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Expression of Dominant-Negative Mutant DP-1 Blocks Cell Cycle Progression in G_1

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Unregulated expression of the transcription factor E2F promotes the G_1-to-S phase transition in cultured mammalian cells. However, there has been no direct evidence for an E2F requirement in this process. To demonstrate that E2F is obligatory for cell cycle progression, we attempted to inactivate E2F by overexpressing dominant-negative forms of one of its heterodimeric partners, DP-1. We dissected the functional domains of DP-1 and separated the region that facilitate heterodimer DNA binding from the E2F dimerization domain. Various DP-1 mutants were introduced into cells via transfection, and the cell cycle profile of the transfected cells was analyzed by flow cytometry. Expression of wild-type DP-1 or DP-1 mutants that bind to both DNA and E2F drove cells into S phase. In contrast, DP-1 mutants that retained E2F binding but lost DNA binding arrested cells in the G_1 phase of the cell cycle. The DP-1 mutants that were unable to bind DNA resulted in transcriptionally inactive E2F complexes, suggesting that the G_1 arrest is caused by formation of defective E2F heterodimers. Furthermore, the G_1 arrest instigated by these DP-1 mutants could be rescued by coexpression of wild-type E2F or DP protein. These experiments define functional domains of DP and demonstrate a requirement for active E2F complexes in cell cycle progression.

Analysis of the E2F family of transcriptional regulators has developed into a model system for studies of the link between transcription factor function and the control of cell cycle progression. The E2F proteins not only have been implicated in control of viral and cellular gene expression but are also thought to play an important role in growth regulatory processes such as the G_1-to-S phase transition of the cell cycle (40, 47, 49). Most of the evidence that E2F plays a key role in G_1 progression has come from experiments showing that the overexpression of E2F promotes cell cycle progression (7, 34, 45, 56, 60, 69) and cause transformation of rodent embryo fibroblasts (7, 19, 35, 62, 70) and from the characterization of proteins that physically interact with E2F, such as the retinoblastoma gene product, pRB (for a review see reference 49).

The E2F (also called DRTF1) proteins were initially identified as a cellular DNA-binding activity required for transcriptional activation of the adenovirus E2 promoter (37, 40, 41, 49). It was subsequently shown that several viruses have evolved strategies to disrupt E2F complexes, and the regions of the viral oncoproteins important for this function overlap those required for viral transformation and related activities (2, 6, 10, 49, 57). The biological rationale for these viruses having evolved mechanisms to regulate E2F activities can be surmised when the natures of cellular E2F targets are considered. Many of these genes are required for cell cycle progression and DNA replication, and their expression is regulated in a periodic fashion throughout the cell cycle, usually being induced near the beginning of or during S phase. Among the genes that are thought to be regulated by E2F in a cell cycle-specific manner are (i) those whose products are required for DNA synthesis (DNA polymerase α, thymidine kinase, dihydrofolate reductase, and thymidylate synthase); (ii) those that encode nuclear oncoproteins (c-Myc, N-Myc, and b-Myb), and (iii) those that encode cell cycle regulators (cyclin A, cyclin E, and cdc2) (for reviews see references 12, 24, 40, and 49). Thus, by activating E2F, the virus promotes cell cycle progression, presumably to allow viral DNA replication. Indeed, microinjection of a dominant-negative E2F-1 mutant lacking the transactivation domain blocked E1A but not serum-stimulated S-phase entry (13). Similarly, expression of an amino-terminal deletion mutant E2F-1 lengthens S phase in fibroblasts (44).

The observation that domains of adenovirus E1A required to activate E2F were identical to those responsible for the interaction with pRB or other related proteins provided further understanding of how the action of viral oncogenes is linked to the derepression of E2F activity (49). The tumor suppressor protein pRB appears to function primarily in G_1 (66). Mutations in pRB that eliminate its ability to impede cell growth also prevent complex formation with E2F, suggesting that one of the levels at which pRB exerts its biological effects is by regulating the transcriptional activity of E2F complexes (10, 49, 54, 57, 63). Support for this idea has come from experiments in which E2F-mediated transcription can be repressed by wild-type pRB (16, 17, 22, 25, 29, 59, 67, 71). It has also been demonstrated that pRB can repress directly E2F-mediated transcription (16, 25, 55). This inhibition is abolished upon phosphorylation of pRB by cyclin A/cdk2 or cyclin E/cdk2 complexes, thus coordinating transcriptional regulation and cell cycle control (16, 38). Furthermore, it has been shown that overexpression of E2F can induce S-phase entry (7, 34, 45, 56, 60, 69) and cause transformation of rodent embryo fibroblasts (7, 19, 35, 62, 70). Overexpression of E2F-1 also leads to p53 dependent apoptosis (56, 60, 69).

Biochemical studies have revealed that E2F is a family of related transcription factors that bind DNA and synergistically regulate transcription as heterodimers composed of two classes of polypeptides, the E2F and DP proteins (4, 21, 27, 32, 39). In mammalian cells, seven members of the broader E2F family (E2F-1 through E2F-5, DP-1, and DP-2) have been cloned (7,

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19, 21, 26, 30, 33, 36, 42, 43, 58, 61, 68, 72). Homologs have also been cloned from *Drosophila melanogaster* and *Xenopus laevis* (15, 20, 51), and members of each group show substantial sequence homology. Functional E2F protein domains include the DNA-binding and dimerization motifs, the transactivation domain, and the binding domain for pRB family members (7, 11, 16, 19, 26, 33, 36, 38, 42, 58, 61, 64). The DNA-binding, dimerization, and transactivation domains are highly conserved among family members. In addition E2F-1, E2F-2, and E2F-3 also contain an interaction site for cyclin A/cdk2 kinase. The DP proteins show structural similarities to E2F in their DNA-binding, heterodimerization, and transactivation domains but have not yet been shown to contact DNA, bind cyclins, or specify binding to pRB family members (4, 5, 21, 27, 35, 39, 50, 68, 72).

The experiments reviewed above strongly suggest that mammalian E2F can promote G_1-to-S phase transition. It has recently been shown that in *Drosophila melanogaster*, in which there is evidence for only a single E2F gene, E2F is required for S phase during embryogenesis (14). Genetic knockout of mammalian E2F function is complicated by the fact that it is a family of at least seven polypeptides (see references above). Furthermore, it appears that many combinatorial interactions are possible, not only at the level of various E2F family members but also in regard to their interaction with the pocket proteins. In the experiments described in this report, we eliminated E2F activity by overexpressing dominant-negative forms (28) of the DP proteins. We systematically dissected the human DP-1 gene and identified a small region of DP-1 that is essential for the heterodimer to bind DNA. The mutants which can bind to all known mammalian E2Fs form inactive heterodimers with E2F polypeptides. Overexpression of the dominant-negative mutants block cell cycle progression in G_1 and provide evidence for a mandatory role of E2F activity in cell cycle progression.

MATERIALS AND METHODS

Plasmid construction. Cytomegalovirus expression vector pCMV-HA is modified from pCMV-neo-Bam (3). All pCMV-HA-DP-1 and pCMV-HA-DP-2 deletion mutants described in this report were constructed in pCMV-HA. DP1 coding sequences, as indicated in Fig. 1A, were amplified by PCR and subcloned into the *Bam*HI site of the pCMV-HA vector. Except for DP-$1^{155\text{-}410}$, DP-$1^{182\text{-}410}$, and DP-$1^{192\text{-}410}$, all constructs are preceded by overlapping *Bam*HI-*Nco*I sites (GGA TCC ATG G) 5′ of the coding sequence. All coding sequences were followed by an in-frame stop codon. DP-2 mutants were constructed in the same way. All PCR products were verified by sequencing. pCMV-E2F1, pCMV-HA-DP1, pCMV-HA-DP2, and pCMV-HA-DP1 deletion mutants 1-204, 1-277, and 1-346 have been described elsewhere (27, 68).

Cell culture and transfections. Human osteosarcoma cell line Saos-2 was purchased from the American Tissue Culture Collections and cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Cells were transfected by using standard calcium phosphate precipitation protocols (1). DNA precipitates were left on cells for 12 h; cells were then supplied with fresh medium and harvested 48 h later.

Detection of DP and E2F proteins. Saos-2 cells were transfected with a total of 20 μg of pCMV DNA, including 10 μg of pCMV-E2F-1 and the indicated pCMV-HA-DP constructs. Whole-cell extracts were prepared 60 h after transfection as described before (27, 68). Fifteen micrograms of protein was subjected to electrophoresis in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and blotted onto Immobilon P membranes (Millipore). The blots were probed with the anti-hemagglutinin (HA) monoclonal antibody 12CA5 or the anti-E2F1 monoclonal antibody KH20. The blots were developed with enhanced chemiluminescence as instructed by the manufacturer (Amersham). To detect interactions between DP-1 and E2F-1, immunoprecipitation-Western blot (immunoblot) analysis was performed. Extracts from transfected Saos-2 cells (50 μg of protein) were first immunoprecipitated with the anti-E2F-1 monoclonal antibody KH95 coupled to protein G-Sepharose beads (23) and then separated on SDS–12% polyacrylamide gels, blotted to Immobilon P membranes, and probed with the anti-HA antibody 12CA5.

DNA binding assays. Gel shift assays were performed as described previously (68). Briefly, whole-cell extracts from transfected Saos-2 cells (4 μg of protein) were incubated with a ^{32}P-labeled 22-bp oligonucleotides (68) containing an E2F-binding site. The protein-DNA complexes were separated by electrophoresis (4% native acrylamide gels). The gels were dried and visualized by autoradiography. The specificity of the protein-DNA interactions was confirmed by competition with wild-type or mutant oligonucleotides (not shown).

Flow cytometry. Asynchronous populations of Saos-2 cells in log phase were transiently transfected with 20 μg of pCMV-HA or pCMV-HA-DP1 mutant along with 2.5 μg of pCMV-CD20. The cells were harvested 60 h posttransfection, and CD20 and DNA staining was carried out as described previously (65, 73). Flow cytometry analysis was performed on a Becton Dickinson FACScan, and data from 40,000 to 80,000 cells per sample were analyzed by Cell Fit cell cycle software. Each DNA histogram contains information from 2,000 to 8,000 CD20-positive cells. For treatment with nocodazole, cells were refed with medium containing 50 ng of nocodazole per ml 40 h posttransfection and harvested 20 and 48 h later.

Transactivation. C33-A, a human cervical carcinoma cell line, was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Transient transfections were carried out as described above. For chloramphenicol acetyltransferase (CAT) assays, cells were transfected with 1.5 μg of $(E2F)_4$BCAT, 1 μg of pRSVluciferase, a total of 1.1 μg of pCMV expression plasmids and pCMV-neo-Bam as indicated in the figure legends, and 15 μg of carrier DNA (pBSK-globin). Cells were harvested 36 h after transfection. CAT assays were performed by CAT enzyme-linked immunosorbent assay and luciferase assays as described by the manufacturer (Boehringer Mannheim), and results are presented as arbitrary values.

RESULTS

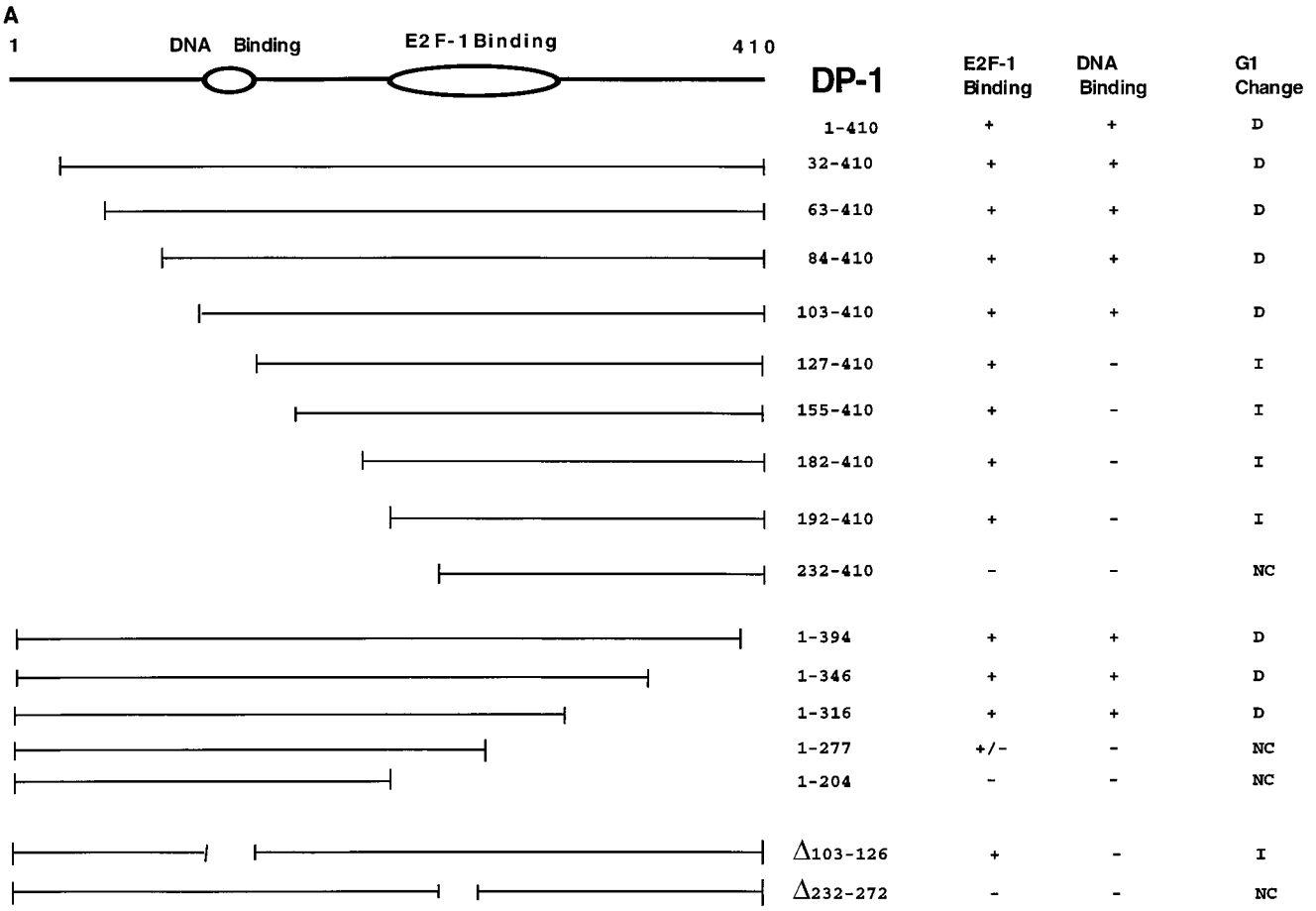
Previous studies have indicated that distinct regions of DP-1 may be involved in DNA binding and E2F dimerization (4, 5, 21, 27, 39, 50, 68, 72), raising the possibility that DP-1 mutants that interact with E2F proteins but prevent DNA binding by the heterodimer could be constructed. The overexpression of such a mutant might be expected to inhibit endogenous E2F activity. The optimal creation of such a mutant requires detailed knowledge of the boundaries of functional DP-1 domains. Therefore, we constructed a panel of deletion mutants of DP-1 that cover the entire protein in short intervals (30 amino acids or so), as illustrated in Fig. 1A.

Mutants were cloned into a cytomegalovirus expression vector that allowed the addition of an HA epitope tag. Following transient transfection of these constructs into Saos-2 cells, expression levels of these proteins were monitored by immunoblotting the cell extracts with anti-HA antibody. Similar levels of protein were detected for all of the mutants described in this study (Fig. 1B). Thereafter, the DP-1 mutants were characterized for their E2F-binding and DNA-binding properties in vivo.

Heterodimer formation by DP-1 mutants. The ability of each of the DP-1 mutants to bind to E2F-1 was determined following cotransfection of wild-type or mutant HA-DP-1 expression vectors with pCMV-E2F-1 into Saos-2 cells. E2F-1 was immunoprecipitated with an E2F-1-specific antibody, and associated HA-DP-1 protein was detected following immunoblotting. Under these conditions, equal amounts of E2F protein are precipitated with the antibody (data not shown).

Extensive deletion of amino-terminal DP-1 sequences was possible without affecting association with E2F-1. DP-$1^{192\text{-}410}$ bound to E2F-1 at levels equivalent to those of the full-length protein, but DP-$1^{232\text{-}410}$ was not coprecipitated with E2F-1. This result indicates that amino acids 1 to 191 of DP-1 are dispensable for binding to E2F-1 and suggest that sequences between 192 and 232 are required for the association (Fig. 2, lanes 4 to 13).

Three carboxyl-terminal deletion DP-1 mutants (DP-$1^{1\text{-}394}$, DP-$1^{1\text{-}346}$, and DP-$1^{1\text{-}316}$) bound to E2F-1 at wild-type levels (Fig. 2, lanes 14 to 16). However, DP-$1^{1\text{-}204}$ failed to interact with E2F-1 in this assay (Fig. 2, lane 18). An intermediate mutant, DP-$1^{1\text{-}277}$, gave a trace level of association with E2F-1 (Fig. 2, lane 17). In previous work, a glutathione *S*-transferase fusion protein containing DP-$1^{1\text{-}277}$ was shown to bind to in vitro-translated E2F-1 (27). We attribute the lower level of


A
1
DNA Binding
E2F-1 Binding
410
DP-1
E2F-1 Binding
DNA Binding
G1 Change
1-410 + + D
32-410 + + D
63-410 + + D
84-410 + + D
103-410 + + D
127-410 + - I
155-410 + - I
182-410 + - I
192-410 + - I
232-410 - - NC
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Δ103-126 + - I
Δ232-272 - - NC

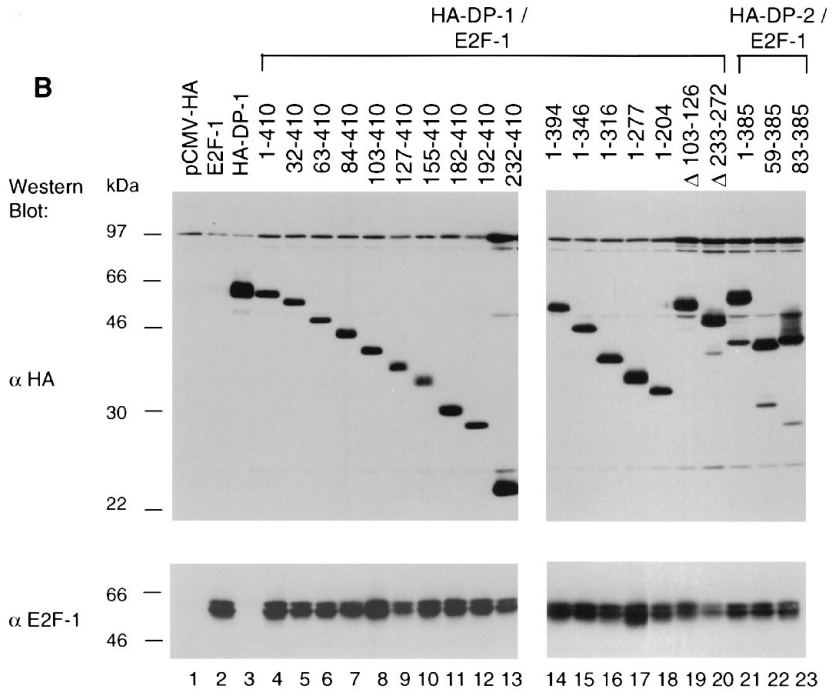
B
HA-DP-1 / E2F-1
HA-DP-2 / E2F-1
pCMV-HA
E2F-1
HA-DP-1
1-410
32-410
63-410
84-410
103-410
127-410
155-410
182-410
192-410
232-410
1-394
1-346
1-316
1-277
1-204
Δ 103-126
Δ 233-272
1-385
59-385
83-385
Western Blot:
kDa
97
66
46
30
22
α HA
α E2F-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

FIG. 1. (A) Schematic illustration of DP-1 mutants. Human DP-1 contains 410 amino acids. The domain essential for E2F-1 interaction (E2F-1 binding) and the amino-terminal boundary of the domain essential for DNA interaction by E2F/DP heterodimers (DNA binding) were mapped in this study. The deletion mutants are indicated with their starting and ending amino acids, and internal deletions are identified by △ symbols. Also shown is a summary of the properties of these mutants as described in Fig. 2 to 4 (D, decrease; I, increase; NC, almost no change). (B) Expression of DP-1 and DP-2 mutants. Saos-2 cells were transfected with 10 μg of the indicated pCMV-HA-DP plasmids and 10 μg of pCMV-E2F-1 (when pCMV-DP or pCMV-E2F-1 was expressed alone, 10 μg of vector was added), and extracts were prepared 60 h after transfection. Twenty-microgram aliquots of the extracts were separated on SDS–12% polyacrylamide gels, blotted and probed with the anti-HA monoclonal antibody 12CA5 (αHA; upper panels) for detection of DP proteins or the anti-E2F-1 monoclonal antibody KH20 (αE2F-1; lower panels). Positions of molecular mass markers are indicated on the left. Three nonspecific bands (96, 50, and 24 kDa) in the upper panel are proteins cross-reacting with 12CA5 and serve as internal controls for equivalent protein loading.

association seen here to the fact that coimmunoprecipitation of in vivo complexes is a more stringent assay for the interaction. Taken together, these data suggest that amino acids between positions 192 and 277 of DP-1 are required for association with E2F-1 and that additional sequences between positions 277 and 316 contribute to high-affinity binding. To confirm that sequences between residues 192 and 277 are required for E2F-1 binding, an internal deletion mutant, DP-$1^{\Delta 233\text{-}272}$, was constructed. As expected, DP-$1^{\Delta 233\text{-}272}$ failed to bind to E2F-1 (Fig. 2, lane 20).

Effect of DP-1 mutants on E2F DNA-binding activity. E2F-1/DP-1 heterodimers bind DNA in a sequence-specific manner. Although purified E2F-1 has been reported to possess low-affinity DNA-binding activity, no specific DNA interaction has been found for purified DP-1 (4, 21, 27, 39). At present, it is unclear whether DP-1 contacts DNA directly or potentiates binding by altering the conformation of E2F-1. To identify domains of DP-1 that are essential for facilitating DNA binding, we assayed the E2F-binding activity generated by coexpression of the DP-1 mutants with E2F-1 in Saos-2 cells. As described previously, little or no DNA-binding activity was generated following transfection with E2F-1 alone or HA-DP-1 alone (Fig. 3, lanes 1 to 3), but their coexpression produced high levels of binding activity (Fig. 3, lane 4).

The amount of DNA-binding activity generated by the carboxyl-terminal deletion mutants closely correlated with their E2F-1-binding properties. Mutants DP-$1^{1\text{-}394}$, DP-$1^{1\text{-}346}$, and DP-$1^{1\text{-}316}$ bound to E2F-1 at wild-type levels and generated levels of DNA binding equivalent to that of the full-length protein (Fig. 3, lanes 14 to 16). DP-$1^{1\text{-}204}$, which was defective for E2F-1 binding, and DP-$1^{1\text{-}277}$, which had trace levels of E2F-1 binding, failed to generate DNA-binding activity in this assay (Fig. 3, lanes 17 and 18).

Four amino-terminal deletion mutants (DP-$1^{32\text{-}410}$, DP-$1^{63\text{-}410}$, DP-$1^{84\text{-}410}$, and DP-$1^{103\text{-}410}$) generated quantities of DNA-binding activity similar to those of the wild-type protein (Fig. 3, lanes 5 to 8). In contrast, DP-$1^{127\text{-}410}$, DP-$1^{155\text{-}410}$, DP-$1^{182\text{-}410}$, and DP-$1^{192\text{-}410}$ failed to produce any DNA-binding activity (Fig. 3, lanes 9 to 12) despite the fact that they associated with E2F-1 at wild-type levels (Fig. 2, lanes 9 to 12). These results indicate that DP-1 binding to E2F-1 is not sufficient to generate a DNA-binding complex in vivo and that sequences in addition to the minimal E2F-1-binding regions are necessary to enable DP-1/E2F-1 heterodimers to bind to DNA. The boundary of the domain providing this function lies between residues 103 and 127 of DP-1. To confirm the requirement of this region, an internal deletion mutant, DP-$1^{\Delta 103\text{-}126}$, was constructed. As expected, DP-$1^{\Delta 103\text{-}126}$ bound efficiently to E2F-1 (Fig. 2, lane 19), but these complexes lacked DNA-binding activity (Fig. 3, lane 19).

These results identify a series of DP-1 mutants (DP-$1^{\Delta 103\text{-}126}$, DP-$1^{127\text{-}410}$, DP-$1^{155\text{-}410}$, DP-$1^{182\text{-}410}$, and DP-$1^{192\text{-}410}$) that bind efficiently to E2F-1 but form complexes that are unable to bind to DNA and are therefore expected to be unable to regulate transcription. These properties make them excellent candidates for dominant-negative mutants of DP-1.

Cell cycle changes caused by DP-1 mutants. The overexpression of E2F-1 and E2F-1-related genes has been shown to drive cell cycle progression (see references above). Similarly, the overexpression of DP-1 was also found to change the cell cycle

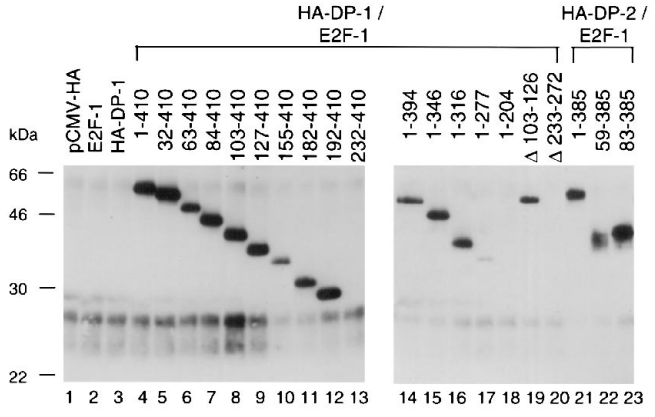

FIG. 2. Heterodimerization between E2F-1 and DP-1 and DP-2 mutants. Extracts from Saos-2 cells transiently transfected with indicated constructs were immunoprecipitated with the anti-E2F-1 monoclonal antibody KH95 coupled to protein G-Sepharose beads, separated on SDS–12% polyacrylamide gels, blotted, and probed with the anti-HA monoclonal antibody 12CA5.

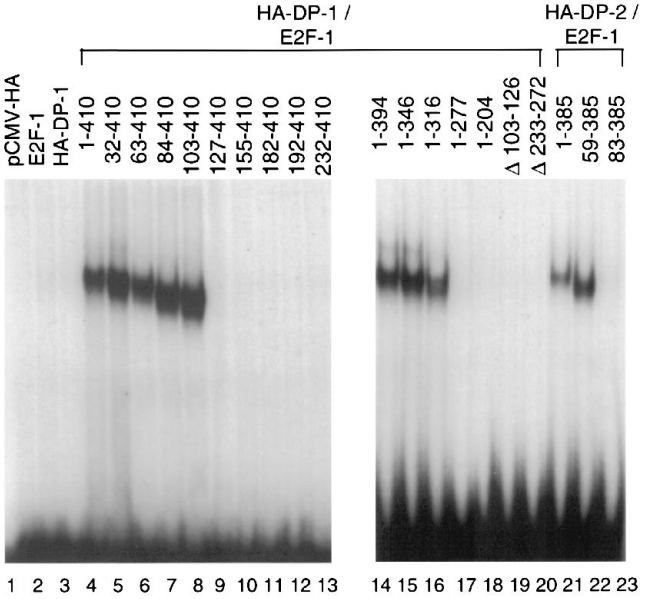

FIG. 3. Effect of DP-1 and DP-2 mutants on E2F DNA-binding activity. Cell extracts from Saos-2 cells transfected with indicated constructs were incubated with an oligonucleotide containing an E2F-binding site. The resulting complexes were separated on native gels and visualized by autoradiography.

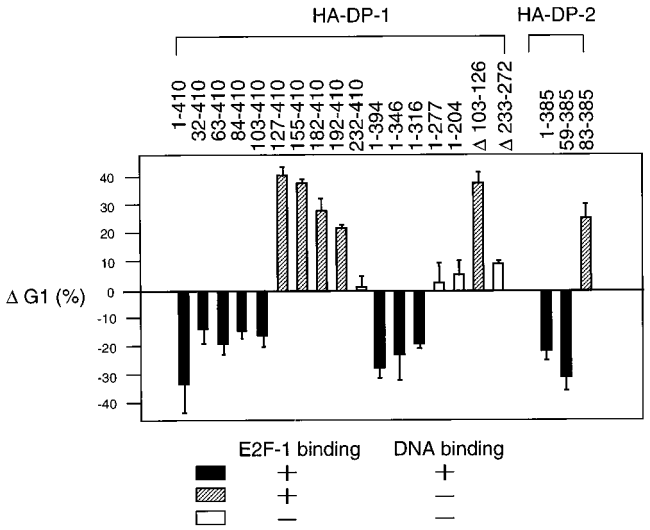

FIG. 4. Effects of DP-1 and DP-2 mutants on cell cycle progression. Saos-2 cells, transfected with 20 μg of the indicated plasmids and 2.5 μg of pCMV-CD20, were analyzed by flow cytometry to determine the cell cycle profiles of CD20-positive cells. The data from this analysis are presented as ΔG_1, the percent change in the proportion of cells in G_1 relative to the vector control sample {ΔG_1 = [(percentage of cells in G_1 in test samples − percentage of control cells in G_1)/percentage of control cells in G_1] × 100}. Each datum point represents the mean value (± standard deviation) from at least three independent experiments.

profile of transfected cells and cause an increase in the number of S-phase cells (7). To investigate the effects of the DP-1 mutants on cell cycle progression, we transfected wild-type or mutant DP-1 into human osteosarcoma cell line Saos-2 together with the cell surface marker CD20. Transfected cells were identified with an anti-CD20 antibody, and their DNA content was assayed by flow cytometry analysis to determine cell cycle distribution.

The results of these experiments are shown in Fig. 4 to 6. The G_1 fraction of asynchronous Saos-2 cells is approximately 50%, and this population was unaffected by cotransfection with pCMV-HA vector. Transfection of full-length DP-1 reduced the fraction of G_1 cells, with concomitant increases in S- and G_2/M-phase cells. These effects are similar to those reported in previous studies (7) and are dose dependent.

The DP-1 mutants could be grouped into three general classes that correlated with their E2F-1-binding and DNA-binding properties (Fig. 4). The first group of mutants (DP-$1^{32\text{-}410}$, DP-$1^{63\text{-}410}$, DP-$1^{84\text{-}410}$, DP-$1^{103\text{-}410}$, DP-$1^{1\text{-}394}$, DP-$1^{1\text{-}346}$, and DP-$1^{1\text{-}316}$) resembled full-length DP-1 and caused a reduction in the G_1 population (Fig. 4). A common feature of this group of mutants was that they all bound efficiently to E2F-1 and generated complexes that bound to DNA (Fig. 2 and 3). A second class of mutants contained those that were unable to bind efficiently to E2F-1 (DP-$1^{232\text{-}410}$, DP-$1^{1\text{-}277}$, DP-$1^{1\text{-}204}$, and DP-$1^{\Delta 233\text{-}272}$), and the expression of these mutants had little or no effect on the cell cycle profile of the transfected cells (Fig. 4).

The third class of DP-1 mutants (DP-$1^{127\text{-}410}$, DP-$1^{155\text{-}410}$, DP-$1^{182\text{-}410}$, DP-$1^{192\text{-}410}$, and DP-$1^{\Delta 103\text{-}126}$) caused a significant increase in the population of cells in G_1 (Fig. 4). Interestingly, this group comprised the mutants that were able to associate with E2F-1 but produced complexes that were unable to bind to DNA (Fig. 2 and 3). As seen for wild-type DP-1, the effects of these mutants were titratable and increased with increasing quantity of transfected plasmid (Fig. 5). To confirm that this increase in the G_1 population was due to the arrest of cells in G_1 rather than acceleration of S or G_2/M phase, transfected cells were incubated with nocodazole, a drug that arrests cells in the M phase. Nocodazole treatment for 20 h (Fig. 6B) or 48 h (data not shown) caused a substantial increase in the G_2/M population of cells transfected with vector alone or with wild-type DP-1 but had no effect on the profile of cells transfected with DP-$1^{127\text{-}410}$ or DP-$1^{\Delta 103\text{-}126}$. This result shows that the cells were not progressing through the cell cycle and indicated that the increase in G_1 caused by the expression of these DP-1 mutants is the result of cell cycle arrest. In addition, transfection of DP-$1^{\Delta 103\text{-}126}$ into C33-A and U2OS cells resulted in a 52 and 54% increases, respectively, in the G_1 populations of these cells.

In conclusion, these data show that the expression of DP-1 mutants which bound E2F-1 and generated DNA-binding complexes promoted S-phase entry. Conversely, mutants of DP-1 that bound to E2F-1 but formed complexes unable to interact with DNA caused G_1 arrest (summarized in Fig. 1A).

DP-2 mutants. Since DP-2 has high sequence homology with DP-1 and, when overexpressed, has similar properties in E2F-1 binding, DNA binding, and transactivation assays (68, 72), we tested whether DP-2 mutants could have similar effects on cell cycle progression. We prepared two DP-2 mutants, DP-$2^{59\text{-}385}$ and DP-$2^{83\text{-}385}$, that contained sequences homologous to those of DP-$1^{103\text{-}410}$ and DP-$1^{127\text{-}410}$, respectively. As predicted, DP-$2^{59\text{-}385}$ bound to E2F-1 (Fig. 2) and generated DNA-binding-competent complexes (Fig. 3). Although DP-$2^{83\text{-}385}$ bound to E2F-1 as efficiently as the wild-type protein (Fig. 2, lanes 21 and 23), the resulting complexes were unable to bind DNA (Fig. 3 lane 23). These mutants had effects on cell cycle progression that resembled those of their DP-1 counterparts. DP-$2^{59\text{-}385}$, like DP-$1^{103\text{-}410}$, caused a decrease in the G_1 population, and DP-$2^{83\text{-}385}$, like DP-$1^{127\text{-}410}$, caused an accumulation of G_1-phase cells (Fig. 4).

Properties of the G_1 arrest caused by DP-1 DNA-binding mutants. Since the DP-1 and DP-2 mutants that caused a G_1 arrest have in common the property of binding to E2F-1 but not supporting DNA binding, a reasonable model for the activity of these mutants is that they compete with endogenous DP-1 for binding to E2F polypeptides. This model makes sev-

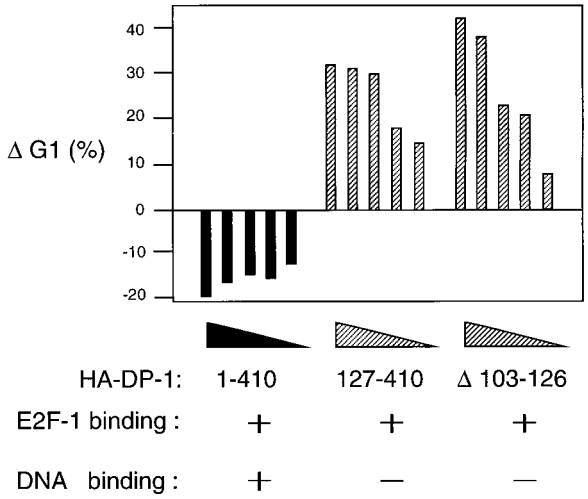

FIG. 5. Dose-dependent effect of dominant-negative mutants of DP-1 on cell cycle progression. Saos-2 cells, transfected with 2.5 μg of pCMV-CD20 and 1, 2.5, 5, 10, and 20 μg of the indicated constructs, were analyzed by flow cytometry, and the results were plotted as described for Fig. 4.

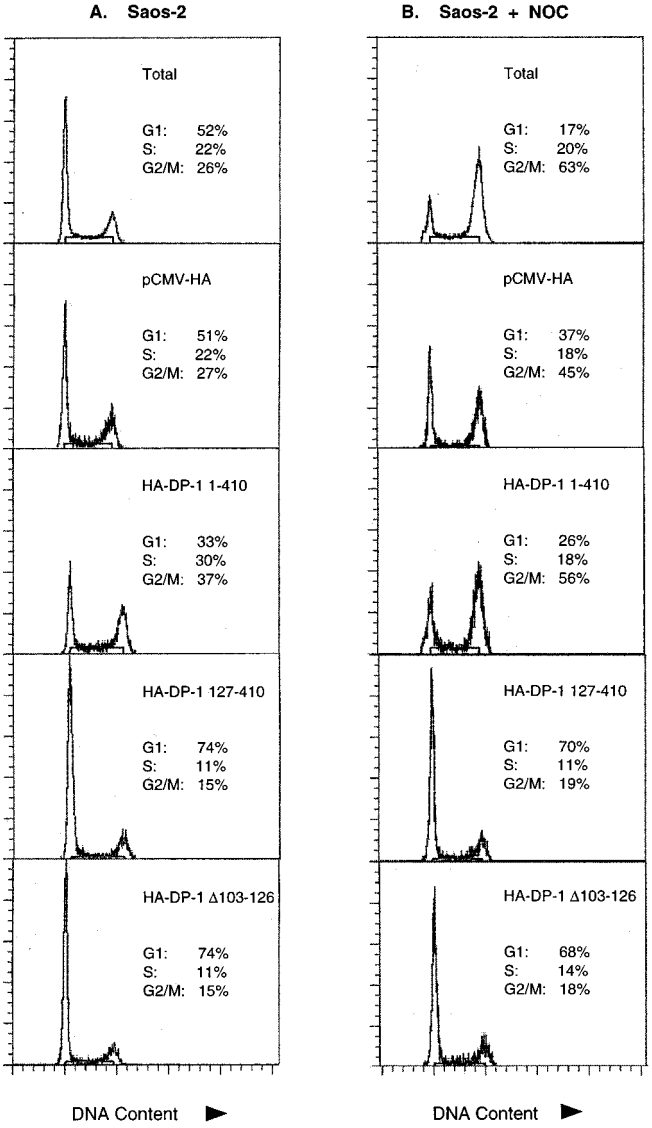

FIG. 6. DNA histograms of cells transfected with wild-type or mutant DP-1. Saos-2 cells were transfected and analyzed as described for Fig. 5. Histograms of DNA contents and cell cycle distribution values are shown. (A) Saos-2 cells transfected with the indicated plasmids and harvested 60 h after transfection; (B) Saos-2 cells transfected with the indicated plasmids and treated with 50 ng of nocodazole (NOC) per ml 40 h posttransfection and harvested 20 h later.

eral predictions: the DP-1 mutants should be unable to promote E2F-dependent transcription; G_1 arrest should be reversible by coexpression of wild-type DP proteins; and G_1 arrest should also be overcome by coexpression of members of the E2F family of proteins. These properties were tested for the DP-$1^{\Delta 103\text{-}126}$ mutant, as shown in Fig. 7 and Table 1. E2F-1 was transfected into C33-A cells with either DP-$1^{\Delta 103\text{-}126}$ or wild-type DP-1, and E2F-dependent transcription was monitored by using the $(E2F)_4$-BCAT reporter construct (27). Cotransfection of wild-type DP-1 greatly increased the transcriptional activity of E2F-1 (Fig. 7). In contrast, the cotransfection of DP-$1^{\Delta 103\text{-}126}$ failed to augment the activity of E2F-1. Instead expression of DP-$1^{\Delta 103\text{-}126}$ resulted in a level of activity somewhat reduced from that generated by transfection of E2F-1 alone. This result is not surprising given the E2F-binding properties and the inability of this mutant to interact with DNA

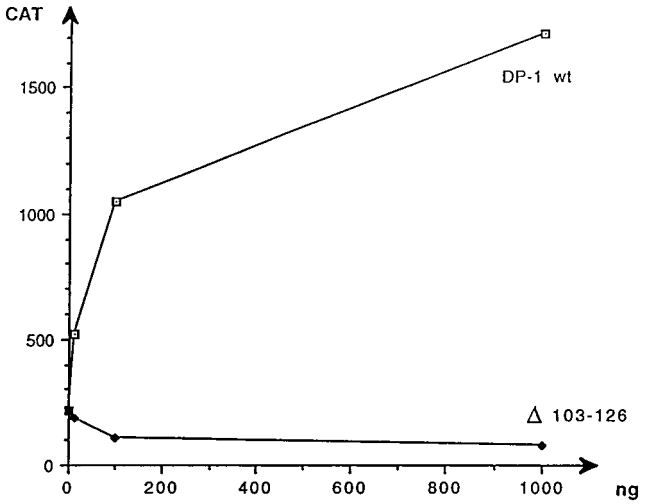

FIG. 7. A dominant-negative form of DP-1 is transcriptionally inactive. CAT assays were performed with C33-A cells transfected with 100 ng of E2F-1 together with 10, 100, and 1,000 ng of wild-type DP-1 (DP-1 wt) or DP-$1^{\Delta 103\text{-}126}$ in triplicate. The reporter plasmid $(E2F)_4$-BCAT contains four E2F-binding sites upstream of the E1B TATAA box and CAT gene. Transfection efficiencies were normalized by comparison with a cotransfected Rous sarcoma virus-luciferase construct, and the data are presented as arbitrary CAT units.

(Fig. 2 and 3). Similar results were obtained with all of the mutants that arrest the cell cycle (DP-$1^{127\text{-}410}$, DP-$1^{155\text{-}410}$, DP-$1^{182\text{-}410}$, DP-$1^{192\text{-}410}$, and DP-$1^{\Delta 103\text{-}126}$) in combination with all E2F genes (data not shown).

Rescue of the G_1 arrest was tested by cotransfection of Saos-2 cells with DP-$1^{\Delta 103\text{-}126}$ and equal amounts of DP-1, DP-2, and E2F-1 expression vectors. In each case, the G_1 arrest induced by DP-$1^{\Delta 103\text{-}126}$ was eliminated by the coexpression of these components (Table 1). The fact that G_1 arrest could be rescued by the expression of either wild-type DP-1 or DP-2 protein or by the expression of the normal binding partner argues that DP-$1^{\Delta 103\text{-}126}$ acts as a bona fide dominant-negative mutant. However, this interpretation is complicated by the fact that these polypeptides stimulate cycling on their own.

DISCUSSION

The ability to understand the overall importance and function of a gene regulatory pathway is often limited by the complexity of the system and the limited knowledge of target genes. For the E2F transcription factor family, there is substantial circumstantial evidence pointing toward a role in cell cycle regulation. Perhaps the best argument for a role for E2F in cell

TABLE 1. Rescue of G_1 arrest[a]

Construct	ΔG1 (%)
HA-DP-$1^{\Delta 103\text{-}126}$	
Alone	33
+ HA-DP-1	−10
+ HA-DP-2	2
+ HA-E2F-1	−2
pCMV-HA + HA-DP-1	−15
pCMV-HA + HA-DP-2	−14
pCMV-HA + HA-E2F-1	−21

[a] Saos-2 cells were transfected with 10 μg of each of the indicated pCMV expression constructs and analyzed as described in the text. ΔG_1 was calculated as in Fig. 4. The data are from one of two independent experiments.

cycle progression comes from studies with *D. melanogaster*, from which only one representative from each class of genes has been isolated (dE2F and dDP). Here, it has been shown that E2F is essential for the activation of the transcriptional program that is required for DNA replication during embryonic development (14). Similar investigations in mammalian cells are complicated by the fact that the E2F transcriptional activity appear to originate from the concerted action of a family of proteins that can be subdivided into two subfamilies, E2F-1 to E2F-5 and DP-1 to DP-2. Furthermore, the transcriptional activity of E2F is in part constrained by its association with pRB and related pocket proteins (10, 49, 54, 57, 63). These interactions are in turn regulated through phosphorylation by cyclin-dependent kinases (16, 38). Phosphorylation of pRB is thought to cause release of E2F and subsequent activation of E2F-dependent transcription. The DP subunit in the heterodimer makes little or no contribution in specifying these interactions.

In this study, we have assayed the role of E2F in cell cycle progression by designing mutant DP-1 and DP-2 proteins that can act as dominant-negative polypeptides. This approach, as discussed by Herskowitz (28), involves the manipulation of a cloned gene to create a mutant polypeptide that when overexpressed disrupts the activity of the wild-type protein. Naturally occurring dominant-negative mutations were originally described for the multimeric repressor proteins affecting the *lac* and *trp* operons and the λ repressor (46). These studies demonstrated that it is possible to inactivate a DNA-binding motif without affecting the oligomerization domain and thereby create an inhibitory polypeptide. There are also some naturally occurring examples found in mammalian cells, exemplified by the helix-loop-helix protein Id, which lacks DNA-binding capacity and negatively regulates other helix-loop-helix proteins by protein-protein interactions (48, 52). There are also some examples of artificially generated dominant-negative proteins that inhibit sequence-specific DNA binding of a heterodimeric partner (8). Another way of creating inhibitory derivatives of DNA-binding proteins is demonstrated by mutation of the activation domain of yeast GCN4, turning this activator into a repressor (31). This strategy, inactivation of activation domains, has been used in a number of studies involving transcriptional regulators such as Jun and serum response factor (9, 18, 53).

Endogenous DP activity is due to the action of two known polypeptides both capable of binding to all five of the known E2F family members, and this interaction leads to high-affinity DNA binding and increased transcriptional activity. Because DP, when overexpressed, shows no specificity for its E2F target and does not significantly contribute to pocket protein interactions, we decided to create DP mutants that would generate E2F complexes unable to bind DNA and thereby unable to affect transcription. These dominant-negative mutants arrested the cell cycle in a dose-dependent manner in the G_1 phase in Saos-2 cells as well as in C33-A and U2OS cells. As with any overexpression system, one should keep in mind that the results could simply be due to nonspecific effects of the vast excess of the protein in question. To control for this possibility, we showed that the overexpression of wild-type DP or mutant proteins that have the ability to interact with E2F and bind DNA does not block nonspecifically but in fact drives cells into S phase.

The ability to arrest the cell cycle corresponded precisely with the ability of the DP dominant-negative mutants to bind to E2F, arguing that the target of the action was E2F itself or some other protein that binds in a similar manner. Consistent with these mapping studies, the G_1 arrest could be rescued by overexpressing DP or E2F. Finally, we found that the dominant-negative mutant proteins also form transcriptionally inactive complexes with E2F1 to E2F5, suggesting that a mechanism for G_1 arrest could be the formation of transcriptionally defective E2F complexes. All of these findings argue that the targets of the dominant-negative mutations were the E2F polypeptides themselves. While this conclusion cannot be proven without a complete knowledge of all possible proteins that might interact with DP, the data suggest that E2F is the likely target.

In summary, we have shown that dominant-negative mutants of DP that prevent DNA binding and transcriptional regulation by E2F cause an arrest of the cell cycle in G_1. These findings complement previous work that showed that overexpression of E2F-1 can drive cells through the cell cycle. Here we have tested the requirement for E2F in G_1 and find that E2F, or perhaps an unknown but structurally related protein, plays an essential role in G_1 control. We are currently expanding the use of these mutants in an attempt to derive variants that will interact specifically with the various E2F family members. These mutants might be useful in future studies to discriminate between the various roles of the E2F family members.

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