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E2F-5, a New E2F Family Member That Interacts with p130 In Vivo

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E2F DNA binding sites are found in a number of genes whose expression is tightly regulated during the cell cycle. The activity of E2F transcription factors is regulated by association with specific repressor molecules that can bind and inhibit the E2F transactivation domain. For E2F-1, E2F-2, and E2F-3, the repressor is the product of the retinoblastoma gene, pRb. E2F-4 interacts with pRb-related p107 and not with pRb itself. Recently, a cDNA encoding a third member of the retinoblastoma gene family, p130, was isolated. p130 also interacts with E2F DNA binding activity, primarily in the G_0 phase of the cell cycle. We report here the cloning of a fifth member of the E2F gene family. The human E2F-5 cDNA encodes a 346-amino-acid protein with a predicted molecular mass of 38 kDa. E2F-5 is more closely related to E2F-4 (78% similarity) than to E2F-1 (57% similarity). E2F-5 resembles the other E2Fs in that it binds to a consensus E2F site in a cooperative fashion with DP-1. By using a specific E2F-5 antiserum, we found that under physiological conditions, E2F-5 interacts preferentially with p130.

E2F is the name given to a group of heterodimeric transcription factors that are composed of E2F-like and DP-like subunits (28). E2F DNA binding sites are present in the promoters of a number of genes whose expression is regulated during the cell cycle, and there is evidence that the presence of these E2F sites contributes to the cell cycle-regulated expression of these genes (13, 27, 38).

E2F DNA-binding activity has been found in complex with the retinoblastoma protein (pRb) and pRb-related p107 and p130 (6, 10, 29, 37). This group of proteins shares a conserved motif, the "pocket," that is involved in binding to both cellular and viral proteins. For this reason, the group of pRb-like proteins is collectively known as the pocket protein family. Complexes between E2F and the various pocket proteins are likely to have different functions in cell cycle regulation, as their times of appearance during the cell cycle differ. E2F in complex with pRb is found mostly in the G_1 phase of the cell cycle (5–7, 11). In contrast, complexes between p107 and E2F persist during the cell cycle but their composition is variable. In G_1, apart from E2F and p107, cyclin E and cdk2 are present. In the S phase, cyclin E is replaced by cyclin A in the E2F-p107 complex (29, 37). The functional significance of the presence of these cyclin-cdk complexes in the p107-E2F complex is not clear. In quiescent cells, a complex between E2F and p130 is the most prominent E2F DNA binding species. This complex disappears as cells emerge from quiescence, suggesting a role for p130-interacting E2F activity in cell cycle entry (10).

The ability of E2F to activate transcription is regulated by complex formation with the pocket proteins. Complex formation between E2F and pRb is subject to regulation by phosphorylation. Only the hypophosphorylated species of pRb interact with E2F, indicating that phosphorylation of pRb by cyclin-cdk complexes controls the interaction between E2F and pRb during the cell cycle (5–7, 11).

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The crucial role of E2F transcription factors in cell cycle regulation is emphasized by the findings that enforced expression of E2F causes cells to progress from G_1 into the S and G_2-M phases of the cell cycle (3) and E2F can stimulate quiescent cells to initiate DNA synthesis (23). Importantly, overexpression of E2F, together with an activated *ras* oncogene, can cause oncogenic transformation of primary rodent fibroblasts (3).

Four different E2F-like polypeptides have already been isolated. E2F-1, E2F-2, and E2F-3 are found only in complexes with pRb, whereas E2F-4 interacts preferentially with p107 (3, 15, 19, 22, 24, 30, 36). How complex formation between E2F and p107 and E2F and p130 is regulated is not known. To begin to address the regulation of E2F-p107 and E2F-p130 complexes, we searched for additional members of the E2F gene family. We report here the cloning of a fifth member of the E2F gene family that interacts preferentially with p130.

MATERIALS AND METHODS

Yeast two-hybrid screen. Yeast strain Y190 (17), containing "bait" plasmid pPC97-p107, encoding the Gal4 DNA binding domain (DBD) fused to the pocket region of p107, was transformed with a day 14.5 CD1 mouse embryo library (8) by the lithium acetate method (34). Two million transformants were selected for growth on plates lacking histidine and supplemented with 25 mM 3-aminotriazole. His^+ colonies were subsequently analyzed for β-galactosidase activity as previously described (12). cDNA library plasmids derived from double-positive yeast colonies were tested for bait specificity by retransformation with different Gal4 DBD fusion plasmids: pPC97-p107, pPC97-*bmi*, and pPC97 without an insert. The partial mouse E2F-5 cDNA was used to screen additional human cDNA libraries. The full-length human E2F-5 cDNA described here was isolated from the T84 colon carcinoma library (Stratagene).

Plasmids. pPC97-p107 was generated by cloning the pocket region of p107 (amino acids 240 to 816) in frame with the Gal4 DBD (amino acids 1 to 147) of pPC97 (8). pGST-E2F-5A and pGST-E2F-5B were constructed by cloning a fragment of human E2F-5 cDNA encoding amino acids 89 to 200 (pGST-E2F-5A) or 89 to 346 (pGST-E2F-5B) in pGEX-2T. For transfection experiments, the following plasmids were used. pSG-Gal4-E2F-1 contains amino acids 284 to 437 of human E2F-1 (19). pJ3-Gal4-E2F-4 and pJ3-Gal4-E2F-5 were obtained by cloning a fragment of the human cDNA of E2F-4 (encoding amino acids 276 to 412) or E2F-5 (encoding amino acids 222 to 346) in frame with the Gal4 DBD in pJ3Ω (33). pJ3-E2F-5 was constructed by cloning the full-length human E2F-5 cDNA (lacking the last 184 nucleotides of the 3′ noncoding sequence) into mammalian expression vector pJ3Ω. pCMV-E2F-5 was constructed by cloning

the full-length human E2F-5 cDNA in a cytomegalovirus promoter-containing expression vector. pCMV-DP-1, pCMV-pRb, pCMV-p107, pCMV-p107DE, and pCMV-pRbΔ22 have been described previously (20, 41).

Cell lines. U2-OS and CAMA cells were cultured in Dulbecco's modified Eagle medium supplemented with 10 or 20% fetal calf serum, respectively. Transfections were performed by the calcium phosphate precipitation method (39).

CAT assays. U2-OS cells were transiently transfected with the expression vectors together with 5 μg of $(Gal4)_5$-CAT (25) or 2 μg of $E2F_4$-CAT (20), 0.2 μg of pRSV-luciferase, and herring sperm carrier DNA to a total amount of 20 μg/10-cm-diameter plate. Cells were assayed for chloramphenicol acetyltransferase (CAT) and luciferase activities as described previously (2, 3).

Northern (RNA) blot analysis. For E2F-5 expression analysis, total cytoplasmic RNA was prepared from a panel of cell lines. Twenty micrograms of total cellular RNA was electrophoresed through a 1% formaldehyde agarose gel as previously described (4), transferred to nitrocellulose, and probed with a ^{32}P-labeled partial human E2F-5 cDNA (nucleotides 666 to 1038). Subsequently, the same filter was probed with a rat α-tubulin cDNA to control for the amount of RNA loaded in each lane.

Immunological reagents and immunoprecipitations. To generate antibodies against E2F-5, the GST-E2F-5A and GST-E2F-5B (see plasmids) proteins were made in *Escherichia coli* and purified with glutathione-Sepharose beads. Both proteins were injected into both rabbits (for polyclonal sera) and mice (for monoclonal antibodies) in equal amounts (50 μg of each protein per immunization). After three rounds of immunization, polyclonal serum was obtained. Monoclonal antibodies were made as previously described (3).

Monoclonal antibodies against E2F-1 (KH20), E2F-4 (RK13), pRb (XZ77), and p107 (SD-4 and SD-9) have been described previously (3, 20, 21, 41). p130 (C20) rabbit polyclonal antiserum was obtained from Santa Cruz Biotechnology Inc. CAMA cells and transfected U2-OS cells were labeled and immunoprecipitated as described previously (3).

Gel retardation assays. Gel retardation assays for transiently transfected U-2 OS cells were performed as described previously (20), with minor modifications. Ten micrograms of whole-cell extract was used in a binding buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4), 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 7% glycerol, 1 mM NaF, and 1 μg of sonicated herring sperm DNA in a 20-μl reaction volume with 0.5 ng of a ^{32}P-labeled oligonucleotide specifying the consensus E2F DNA binding site (Santa Cruz Biotechnology). DNA-protein complexes were allowed to form during incubation for 20 min at room temperature. For antibody supershift experiments, 1 μl of serum was preincubated with cell extracts for 10 min at room temperature, after which the oligonucleotide was added and the mixture was incubated for another 20 min at room temperature. The reaction products were separated on a 3.5% polyacrylamide gel in 0.25× TBE (1× TBE contains 89 mM Tris-borate [pH 8.3] and 1 mM EDTA) at 100 V at room temperature for 3 h. The gel was then dried and exposed to film.

Nucleotide sequence acession number. The GenBank accession number of the E2F-5 sequence shown in Fig. 1 is X86097.

RESULTS

Cloning of E2F-5. To identify cDNAs encoding polypeptides that interact with p107, a yeast two-hybrid screen was performed (14). Yeast strain Y190 (17), which contains two chromosomally located Gal4-inducible reporter genes, *HIS3* and *lacZ* (12), was transformed with the bait plasmid containing the pocket region (amino acids 240 to 816) of p107 fused to the Gal4 DBD. The bait-containing strain was subsequently transformed with a day 14.5 CD1 mouse embryo cDNA library in which each cDNA is individually fused to the transactivation domain of Gal4 (8). Two million transformants were placed on histidine selection. Eighty-seven colonies appeared on plates lacking histidine and were subsequently screened for expression of β-galactosidase. Of the 87 His^+ colonies, 16 also expressed β-galactosidase. Specificity of p107 interaction was confirmed by retransformation with plasmids encoding unrelated Gal4 DBD fusions. DNA sequence analysis showed that the 16 cDNA library plasmids rescued from the yeasts were derived from 10 different genes. Three cDNAs were derived from the same gene and showed significant homology to the four known E2Fs. Because of this, we named the protein encoded by this cDNA E2F-5.

The partial mouse E2F-5 cDNA was then used to obtain a full-length human cDNA clone by screening a human colon carcinoma cDNA library. The longest cDNA (2.1 kb) was sequenced and contained a 1,038-bp open reading frame encoding a 346-amino-acid protein with a predicted molecular mass of 38 kDa. Figure 1A shows the E2F-5 cDNA sequence and the deduced amino acid sequence.

E2F-5 is more closely related to E2F-4 (78% similarity) than to E2F-1 (57% similarity). In comparison with E2F-1 and E2F-4, three regions of homology can be observed in E2F-5 (Fig. 1B). The DBD (amino acids 43 to 115 of E2F-5) shares 93% similarity with the E2F-4 DNA binding region, whereas the juxtaposed DP-1 dimerization domains of E2F-4 and E2F-5 are 81% similar. Finally, the carboxyl-terminal pocket protein interaction domain of E2F-4 shares 83% similarity with the carboxyl terminus of E2F-5. E2F-4 and E2F-5 differ from E2F-1 in that both proteins lack the amino-terminal motif of E2F-1 that is involved in cyclin A binding. E2F-5 differs from E2F-4 in that it lacks the serine repeat region of E2F-4.

To analyze mRNA expression levels of E2F-5, a human E2F-5 cDNA was used to probe a Northern blot containing total cytoplasmic RNAs from a number of human cell lines. The E2F-5 probe detected a low level of a single 2.1-kb transcript in most cell lines. The human CAMA breast carcinoma cell line expressed somewhat higher levels of E2F-5 (Fig. 2).

E2F-5 contains a carboxyl-terminal transactivation domain. E2F-1 and E2F-4 contain a carboxyl-terminal transactivation domain that overlaps with the pocket protein binding site (3, 18). To test whether E2F-5 also contains a transactivation domain, we fused the carboxyl terminus of human E2F-5 to the Gal4 DBD in mammalian expression vector pJ3Ω. U2-OS osteosarcoma cells were transiently transfected with a CAT-encoding reporter gene harboring five upstream Gal4 sites or cotransfected with the reporter gene and Gal4 E2F expression vectors. Figure 3 shows that cotransfection of the Gal4 reporter plasmid with the Gal4 E2F-5 expression vectors resulted in 50-fold activation of the CAT-encoding reporter gene. Cotransfection with Gal4 E2F-1 or E2F-4 resulted in two- to threefold higher activation of the CAT reporter gene, whereas the Gal4 DBD alone failed to activate the reporter gene (Fig. 3). We concluded that E2F-5 contains a potent carboxyl-terminal transactivation domain.

E2F-5 requires DP-1 for DNA binding. Both E2F-1 and E2F-4 require dimerization with DP-1 for efficient DNA binding (1, 3, 20, 26). To investigate whether E2F-5 can bind to a consensus E2F DNA binding site and whether E2F-5 requires DP-1 dimerization in order to bind DNA, we performed a transient transfection experiment. Human U2-OS osteosarcoma cells were transfected with a CAT reporter plasmid in which a core promoter was linked to four upstream E2F sites. Figure 4 (track 1) shows that the E2F-CAT reporter plasmid only has low activity when transfected alone in osteosarcoma cells. Transfection of the DP-1 and E2F-5 expression vectors separately did not result in activation of the E2F-CAT reporter (Fig. 4, tracks 2 and 6). Cotransfection of the DP-1 and E2F-5 expression vectors resulted in strong dose-dependent, synergistic activation of the CAT reporter (Fig. 4, tracks 3 to 5). These data indicate that E2F-5 can bind the consensus E2F site and that DNA binding is DP-1 dependent. On the basis of these results, we conclude that E2F-5 is a genuine member of the E2F gene family.

E2F-5 transactivation is suppressed by pocket proteins. Transactivation of E2F-1 and E2F-4 is suppressed by pocket protein binding because the transactivation domain of these E2Fs overlaps with the pocket protein interaction surface. To test the effect of pocket protein expression on E2F-5 transactivation, we used a transient transfection assay. Since E2F-1 and E2F-4 both require DP-1 dimerization for efficient binding to their respective pocket proteins (3, 20), we measured the

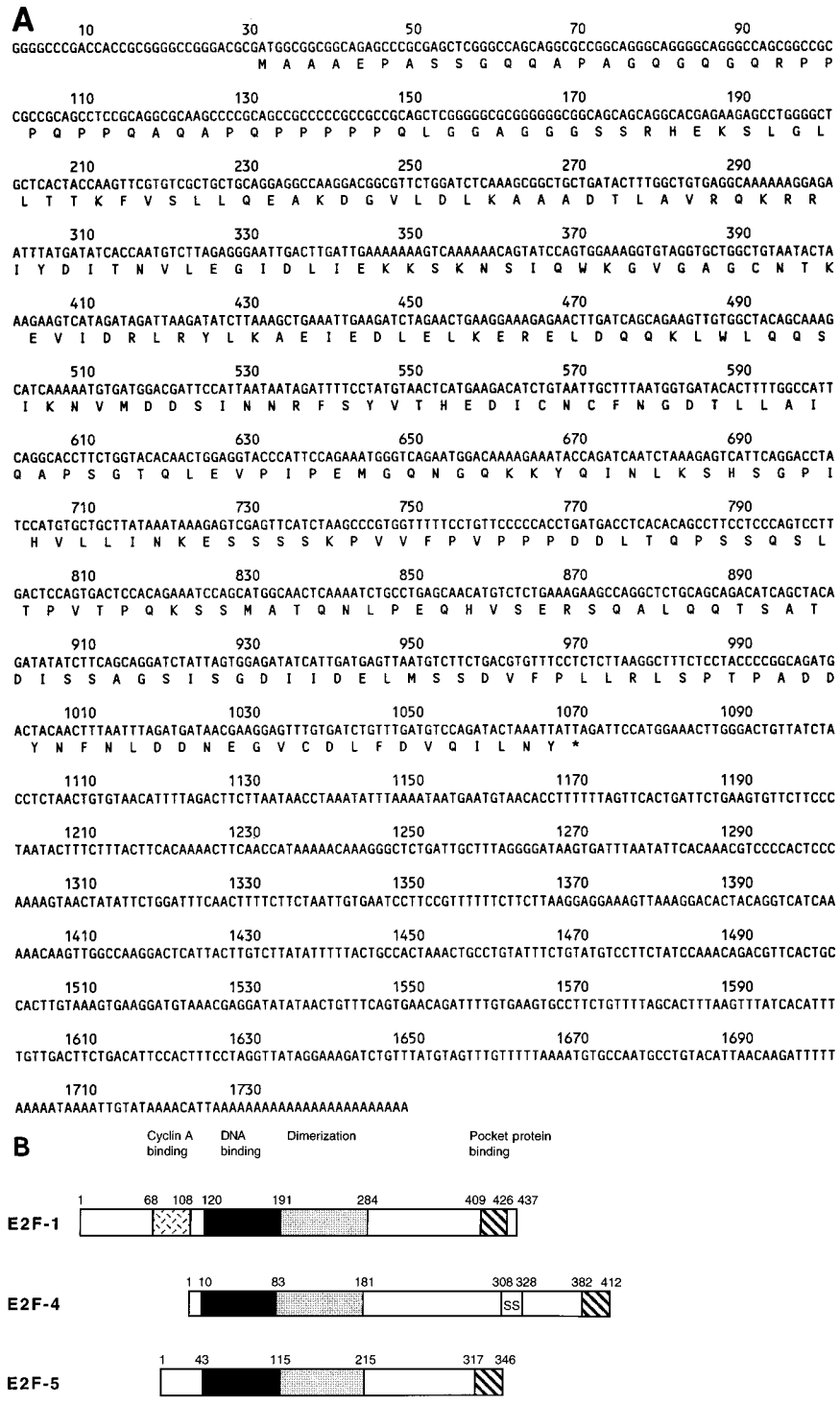

FIG. 1. Human E2F-5 structure. (A) Nucleotide sequence and deduced amino acid sequence of the human E2F-5 cDNA. (B) Schematic representation of E2F-5 in comparison with E2F-1 and E2F-4. The borders of the conserved domains are indicated by amino acid numbers. SS in E2F-4 indicates the serine-rich motif.

effect of pocket protein expression on transcription activated by E2F-5 plus DP-1. U2-OS cells were transfected with the E2F-CAT reporter plasmid together with E2F-5 and DP-1. Figure 5 (track 3) shows that cotransfection of E2F-5 and DP-1 resulted in greater than 100-fold activation of the E2F-CAT reporter gene. E2F-5-stimulated transcription was inhibited by cotransfection with expression vectors pRb, p107, and p130 in a dose-dependent fashion. pRb (pRbΔ22) and p107 (p107DE) mutant plasmids that lack an intact pocket domain were unable to suppress E2F-5 transactivation (Fig. 5, tracks 6 and 9). Significantly, these mutant forms of pRb and p107 also lack growth inhibitory activity (41). Thus, although this experiment

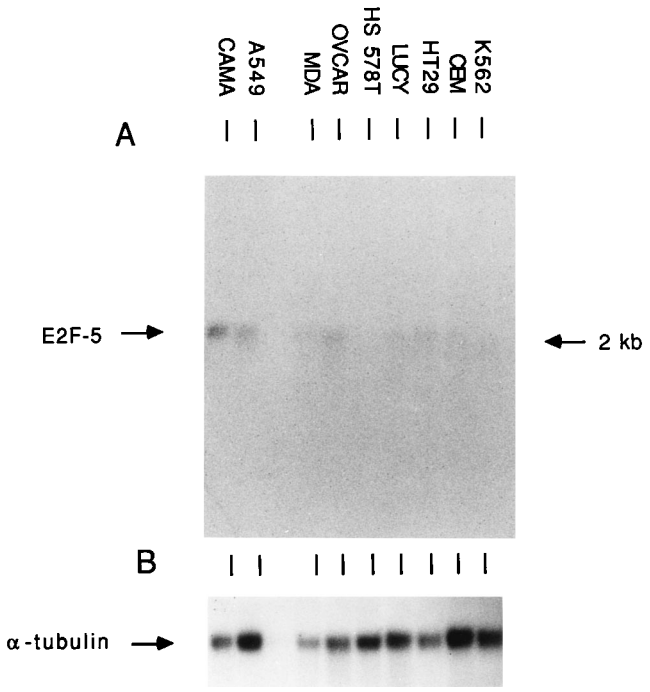

FIG. 2. Expression patterns of E2F-5 in human cell lines. (A) Northern blot containing total cytoplasmic RNAs from the human cell lines indicated hybridized to a human E2F-5 cDNA probe. RNAs from the following human cell lines were used: CAMA, human breast carcinoma; A549, lung carcinoma; MDA, MDA-MD157 breast carcinoma; OVCAR, ovarian carcinoma; HS 578T, breast carcinoma; LUCY, ovarian carcinoma; HT29, colon carcinoma; CEM, T-cell leukemia; K562, erythroleukemia. (B) The same filter hybridized with a rat α-tubulin probe.

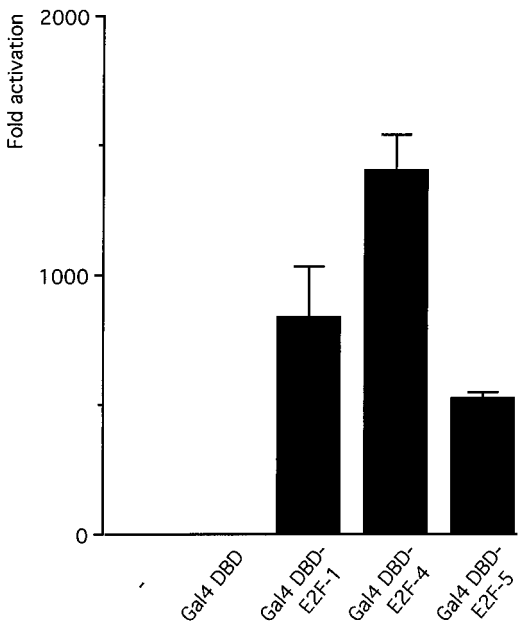

FIG. 3. E2F-5 has a carboxyl-terminal transactivation domain. U-2 OS cells were transfected with a CAT reporter plasmid harboring upstream Gal4 sites (5 μg) in the presence or absence (−) of the Gal4 DBD or E2F expression vector (1 μg). pRSV-Luciferase (0.2 μg) was added as an internal control for transfection efficiency. CAT activity was assayed 2 days posttransfection and was normalized to the luciferase activity of each sample. The fold activation of Gal4 E2F over the CAT reporter gene alone is represented. The data are representative of at least three independent experiments, each done in duplicate.

did not allow unambiguous identification of the preferred binding partner of E2F-5, it did indicate that E2F-5 transactivation is inhibited by pocket protein binding and that a close correlation exists between the ability of pRb and p107 to cause a growth arrest and their ability to inhibit E2F-5 transactivation. It is important to point out that the U2-OS cells used in this experiment are insensitive to pRb- or p107-induced growth arrest (41). The observed effects on E2F-5 transactivation are therefore unlikely to be due to nonspecific cell cycle effects of pRb or p107.

E2F-5 interacts preferentially with p130 in a band shift assay. To further investigate the specificity of pocket protein binding by E2F-5, we performed an electrophoretic mobility shift assay. U2-OS cells were transiently transfected with the DP-1 and E2F-5 expression vectors with or without the pRb, p107, or p130 expression vector. Two days after transfection, whole-cell extracts were prepared from transfected cells and incubated with a ^{32}P-labeled oligonucleotide that specifies a consensus E2F site. DNA-protein complexes were separated on a polyacrylamide gel and visualized by radiography. Figure 6A shows that transfection of the E2F-5 and DP-1 expression vectors led to the appearance of a novel complex that was not observed in mock-transfected cells (Fig. 6A, compare lanes 1 and 2). This novel complex could be supershifted by addition of a rabbit E2F-5 antibody (see below), thus confirming that this complex contained E2F-5 (Fig. 6B, lanes 4 and 5). The E2F-5–DP-1 complex could also be supershifted by cotransfection with the p130 expression vector and to a lesser extent by cotransfection with p107 but not with the pRb expression vector (Fig. 6A, lanes 3 to 5). To confirm that p130 and E2F-5 were both present in the slowly migrating complex, we incubated extracts from E2F-5-, DP-1-, and p130-transfected U2-OS cells with antibodies to E2F-5 and p130. Figure 6B (lanes 7 to 9) shows that both the E2F-5 antibody and the p130 antibody interfered with the DNA binding of the slowly migrating complex, thus confirming that the slowly migrating complex contained both E2F-5 and p130. Taken together, these data indicate that in this assay, of the three pocket proteins tested, p130 had the highest affinity for the E2F-5–DP-1 heterodimer.

E2F-5 interacts preferentially with p130 in vivo. Under physiological conditions, E2F-1 binds preferentially to pRb and E2F-4 binds preferentially to p107 (3, 15, 19, 24). In transient transfection experiments, however, both E2F-1-activated gene expression and E2F-4-activated gene expression can be suppressed by both pRb and p107 (3, 40). This loss of specificity is probably caused by the overexpression of these proteins. To address which of the three members of the retinoblastoma protein family interacts with E2F-5 under physiological conditions, we generated both a rabbit polyclonal antiserum and a mouse monoclonal antibody against human E2F-5. Initial immunoprecipitation experiments using in vitro-transcribed and translated E2F-1, E2F-4, and E2F-5 indicated that the polyclonal anti-E2F-5 serum and the monoclonal antibody to E2F-5 specifically recognized E2F-5 (data not shown). To characterize the E2F-5 protein species that are recognized by these antibodies, we transiently transfected U2-OS osteosarcoma cells with an E2F-5 expression vector and immunoprecipitated lysates from metabolically labeled transfected cells with both the polyclonal and monoclonal anti-E2F-5 antibodies. Figure 7 shows that both the polyclonal and monoclonal anti-E2F-5 antibodies recognized three closely migrating protein species of around 45 kDa in transfected U2-OS cells that are not present in mock-transfected cells. We pre-

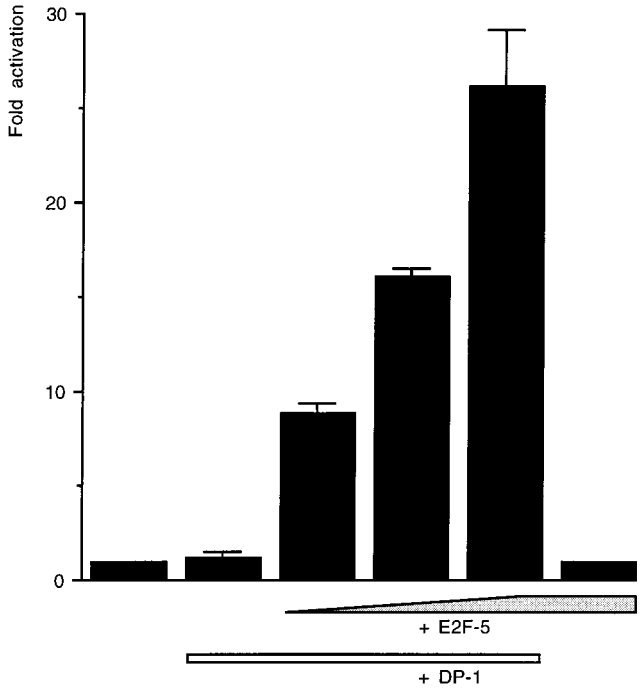

FIG. 4. E2F-5 and DP-1 cooperate in transactivation. U2-OS osteosarcoma cells were transfected with increasing amounts of the pJ3-E2F-5 expression vector (1, 2, or 5 μg) together with 100 ng of pCMV-DP-1, as indicated. In each transfection, 2 μg of the reporter construct $E2F_4$-CAT and 0.2 μg of pRSV-Luciferase were added. CAT activity was normalized to the luciferase activity of each sample. Fold activation was calculated relative to the basal level of $E2F_4$-CAT, which was set to unity (1.0). The data are representative of at least three independent experiments done in duplicate.

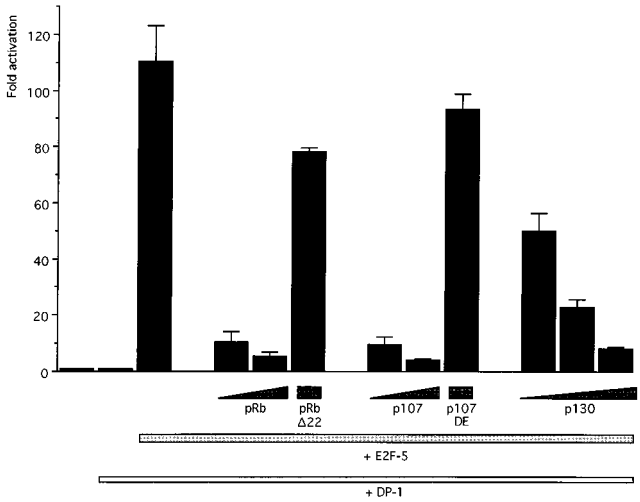

FIG. 5. Inhibition of E2F-5 transactivation by pocket proteins. U2-OS cells were transfected with 5 μg of pJ3-E2F-5 and 100 ng of pCMV-DP-1 in combination with pCMV-Rb (50 and 100 ng), pCMV-RbΔ22 (100 ng), pCMV-p107 (50 and 100 ng), pCMVp107DE (100 ng), or pCMV-HA-p130 (50, 100, and 500 ng). Together with the expression plasmids, the cells were transfected with 2 μg of $E2F_4$-CAT and 0.2 μg of pRSV-Luciferase. CAT activity was normalized to the luciferase internal control. The data are representative of at least three independent experiments done in duplicate.

sume that by analogy with E2F-4, these products are the result of differential phosphorylation of E2F-5 (3, 15).

To investigate which members of the pRb protein family E2F-5 associates with in nontransfected cells, we used the E2F-5 antiserum in a sequential immunoprecipitation experiment. CAMA breast carcinoma cells were metabolically labeled with $^{32}P_i$, and nonionic detergent lysates were prepared. These lysates were subjected to immunoprecipitation with nonimmune serum, pRb-specific antibody, p107 antibody, or p130-specific antiserum. Proteins that were immunoprecipitated with these sera were released by boiling in sodium dodecyl sulfate (SDS)-containing buffer, diluted, and reimmunoprecipitated with E2F-5-specific antiserum or with nonimmune control serum. Figure 8A shows that three protein species of around 45 kDa could be specifically reimmunoprecipitated with E2F-5 antiserum from the p130 immunoprecipitate but not from the pRb, p107, or control nonimmune serum immunoprecipitate. Significantly, these three protein species virtually comigrated with those seen in E2F-5-transfected U2-OS cells (Fig. 6A, compare lanes 8 and 10). The minor differences in electrophoretic mobility between the p130-associated E2F-5 species in CAMA cells and the E2F-5 species present in U2-OS cells (Fig. 6A, lanes 8 and 10) are most likely due to a difference in E2F-5 phosphorylation status between these two cell types. As a further control, we verified whether the pRb and p107 immunoprecipitates contained their respective E2Fs. Figure 8B shows that pRb did, indeed, coimmunoprecipitate E2F-1 and that p107 brought down E2F-4. Taken together, these data indicate that E2F-5 preferentially interacts with p130 in vivo.

DISCUSSION

We report here the isolation of a fifth member of the E2F gene family. E2F-5 has all of the hallmarks of a genuine E2F family member: it contains a highly conserved DBD, a DP-1 dimerization domain, and a carboxyl-terminal transactivation domain. Furthermore, E2F-5 binds a consensus E2F DNA binding site in a cooperative fashion with DP-1 and can activate the expression of an E2F site-containing reporter gene.

We performed three types of experiments to determine with which of the three pocket proteins E2F-5 interacts preferentially in vivo. In transient transfection experiments, E2F-5 transactivation could be suppressed by all three members of the retinoblastoma protein family, pRb, p107, and p130. In this respect, E2F-5 resembles E2F-1 and E2F-4, since transactivation of both E2F-1 and E2F-4 can be inhibited in transient transfection assays by pRb, as well as p107 (3, 40). This apparent lack of specificity in a transient transfection assay is probably the result of the high expression levels of both E2F and the pocket proteins in the transiently transfected cells. Two additional experiments were performed to address the pocket protein specificity of E2F-5. In the first experiment, cells were transiently transfected with the E2F-5 and DP-1 expression vectors in the presence or absence of expression vectors for all three pocket proteins. Subsequently, band shift assays were performed with extracts from the transfected cells and an oligonucleotide specifying a consensus E2F binding site. Cotransfection of p130 could effectively supershift the E2F-5–DP-1 complex, although lower affinity for p107 could also be detected in this assay (Fig. 6). In the band shift experiment, only complexes between pocket proteins and E2F-5 that are stable for prolonged periods of time were detected as E2F-pocket protein supershifted complexes. Thus, although all three pocket proteins could suppress E2F-5 transactivation, the complex between E2F-5 and p130 was most stable in the band shift assay (Fig. 6). In a similar experiment, we were able to supershift an E2F-4 DNA binding complex with p107 but not with pRb (1a). Finally, and most significantly, in nontransfected,

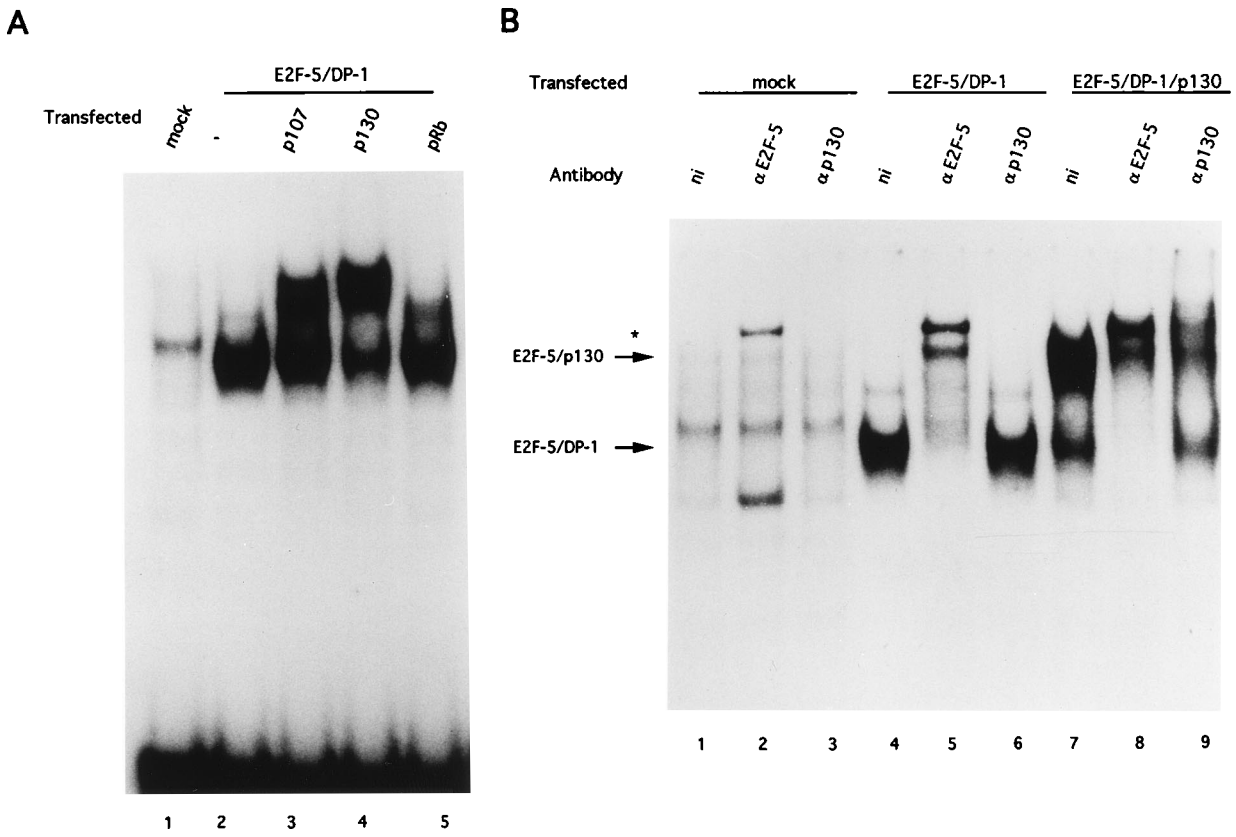

FIG. 6. E2F-containing complexes in transiently transfected U2-OS cells. (A) U2-OS osteosarcoma cells were transiently transfected with expression vectors pCMV-E2F-5 and pCMV-DP-1 in the presence or absence (−) of expression vector pRb-p107 or p130, as indicated. After 2 days, whole-cell extracts were prepared, incubated with a ^{32}P-labeled oligonucleotide containing a consensus E2F DNA binding site, and subjected to gel electrophoresis. (B) U2-OS osteosarcoma cells were transiently transfected with expression vectors E2F-5 and DP-1 in the presence or absence of expression vector p130, as indicated. After 2 days, whole-cell extracts were prepared, incubated in the presence of the antibodies indicated with a ^{32}P-labeled oligonucleotide containing a consensus E2F DNA binding site, and subjected to gel electrophoresis. The positions of the E2F-5–DP-1 DNA complex and the E2F-5-–p130 complex are indicated. The asterisk indicates a nonspecific complex that is the result of adding the E2F-5 antibody. ni, nonimmune serum.

metabolically labeled CAMA breast carcinoma cells, E2F-5 could be coimmunoprecipitated with p130 and not with p107 or pRb (Fig. 8). These data indicate that under physiological conditions, E2F-5 preferentially associates with p130. We cannot exclude the possibility that in assays more sensitive than the one used here, E2F-5 can also be detected in complex with p107.

The finding that E2F-5 interacted with p130 but not with p107 was somewhat unexpected because p130 and p107 are structurally closely related and, indeed, p107 and p130 share the ability to bind cyclins A and E (16, 32, 41). On the other hand, p107 and p130 differ in the ability to interact with D-type cyclins in vivo, as only p107, and not p130, coimmunoprecipitates with anti-D-type-cyclin antibodies (32). Importantly, the times of appearance of the p130-E2F and p107-E2F complexes in the cell cycle differ (9, 10, 29, 35, 37). This suggests that p107 and p130 have distinct functions during the cell cycle. The preferential binding of E2F-5 by p130 is consistent with such a distinct role for p130 in cell cycle regulation.

Our finding that E2F-5 can bind to a consensus E2F site by no means rules out the possibility that E2F-5 interacts in vivo with a discrete subset of E2F sites that is distinct from the E2F sites that are bound by the other members of the E2F gene family. Consistent with such a binding site preference of the different E2Fs is the finding that the E2F sites that are present in the thymidine kinase gene promoter and in the b-*myb* pro-

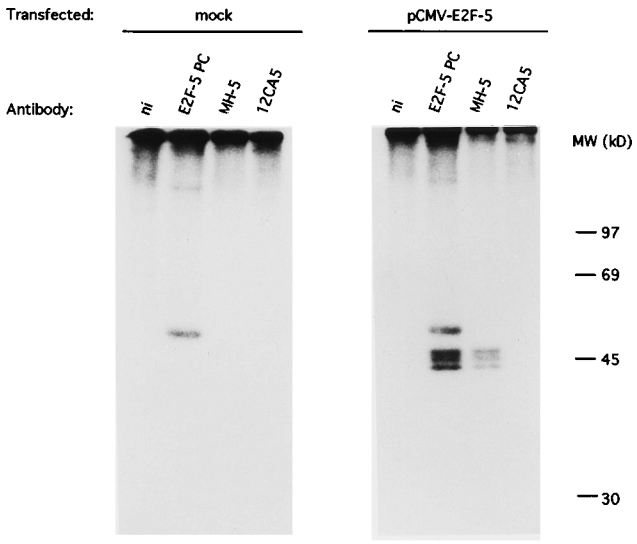

FIG. 7. Immunoprecipitation of E2F-5 from transfected cells. U2-OS osteosarcoma cells were transiently transfected with an E2F-5 expression vector. After 2 days, cells were labeled with $^{32}P_i$ and immunoprecipitated with either nonimmune serum (ni), polyclonal rabbit anti-E2F-5 serum (E2F-5 PC), monoclonal anti-E2F-5 antibody (MH-5), or a control monoclonal antibody (12CA5). Immunoprecipitated proteins were separated on an SDS–7.5% polyacrylamide gel and detected by autoradiography. MW, molecular mass; kD, kilodaltons.

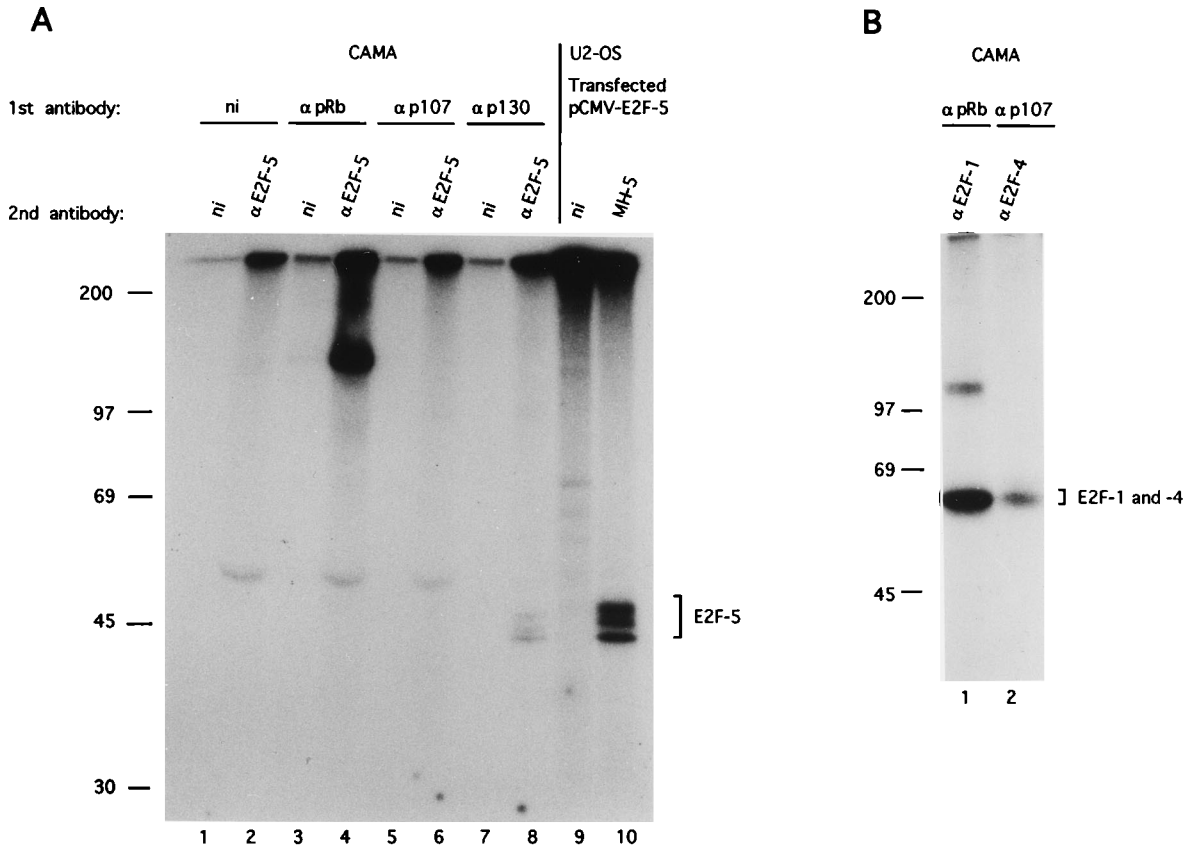

FIG. 8. E2F-5 preferentially interacts with p130 in vivo. (A) Human CAMA breast carcinoma cells were labeled with $^{32}P_i$ and nonionic detergent lysates were subjected to sequential immunoprecipitation. In a first immunoprecipitation, lysates were incubated with nonimmune serum (ni) or pRb, p107, or p130 antibody, as indicated. Associated proteins were released from the immunoprecipitated pocket proteins by boiling in SDS-containing buffer and reimmunoprecipitated with control nonimmune serum or E2F-5-specific polyclonal antiserum (αE2F-5). As a control, U2-OS cells were transiently transfected with expression vector E2F-5 as described in the legend to Fig. 7, labeled with $^{32}P_i$, and immunoprecipitated with nonimmune serum or E2F-5 antibody (lanes 9 and 10, respectively). As a further control, proteins released from pRb and p107 immunoprecipitates were reimmunoprecipitated with anti-E2F-1 (KH20) or anti-E2F-4 (RK13) antibody (B). Immunoprecipitated proteins were separated on an SDS–7.5% polyacrylamide gel and detected by autoradiography. The numbers to the left of each panel are molecular masses in kilodaltons.

moter interact preferentially with E2F-p107 complexes (27, 31). Since complexes between E2F and p130 are found mostly in quiescent cells and disappear quickly after cells emerge from quiescence, it is likely that E2F-5-responsive genes are involved in the early responses of resting cells to growth factor stimulation (10). The availability of p130-interacting E2F-5 should allow us to identify E2F-5-responsive genes.

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