity and that the interaction of two of these gene products, E2F1 and DP1, generates a heterodimer with DNA binding and transcriptional activating capacity. Previous experiments have shown that the adenovirus E4 19-kDa protein facilitates the formation of a stable E2F dimer on the adenovirus E2 promoter. We now show that coexpression of the E2F1 and DP1 products in transfected SAOS-2 cells, together with the E4 product, generates a multicomponent complex with specificity to the adenovirus E2 promoter. Using a yeast two-hybrid assay system, we find that the E2F1 hydrophobic heptad repeat (E2F1 amino acid residues 206 to 283) allows interaction with a corresponding domain of the DP1 protein (amino acids 196 to 245). We also find that the adenovirus E4 protein interacts with the DP1 hydrophobic heptad repeat domain, but we could not detect a direct interaction between E2F1 and E4. Additional assays demonstrate that the E4 protein can dimerize. Since our previous experiments have shown that mutations within the E2F1 hydrophobic heptad repeat element abolish the E4-mediated transcription enhancement in transfection assays, we conclude that the E4 protein likely interacts with the E2F1-DP1 heterodimer by directly binding to the DP1 product. As a consequence of the ability of E4 to dimerize, we propose that the stable complex formed on the two E2F sites within the E2 promoter is composed of two E2F1-DP1 heterodimers held together by an E4 dimer.

Much attention has focused on the E2F transcription factor as a functional target for the growth-suppressing action of the retinoblastoma gene product (Rb) as well as other members of the Rb family, and a variety of studies have shown that the ability of E2F to stimulate transcription coincides with its release from such interactions (30). The normal targets for E2F action appear to include a group of genes expressed at the beginning of S phase that encode activities essential for the replication of DNA, such as the dihydrofolate reductase gene (35). At least part of the process of S-phase entry would appear to involve the accumulation of active E2F, either as a function of normal G_1 regulation or as a consequence of the action of viral oncoproteins that can release E2F from inhibitory complexes, since the overexpression of E2F can drive quiescent cells into S phase (22).

A series of recent experiments have shown that the previously defined E2F activity is composed of a family of polypeptides of related sequence and structure. The initial isolation of the E2F1 clone (14, 23, 34), followed by the isolation of the DP1 clone (11), has now led to the isolation of several additional clones that encode E2F-related polypeptides (15, 21, 27). Previous assays had provided biochemical evidence that the E2F activity resulted from heterodimer formation involving two distinct polypeptides (20), and subsequent experiments demonstrated that the products of the E2F1 and DP1 genes represented two such polypeptides. These two cDNAs encode proteins that physically interact both in vitro and in vivo, resulting in enhanced E2F DNA binding and transcriptional activating ability (2, 15, 26). The interaction of E2F1 and DP1 involves putative hydrophobic heptad repeats in both proteins, potentially allowing the formation of a coiled-coil structure. Additional sequences in both proteins are essential for DNA binding activity. Finally, other sequences in the C-terminal region of the E2F1 polypeptide are essential for transcriptional activating capacity as well as the binding to Rb and related proteins (7, 14, 23, 34).

In addition to the adenovirus E1A protein, which releases E2F from inhibitory complexes, the virus encodes another protein, a 19-kDa product of the early E4 gene, that alters the DNA binding properties of E2F. The E4 protein has been shown to physically interact with E2F and facilitate the formation of a very stable DNA-protein complex on the adenovirus E2 promoter (12, 13, 19, 28, 29). Our previous experiments have shown that the hydrophobic repeat sequences in the E2F1 polypeptide are essential for the adenovirus 19-kDa E4 protein to coactivate transcription with E2F1 (7). We have now further analyzed the capacity of the adenovirus E4 protein to stimulate E2F-dependent transcription. We demonstrate that the E2F1-DP1 heterodimer is a target for the 19-kDa adenovirus E4 protein. Furthermore, we have used a yeast two-hybrid system to further dissect the interactions of E2F1, DP1, and adenovirus E4. We find that the DP1 heptad repeat domain can bind to the E4 protein, which itself can dimerize, leading to the formation of a multisubunit protein complex at the adenovirus E2 promoter containing E2F1, DP1, and E4.

MATERIALS AND METHODS

Construction of yeast vectors. The yeast two-hybrid expression plasmids pHB44 (4), pHB18 (4), and pGAD10 (6) were gifts from Hal Bogerd and Bryan Cullen. Plasmid pHB44 is predicted to express a fusion protein consisting of the GAL4 DNA-binding domain (G4DBD) (25) fused N terminally to the test protein. Plasmid pHB18 is predicted to express a fusion protein consisting of a protein nuclear localization signal (24) fused to the acidic activation domain of VP16 (VPAD) (36) fused, in turn, to the test protein. Plasmid pGAD10 (6) is predicted to express a fusion protein consisting of the GAL4 transcriptional activation domain (G4AD) fused to the test protein.

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Full-length E2F1 was cloned into the *Eco*RI sites of pHB44 and pHB18 by excising a *Bam*HI E2F1 cDNA fragment from pCMV-E2F1 (7), adding an adaptor to generate an in-frame *Eco*RI site, cutting with *Eco*RI, and ligating into the *Eco*RI cut vectors. Plasmid pHB18-E2F1$_{full\ length}$ appeared to be toxic to *Saccharomyces cerevisiae* GGY::171, perhaps as a result of overexpression of a protein containing two very strong activation domains (3). To circumvent this problem, full-length E2F1 was cloned into pGAD10, which is expected to possess a weaker promoter than pHB18 (4, 6), to yield plasmid pGAD-E2F1$_{full\ length}$, which expresses full-length E2F1 as a fusion with the GAL4 transcriptional activation domain, which is not growth inhibitory to GGY::171. Various E2F1 subfragments were also cloned into the various expression vectors by using artificial *Eco*RI sites which have been described previously (7). A cDNA encoding human DP1 amino acids 24 to 410 was cloned into pHB44 and pHB18 by cutting pBSK-hDP1 (15) with *Sma*I, ligating an *Eco*RI linker (12 bp; Boehringer Mannheim), cleaving with *Eco*RI, and cloning into the *Eco*RI sites of each of the vectors. Insert orientations of constructs were determined by restriction analysis, and the cloning junctions of all constructs were sequenced to ensure that the proper reading frames were intact. pHB18-DP1$_{152\text{-}245}$ and other DP1 deletion mutants were generated by PCR. The adenovirus E4 constructs (and DP1 deletion mutants) were generated by PCR and were sequenced (33) to determine that no PCR-generated mutations were present. The forward PCR primers used to generate DP1 deletions were DP1$_{F152}$ (CCGAGATCTCTTCT AGACACATCTTACCAAACGAG), DP1$_{F163}$ (CCGAGATC TCTTCTAGAATGAAAAACATAAGACGG), DP1$_{F180}$ (CC GAGATCTCTTCTAGAATGAACATCATCTCCAAG), and DP1$_{F196}$ (CCGAGATCTCTTCTAGAATGCCCACCAACTC GGCT). The two reverse primers were DP1$_{R204}$ (CGGGAAT TCGTTCTGACATTCCTGAGC) and DP1$_{R245}$ (CGGGAAT TCCTGCTCCGCATGCCGGTT). The sequences of the internal primers used along with DP1$_{F152}$ and DP1$_{R245}$ in four-primer mutagenesis (17, 18) to generate DP1 2×KE, in which DP1 lysine residues 217 and 219 are changed to glutamate, were CTTGAAAGAATAGAACAGGAACAGTCTC AAC (forward) and GTTGAGACTGTTCCTGTTCTATTCT TTCAAG (reverse).

pYEP51 NcoI (5) and pJLBlacZ (9) were gifts from Scott Hiebert. pE2lacZ was constructed by cloning a double-stranded oligonucleotide containing the two E2F sites of the adenovirus E2 promoter into the *Sal*I sites of pJLBlacZ. pYEP51-E2F1$_{full\ length}$, which is predicted to express full-length E2F1 from a galactose-inducible promoter, was constructed by excising a *Bam*HI E2F1 cDNA fragment from pCMV-E2F1 (7), adding an adaptor to generate an in-frame initiator methionine and *Nco*I site, cutting with *Nco*I, and ligating into *Nco*I-cut pYEP51 NcoI.

Maintenance and transfection of yeast strains. Yeast strains GGY::171 (*gal4 gal80 his3 leu2*) and SGP3 (*ura3 leu2 ade8*), gifts from Hal Bogerd and Steve Garrett, respectively, were each maintained in YEPD medium and were transformed by using an alkali-cation yeast transformation kit (Bio 101, Inc., La Jolla, Calif.). Transformants were selected on supplemented dextrose plates lacking the appropriate amino acids. To estimate β-galactosidase activities, at least three transformant colonies from each transfection were restreaked and then inoculated into 5 ml of an appropriate liquid medium. Equal optical density units of liquid cultures were assayed for β-galactosidase activity, using a permeabilized cell assay and chlorophenol red-β-D-galactopyranoside as the colorimetric substrate (1).

Transfection assays and cell culture. The SAOS-2 cell line (obtained from the American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were carried out by calcium phosphate coprecipitation. Each 100-mm-diameter plate received 8 μg of E2F1- and/or DP1-expressing plasmids. Cells were exposed to DNA precipitates for 8 h and were then washed and maintained in Dulbecco's modified Eagle's medium including 10% fetal calf serum for 24 h. Cells were then washed with phosphate-buffered saline (PBS), scraped into 1 ml of PBS, pelleted, resuspended in lysis buffer, and then lysed by freeze-thawing. Lysis buffer contained 10% (wt/vol) glycerol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.4 mM NaF, 0.4 mM Na_3VO_3, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor, each at 50 ng/ml). Insoluble cellular material was pelleted by centrifugation, and the protein concentration of the supernatant was determined by a dye-binding protein assay (Bio-Rad).

DNA binding assays. A plasmid DNA fragment from the adenovirus E2 promoter containing two E2F recognition sites but lacking the ATF site was used as probe in DNA binding assays, which were performed essentially as described previously (29, 37). Whole-cell extracts were prepared from SAOS-2 cells transfected with appropriate E2F1 (7) and DP1 (15) expression plasmids as described above. Binding reaction mixtures received 2.0 μg of total cellular protein. When included in DNA binding assays, E4 protein was generated in vitro by a coupled transcription-translation protocol (Promega TNT) (29).

RESULTS

Formation of the E2F1-DP1 heterodimer allows interaction with the adenovirus E4 protein. A variety of experiments have shown that the adenovirus E4 protein can alter the DNA binding properties of the E2F transcription factor (12, 13, 19, 28, 31, 32). Recent experiments have shown that the E2F1 product can form a heterodimer with the DP1 product, resulting in greatly enhanced DNA binding and transcriptional activating capacity (2, 15, 26). In addition, cotransfection assays have allowed an identification of sequences within the E2F1 product that are essential for DNA binding (7, 14, 23, 34) as well as interaction with the DP1 product (2, 15, 26). We have used such an assay for E2F1-DP1 heterodimer formation so as to define the role of this heterodimer as a target for the adenovirus 19-kDa E4 protein.

SAOS-2 cells were cotransfected with E2F1 alone, DP1 alone, and E2F1 and DP1 together. Cells were harvested 24 h after transfection, and whole-cell extracts were prepared. These extracts were assayed for E2F DNA binding activity with a DNA probe from the adenovirus E2 promoter, using an electrophoretic mobility shift assay as previously described (37). Whereas the level of E2F-specific DNA binding activity in extracts of cells transfected with either E2F1 or DP1 alone is very low under the conditions of this assay, there is a dramatic increase in total cellular E2F activity upon cotransfection of E2F1 and DP1 (Fig. 1A, lane 6), similar to the recent results of others (15, 26). When the DNA binding specificity of the E2F1-DP1 complex was tested by a competition analysis, the E2F1-DP1 complex was greatly diminished by the addition of unlabeled wild-type competitor but unaffected by the presence of an equal excess of the mutant (nonbinding [16] oligonucleotide) (Fig. 1B, lanes 4 to 6).

Further evidence for the formation of an E2F1-DP1 het-

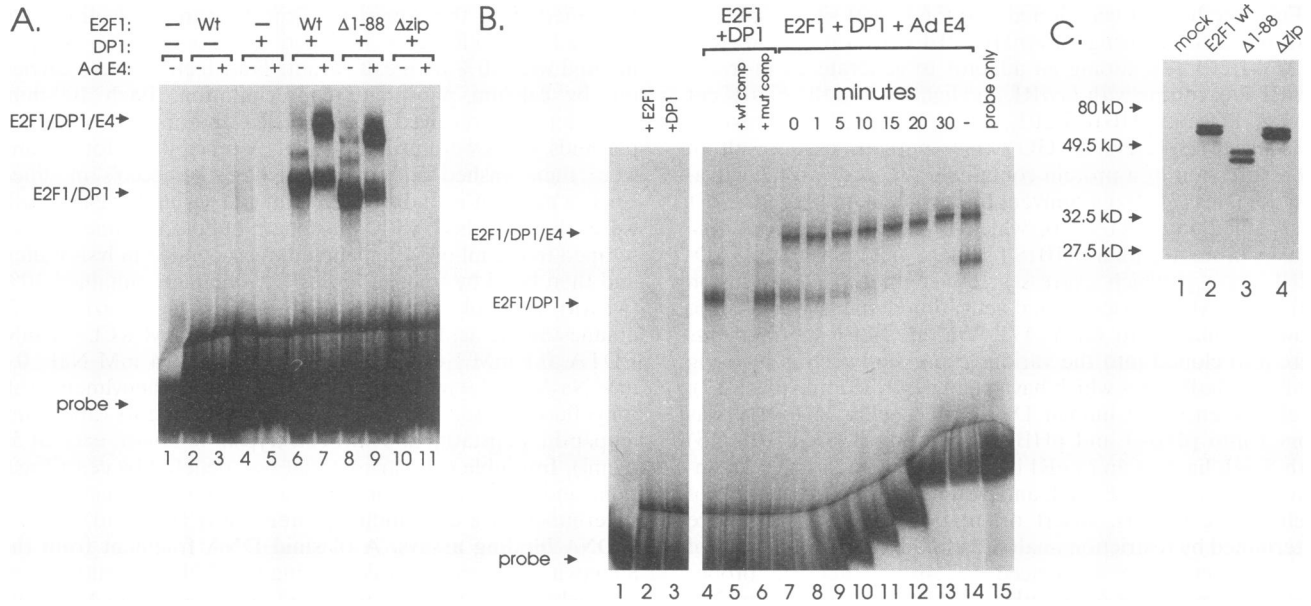
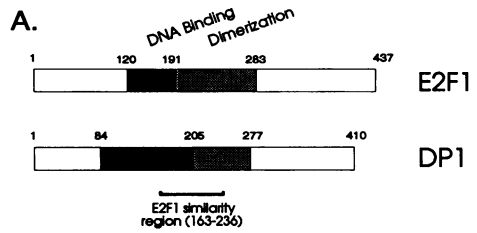


FIG. 1. Formation of an E2F1-DP1-E4 DNA complex is dependent on the E2F1 hydrophobic heptad repeat. (A) An electrophoretic mobility shift DNA binding assay was performed with a fragment of the adenovirus (Ad) E2 promoter and extracts of mock-transfected SAOS-2 cells (lane 1) or SAOS-2 cells transfected with plasmid expressing full-length E2F1, a derivative of E2F1, and/or full-length human DP1 (lanes 2 to 11). The assays depicted in lanes 3, 5, 7, 9, and 11 also received 0.1 μl of in vitro-translated adenovirus E4 protein. The positions of the E2F1-DP1 and E2F1-DP1-E4 complexes are indicated with arrows. The mutants designated Δ1-88 and Δzip are identical to *dl*1-88 and *dl*206-220, respectively, described in reference 7. The DNA fragment used in the mobility shift assay contained E2 promoter sequence between positions −28 and −85, which includes two E2F sites (TTTCGCGC) centered two helical turns apart on the DNA (specifically, TTTCGCGCCCTTTCTCAAATTTA AGCGCGAAA). Wt, wild type. (B) Competition and dissociation rate assays. Assays included extract from mock-transfected cells (lane 1) or extract from E2F1-transfected (lane 2) or DP1-transfected cells (lane 3). Assay of the E2F1-DP1 complex (same as lane 6 in panel A) is shown in lane 4. Competition assays used 10 ng (about a 50- to 100-fold excess over the labeled probe) of wild-type (wt; lane 5) or mutant (mut; lane 6) competitor fragments are added just prior to the addition of extract to the binding assay. For the dissociation rate assays (lanes 7 to 13), binding reaction mixtures were incubated for 30 min prior to the addition of 100 ng of unlabeled competitor. The binding reaction mixtures were then loaded on the running gel at the indicated times after the addition of competitor. Lane 14 was not challenged with unlabeled competitor but was incubated and loaded along with the 30-min-plus-competitor time point. (C) Western blot analysis. Thirty micrograms of the protein extracts used in panel A from SAOS-2 cells transfected with the indicated plasmids was resolved in a sodium dodecyl sulfate–10% acrylamide gel, transferred to nitrocellulose, and probed with a commercially available monoclonal antibody raised against the E2F1 C-terminal sequences (Santa Cruz Biotechnology).

erodimer was provided by assays of E2F1 derivatives. Previous studies have used bacterially produced fusion or in vitro-translated (26) proteins to demonstrate E2F1-DP1 interactions in vitro and to map the interacting regions (2, 15). This analysis implicated a direct interaction between E2F1 and DP1 through the E2F1 hydrophobic heptad repeat element. To confirm the importance of this region in E2F1-DP1 interactions in mammalian cells, we cotransfected two previously described E2F1 deletions (7) along with DP1 into SAOS-2 cells and assayed for an increase in total cellular E2F activity. As shown in Fig. 1A, cotransfection of DP1 and an E2F1 derivative lacking N-terminal amino acids 1 to 88 greatly increases total E2F activity (lanes 8 and 9). As expected, the $E2F1_{\Delta 1\text{-}88}$-DP1 complex exhibits an increased electrophoretic mobility relative to the full-length E2F1-DP1 complex, consistent with the smaller size of the mutant E2F1 polypeptide. This result is in contrast to the deletion of two helical turns of the putative E2F1 hydrophobic heptad repeat, $E2F1_{\Delta zip}$, which abolished the cooperative interaction with DP1 (lanes 10 and 11), even though this E2F1 mutant retains about 40% of the transcriptional activating capacity of wild-type E2F1 (7) and is expressed at a level indistinguishable from that of the wild-type protein, as determined by Western blot (immunoblot) analysis (Fig. 1C).

Using these conditions to assay for the function of the E2F1-DP1 heterodimer, we measured the interaction of the adenovirus 19-kDa E4 protein by addition of in vitro-translated adenovirus 19-kDa E4 protein to the extracts of cells transfected with E2F1 and DP1 (Fig. 1A). The addition of the E4 protein resulted in the shift of the E2F1-DP1 complex to a slower mobility (lanes 6 and 7), consistent with previous assays for cellular E2F activity (28, 29). A variety of previous experiments have shown that the interaction of E2F alone with DNA is unstable, whereas the E2F-E4 complex formed on the E2 promoter, dependent on the precise spacing and orientation of the E2F binding sites in this promoter, is very stable (28, 29). This property is also exhibited by the E2F1-DP1 heterodimer together with the adenovirus E4 protein. As shown in Fig. 1B, the E2F1-DP1 heterodimer rapidly dissociates from the DNA probe, whereas the E2F1-DP1-E4 complex is undiminished for at least 30 min. We conclude from these results that the interaction of the adenovirus E4 protein with the E2F1-DP1 heterodimer produces a DNA binding complex that exhibits the properties of the previously described E2F-E4 interaction on the E2 promoter.

A yeast two-hybrid assay reveals protein-protein interactions involving E2F1, DP1, and adenovirus E4. To study the interactions between the E2F1, DP1, and E4 polypeptides in more detail, we have used a two-hybrid assay system which can identify specific protein-protein interactions of fusion proteins expressed in the yeast *S. cerevisiae* (8). This assay thus allows



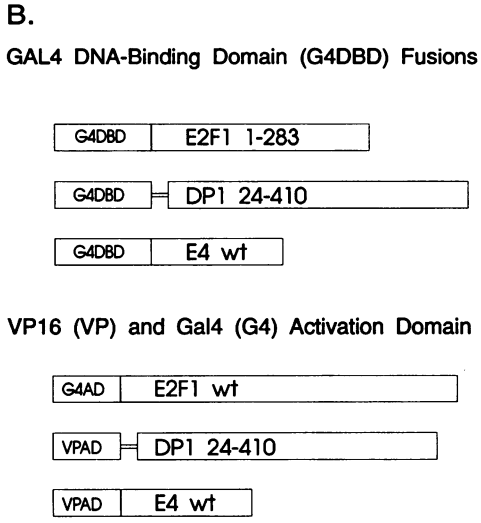


FIG. 2. Structures of E2F1 and DP1 fusion proteins. (A) Functional elements of the E2F1 and DP1 products. The indicated functional elements have been previously defined (2, 7, 14, 15, 21, 23, 26, 34). (B) Two-hybrid fusion protein constructs. VPAD, G4AD, and G4DBD fusion proteins were expressed in GAL4-LacZ reporter yeast strain GGY::171, using the previously described vectors pHB18 (4), pGAD10 (6), and pHB44 (4), respectively. Full-length E2F1 is predicted to be 437 amino acids in length (14, 23) and that numbering designation has been used. Full-length E4 is predicted to be 150 amino acids long (28). Full-length mouse or human DP1 is predicted to be 410 amino acids long (11, 15), and we have used that numbering designation in this report. The relative β-galactosidase activities in the various double-transformant colonies are presented in Table 1. wt, wild type.

one to distinguish between DNA binding effects and protein-protein interaction effects. The assay utilizes a reporter yeast strain which contains a chromosomally inserted *GAL1-lacZ* fusion gene (10). This reporter strain is cotransformed with two selectable plasmids that each express fusion proteins. The first plasmid expresses a given protein as a fusion with the GAL4 DNA-binding domain, thus tethering this fusion to the GAL4 sites in the *GAL1-lacZ* reporter gene. The second plasmid expresses a given protein as a fusion with a transcriptional activation domain. Interaction of the two test proteins recruits the transcriptional activation domain to the promoter of the *GAL1-lacZ* reporter gene and produces an increase in the expression of β-galactosidase.

The initial series of E2F1, DP1, and E4 two-hybrid constructs that were used are shown in Fig. 2. These plasmids were cotransformed into the yeast reporter strain GGY::171, and the average level of β-galactosidase which accumulated in the double-transformant colonies was measured. As expected from previous domain mapping experiments (34), the fusion of full-length E2F1 to the GAL4 DNA-binding domain resulted in a strong activation of the β-galactosidase reporter as a result

TABLE 1. E2F1, DP1, and adenovirus E4 interactions[a]

G4DBD	Fold increase in β-galactosidase activity[b]			
	Vector only	G4AD-E2F1	VPAD-DP1	VPAD-E4
$E2F1_{1-283}$	1	1	550	1
$DP1_{24-410}$	1	310	1	2
E4	1	1	55	9

[a] The two-hybrid fusion protein constructs represented in Fig. 2 were cotransformed in various combinations into the reporter yeast strain GGY::171. β-Galactosidase activities were normalized to the amount of β-galactosidase expressed in cells transformed with the G4DBD fusion and the empty activation domain vector pHB18 (vector only).

[b] Relative to the GAL4 fusion alone.

of the presence of an acidic activation domain at the C terminus of E2F1 (data not shown). In contrast, the fusion of DP1 to the GAL4 DNA-binding domain did not appear to activate transcription, suggesting that DP1, unlike E2F1, does not have an activation domain which is functional in yeast cells, at least under the conditions of this assay.

To circumvent the fact that the full-length E2F1 fused to GAL4 activated transcription, a fusion of the GAL4 DNA-binding domain with the $E2F1_{1-283}$ deletion mutant, which lacks the E2F1 activation domain, was constructed. Indeed, the β-galactosidase assays reveal that the G4DBD-$E2F1_{1-283}$ fusion activates transcription at only a very low level (Table 1). However, expression of this fusion protein together with a protein containing the VP16 activation domain fused to DP1 sequences (VPAD-$DP1_{24-410}$) resulted in substantial transcription activation, yielding a 550-fold increase over the level with G4DBD-$E2F1_{1-283}$ alone. Additional assays demonstrated that there was no activation of transcription in cells expressing proteins carrying the same fusion, either E2F1 or DP1 (Table 1). Thus, there is no indication that either E2F1 or DP1 was capable of forming a homodimer, at least in this assay system. These results therefore demonstrate a capacity for the E2F1 and DP1 products to heterodimerize but not to form homodimers, consistent with in vitro binding assays as well as cotransfection experiments in mammalian cells (2, 15).

These assays also presented the opportunity to measure the ability of E2F1 or DP1 to interact with the adenovirus E4 protein. Using fusions containing the E2F1 sequences fused to either the DNA-binding domain or the activation domain, we found no evidence for an interaction between E2F1 and the adenovirus E4 protein. In contrast, DP1 does appear to interact directly with E4, as evidenced by the 55-fold increase in β-galactosidase activity in the presence of VPAD-DP1. Although we did not observe a strong activation when full-length DP1 (amino acids 24 to 410) was expressed as the DNA-binding-domain fusion and E4 was expressed as the activation domain fusion, we did observe an activation of approximately 10-fold when a shorter DP1 fusion (containing amino acids 152 to 245) was used as the G4DBD fusion (data not shown). From these results, we conclude that E4 can make direct contact with DP1 but not with E2F1.

We have also assayed for the ability of the E4 protein to dimerize in the two-hybrid assay system. In contrast to the results with E2F1 and DP1, we did find evidence for dimerization of the E4 protein. The G4DBD-E4 fusion protein did not activate transcription strongly when expressed alone, but coexpression of a VPAD-E4 fusion protein resulted in a ninefold increase in β-galactosidase activity. From these results, we conclude that the E4 protein can interact with DP1 but not E2F1 and that the E4 protein can self-dimerize.

The E2F1-DP1 and DP1-E4 interactions are both mediated

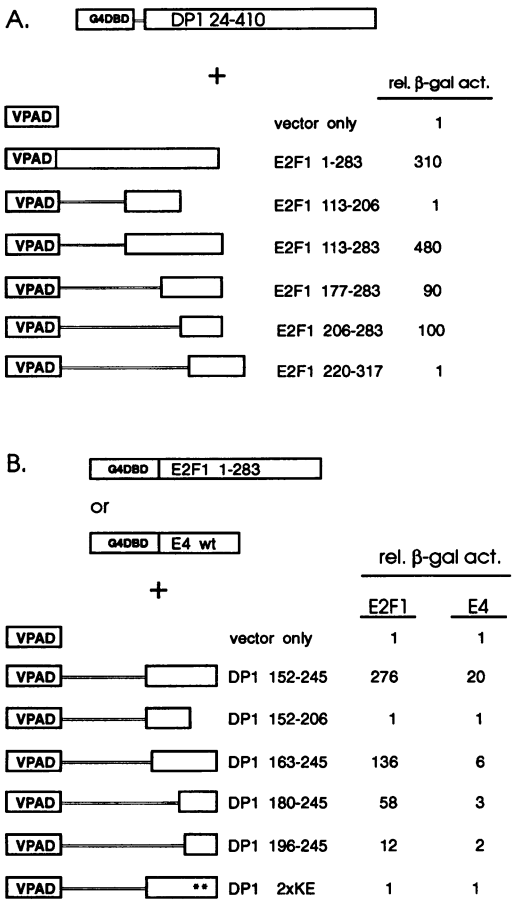


FIG. 3. Two-hybrid E2F1 and DP1 deletion mutants. (A) Mapping of E2F1. A series of VPAD-E2F1 fusions were constructed and tested against G4DBD-$DP1_{24-410}$ in the two-hybrid assay to map interacting domains. The resulting fold increases in β-galactosidase (β-gal) activities, relative to the activity of G4DBD-$DP1_{24-410}$ cotransformed with the VPAD vector alone, are presented on the right. (B) Mapping of DP1. A series of VPAD-DP1 fusions were constructed and tested against G4DBD-$E2F1_{1-283}$ and G4DBD-E4 in the two-hybrid assay to map the E2F1- and E4-binding regions of DP1. The resulting fold increase in β-galactosidase activities, relative to the activities of the respective G4DBD fusion constructs cotransformed with the VPAD vector alone, are presented on the right. **, substitution of DP1 residues K217 and 219 with glutamate in the context of amino acids 152 to 245. wt, wild type.

through a putative DP1 hydrophobic heptad repeat element. We next sought to define the regions of E2F1 and DP1 that were responsible for the various interactions by subcloning various subfragments of E2F1 and DP1 into the yeast two-hybrid plasmids. Figure 3A shows a series of VPAD-E2F1 fusion proteins that were tested for interaction with G4DBD-$DP1_{24-410}$. The resulting levels of β-galactosidase activity in the double-transformant colonies revealed that the two-hybrid interaction between E2F1 and DP1 was dependent on the putative E2F1 hydrophobic heptad repeat element localized to residues 206 to 283. For instance, the 113-206 fusion was inactive, whereas the 113-283 fusion gave high activity. Deletion mutants of E2F1 containing amino acids 177 to 283 and 206 to 283 (corresponding to deletion of the independent E2F1 DNA-binding domain; see Fig. 4) retained DP1 interaction although the β-galactosidase signal was reduced, perhaps indicating that this region contributes to the E2F1-DP1 interaction. N-terminal deletion of E2F1 beyond amino acid 206 eliminated the interaction between E2F1 and DP1.

The region of E2F1 that interacts with DP1 exhibits homology to DP1 amino acids 152 to 245 (11), and as shown in Fig. 3B, this region of DP1 appears to be fully capable of interactions with E2F1. In contrast, a shorter version containing DP1 residues 152 to 206, which deletes the putative hydrophobic heptad repeat, did not interact with E2F1. Gradual elimination of N-terminal residues beginning at amino acid 152 diminished the signal but did not eliminate interactions. Finally, changing two lysine residues (K217 and K219) located in the middle of the hydrophobic heptad repeat to glutamic acid residues, creating VPAD-$DP1_{2\times KE}$, abolished the interaction with E2F1.

Assays of these same DP1 fusions for interaction with the adenovirus E4 protein revealed the same pattern as seen for E2F1 interaction. Thus, we conclude from these results that the putative DP1 hydrophobic repeat element mediates both the interaction with E2F1 and the interaction with E4.

The E2F1 DNA-binding domain specifically recognizes E2F elements in yeast cells independently of DP1. Previous analyses mapped the regions of E2F1 and DP1 that were responsible for E2F site-dependent DNA binding (11, 14, 15, 21, 23, 34). However, these analyses used bacterially expressed proteins, and extremely high concentrations of proteins were necessary to observe specific binding. As an alternative test for DNA binding capacity, the full-length E2F1 cDNA was cloned into a yeast vector (pYEP51 NcoI [5]) to express E2F1 under the control of a galactose-inducible promoter. This plasmid was then cotransfected into yeast strain SGP3 (*leu2 ura3 ade8*) with reporter plasmids in which the β-galactosidase gene was placed under control of a wild-type or mutant adenovirus E2 promoter. Double-transformant colonies were then grown in inducing and noninducing selective media, and the resulting β-galactosidase activities were scored (Fig. 4A). In the absence of exogenous E2F1, the wild-type E2 reporter yielded more activity than the mutant promoter, suggesting an endogenous activity in SGP3 cells that can activate transcription through these elements. Nevertheless, transcription of the reporter plasmid is further activated upon expression of wild-type E2F1 as a function of galactose induction, suggesting that E2F1 can specifically bind to the E2 promoter element and activate transcription in the absence of DP1.

To map the region of E2F1 that was responsible for specific DNA binding, we used the VPAD-E2F1 fusions. VPAD-$E2F1_{1-283}$ strongly activates the wild-type promoter but not the mutant promoter (data not shown). Figure 4B illustrates the activities measured when a series of E2F1 deletions, fused to the VP16 activation domain, were cotransfected with the E2-*lacZ* reporter plasmid. These assays reveal that the minimal DNA-binding domain of E2F1 can be defined as amino acid residues 113 to 206. Thus, the region of E2F1 that is required for the interaction with DP1 is not required for specific DNA binding. We also note that VPAD-$DP1_{24-410}$ does not activate the E2-*lacZ* plasmid even though it must be expressed at a functional level since it is active in the two-hybrid assay (Table 1).

DISCUSSION

A series of recent experiments have demonstrated that the E2F DNA binding activity is at least in part due to the formation of a heteromeric protein complex (20) and that the E2F1 and DP1 gene products can interact to form such a heterodimer (2, 15, 26). This heterodimeric complex has a

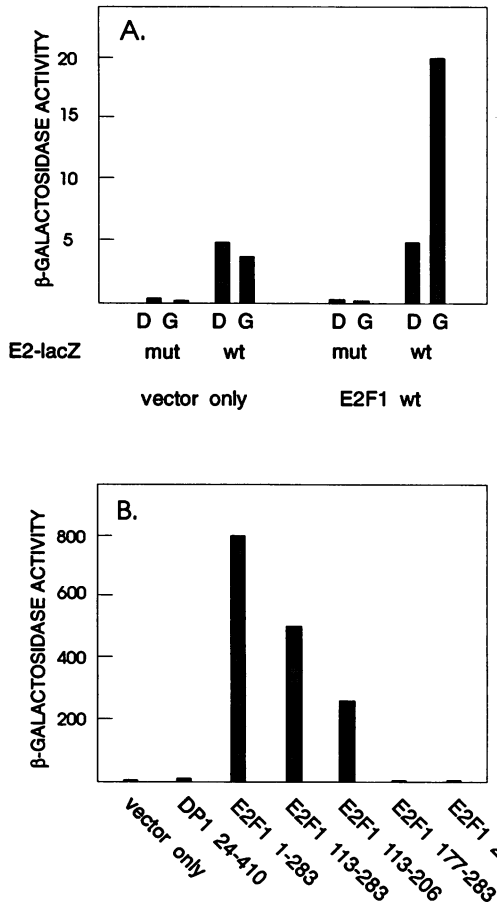


FIG. 4. E2F1 can bind DNA independently of the DP1 interaction domain. (A) Full-length E2F1 can specifically bind and activate an E2 site-driven promoter in yeast cells. Full-length E2F1 was expressed in yeast cells under the control of a galactose-inducible promoter (*YEP51* [5]) along with a reporter plasmid containing either a wild-type (wt) or mutant (mut; nonbinding [16]) E2 promoter upstream of a *cyc1-lacZ* reporter gene (see Materials and Methods for a description). Double-transformant colonies were selected initially on glucose (repressing) medium, and four colonies for each E2F1 derivative were restreaked onto glucose-containing medium. The four double-transformants were then inoculated into liquid medium containing glucose or galactose (inducing) as the carbon source. Average β-galactosidase activities ($\Delta A_{595} \times 1{,}000$/min), normalized to cell suspension optical densities, for the four transformants are shown. (B) The E2F1 DNA-binding domain maps to E2F1 amino acids 113 to 206. VPAD-$DP_{24\text{-}410}$ and a series of VPAD-E2F1 fusion proteins schematically presented in Fig. 3A were tested for activation on an E2-*lacZ* reporter plasmid in order to map the minimal E2F1 region which could recognize the E2 site in yeast cells. Cells were grown in glucose medium.

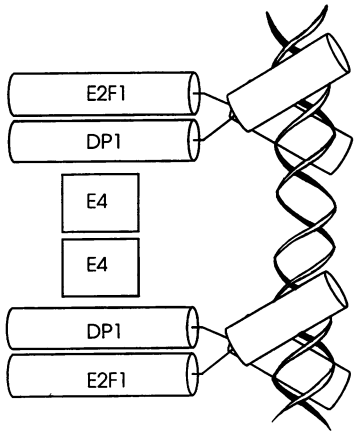


FIG. 5. A model for the interaction of E2F1-DP1-E4 with the adenovirus E2 promoter. The distinct functional domains of E2F1 and DP1 involved in DNA binding and dimerization are represented as cylinders connected by thin lines. The E2F1-DP1 interface has been modeled as a coiled coil (11, 23). The E4 protein has been modeled as a helix-loop-helix (29).

higher affinity for DNA and is more active in transcriptional activation, likely because of the enhanced DNA binding. In addition, the E2F1-DP1 heterodimer also exhibits a greater affinity for Rb than does E2F1 or DP1 alone. The experiments reported here demonstrate that the E2F1-DP1 heterodimeric complex is also a target for the adenovirus E4 protein and that the E2F1-DP1-E4 complex binds specifically and with high affinity to the adenovirus E2 promoter.

The yeast two-hybrid assay system allows an in vivo analysis of protein-protein interactions without the necessity of generating DNA binding activity or transcriptional activity by the E2F molecules. From these assays, we conclude that the E4 protein makes direct contact with the DP1 polypeptide rather than the E2F1 polypeptide, although we cannot rule out the possibility that E4 and E2F1 physically interact in the presence of DP1. Our previous assays showed that the E2F1 hydrophobic heptad repeat element was essential for the E4-mediated coactivation of transcription (7). From the results presented in this report, we would conclude that the requirement of the E2F1 heptad repeat domain is indirect, reflecting the necessity of these sequences to allow heterodimer formation with DP1 and the fact that E4 appears to directly interact with DP1. Previous assays suggest that the E4 protein interacts with most if not all of the E2F activity found in the cell (12, 13, 19, 28). Moreover, although we could find clear evidence for the formation of a heterodimer involving E2F1 and DP1, neither of the proteins could form a homodimer in this assay. It is thus possible that the primary form of E2F in the cell, with respect to E2F1 and DP1, is the heterodimer. Given the fact that E4 appears to make direct contact with the DP1 polypeptide, these results suggest that DP1 is a component of most of the E2F activity in the cell, a conclusion consistent with assays using a DP1-specific antibody (11).

Our results also demonstrate that the E4 protein can form a homodimer in the yeast two-hybrid system, suggesting that E4 may provide a dimerization interface for an E2F1-DP1-E4 trimolecular complex. Combining these results, we propose a general model for the E2F1-DP1-E4 complex on the adenovirus E2 promoter, as depicted in Fig. 5, involving a six-subunit protein complex containing two molecules each of E2F1, DP1, and E4. In this model, E4 provides a dimerization interface which positions two E2F1-DP1 heterodimers to interact directly with the two E2F recognition sites. Previous experiments have shown that the sites must be spaced two helical turns apart at their centers to allow the E2F-E4 complex to interact in the stable complex (12, 31). This would allow the E2F1-DP1 dimers to approach from the same side of the helix and in nearly the same plane. Moreover, the observation that the E2F-binding species is a heterodimer, together with the fact that the E4 protein directly interacts with one component of the heterodimer, provides a stereochemical explanation for why the two E2F sites must be inverted relative to each other

in order to form the E4-specific complex. The requirement for inverted recognition sites imparts asymmetry to the interaction, which can be seen in the asymmetry of the E4 interaction with the E2F1-DP1 heterodimer.

Finally, the fact that the adenovirus E4 protein is able to alter the binding properties of E2F raises the possibility that a cellular protein equivalent to E4 could also alter the binding properties of E2F in a promoter-dependent fashion. The lack of evidence for such an activity could simply reflect the use of a proper promoter target, particularly given the exquisite specificity for the arrangement of binding sites in the adenovirus E2 promoter. Nevertheless, it is also quite possible that the E4 activity is indeed a virus-specific event, reflecting a viral function that maximizes the utilization of a cellular transcription factor to allow efficient viral transcription without regard to a need for regulated expression or the need to later reutilize the factor.

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