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The Retinoblastoma Protein Binds to a Family of E2F Transcription Factors

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E2F is a transcription factor that helps regulate the expression of a number of genes that are important in cell proliferation. Recently, several laboratories have isolated a cDNA clone that encodes an E2F-like protein, known as E2F-1. Subsequent characterization of this protein showed that it had the properties of E2F, but it was difficult to account for all of the suggested E2F activities through the function of this one protein. Using low-stringency hybridization, we have isolated cDNA clones that encode two additional E2F-like proteins, called E2F-2 and E2F-3. The chromosomal locations of the genes for E2F-2 and E2F-3 were mapped to 1p36 and 6q22, respectfully, confirming their independence from E2F-1. However, the E2F-2 and E2F-3 proteins are closely related to E2F-1. Both E2F-2 and E2F-3 bound to wild-type but not mutant E2F recognition sites, and they bound specifically to the retinoblastoma protein in vivo. Finally, E2F-2 and E2F-3 were able to activate transcription of E2F-responsive genes in a manner that was dependent upon the presence of at least one functional E2F binding site. These observations suggest that the E2F activities described previously result from the combined action of a family of proteins.

The retinoblastoma gene (*RB-1*) is one of the best-studied tumor suppressor genes (reviewed in reference 53). Its characterization and cloning were made possible by the frequent mutation of *RB-1* in the development of retinoblastomas (15, 16, 34). All retinoblastomas studied to date contain mutations in both *RB-1* alleles, and these mutations lead to the loss or functional inactivation of the retinoblastoma protein (pRB). Subsequent studies have identified *RB-1* mutations in a wide variety of other tumors, including osteosarcomas, small-cell lung carcinomas, breast carcinomas, prostate carcinomas, and bladder carcinomas (53). Reintroduction of the wild-type *RB-1* gene into a number of *RB-1*-negative cell lines appears sufficient to reverse, or at least reduce, their tumorigenicity (4, 28, 49, 50, 52). These data suggest that the product of this tumor suppressor gene contributes to the regulation of cellular proliferation in a broad range of tissues.

Genetic studies have shown that an essential portion of the transforming activity of the small DNA tumor viruses is the ability of their oncoproteins (adenovirus E1A, simian virus 40 large T antigen, or human papillomavirus E7) to bind and inactivate pRB (reviewed in reference 12). Together, these observations suggest that loss of pRB function is one way in which abnormal cellular proliferation is frequently induced.

Recent experiments have shown that pRB binds to a number of cellular proteins and that this binding is disrupted by the viral oncoproteins (reviewed in reference 41). One of the best characterized of these pRB-associated proteins is the transcription factor E2F. E2F was originally identified as a DNA-binding protein that is essential for the E1A-dependent activation of the adenovirus E2 promoter (33). However, E2F binding sites have subsequently been found in the promoters of many cellular genes whose products are important for cell proliferation (41). Many of these genes are expressed at the G_1/S transition and encode proteins that are essential for DNA synthesis. However, there are a number of other E2F-responsive genes that are expressed at other stages of the cell cycle, including the immediate-early gene c-*myc* (26, 51).

The activity of E2F appears to be regulated, at least in part, by its association with proteins such as pRB. pRB binds directly to E2F and inhibits its transactivation (19, 21, 22, 25, 54). This inhibition of E2F transactivation appears to be cell cycle regulated. In G_1 cells, E2F is associated with unphosphorylated or underphosphorylated pRB (2, 3, 6). As cells pass the G_1/S transition, pRB becomes phosphorylated, and unbound E2F is found. This free E2F is presumed to be transcriptionally active.

In addition to pRB, E2F is also known to interact independently with the pRB-related protein p107 (5, 9, 46). There are two distinct forms of the E2F-p107 complex, one containing the cell cycle kinase cyclin A/cdk2 and the other containing the kinase cyclin E/cdk2 (5, 9, 35, 38). The formation of these p107-containing complexes is also cell cycle regulated, and their appearance correlates with the timing of formation of the active kinase complexes (13, 35, 46). The role of these p107-containing complexes is less well defined, but p107 can inhibit E2F-mediated transactivation (44, 58, 59), and the p107-E2F complexes are also selectively targeted by the transforming proteins of the small DNA tumor viruses. These data suggest that pRB, p107, and cell cycle-dependent kinases may act in concert to confine the activation of E2F and E2F-responsive genes to precise stages of the cell cycle.

Using pRB to screen expression libraries, we and others have recently isolated a cDNA clone, called E2F-1, that has many of the properties of E2F (22, 31, 45). E2F-1 binds to unphosphorylated pRB both in vitro and in vivo, and this binding can be competed for by adenovirus E1A. It also interacts with E2F recognition sites in a sequence-specific manner. E2F-1 stimulates the transcription of E2F-responsive promoters, and this activation can be inhibited by its direct association with pRB (21).

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Despite these findings, a number of experiments suggested that E2F-1 is unable to account for all of the observed E2F activity (11, 22, 31). First, antibodies to E2F-1 are unable to supershift or abolish all of the E2F DNA binding complexes seen in gel shift experiments. Second, the major E2F-like proteins associated with pRB and p107 give different proteolytic patterns (11). These data suggest that the observed E2F activity might result from the concerted action of several E2F-1-related proteins. To test this notion, we have used low-stringency hybridization to screen for E2F-1-related genes. Here, we describe the characterization of two proteins, designated E2F-2 and E2F-3, which bind to E2F DNA recognition sites, associate with pRB in vivo, and transactivate E2F reporter constructs. Similar findings are reported in the accompanying paper by Ivey-Hoyle and colleagues (30). The isolation of E2F-2 and E2F-3 indicates that the activity previously described as E2F is actually the combined activity of a family of proteins with related properties.

MATERIALS AND METHODS

Isolation of E2F-2 and E2F-3. Initially, 1 million plaques from a human cDNA library, NALM-6 (in ZAPII), were screened with a fragment of the E2F-1 cDNA, encoding amino acids 89 to 214, that had been labeled with [α-^{32}P]dCTP by random priming (14). Subsequently, a further 2 million plaques from both a NALM-6 library and a human fetal brain library (in ZAPII; Stratagene) were screened with a mixture of the E2F-1 probe and a randomly primed fragment of E2F-2 encoding amino acids 85 to 200. Finally, these filters were rescreened with a randomly primed fragment of E2F-3 that encoded amino acids 132 to 230. Hybridization was performed at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–5× Denhardt's solution–30% formamide–5% dextran sulfate–0.5% sodium dodecyl sulfate (SDS)–150 μg of sonicated salmon sperm DNA per ml. The filters were washed at room temperature three times for 20 min each time in 2× SSC–0.1% SDS. Positive clones were detected by autoradiography and plaque purified. cDNA clones corresponding to E2F-1 were identified by sequencing with a 20-bp primer, pC15.1 (GTGCTGAAGGTGCAGAAGCG), encoding amino acids 159 to 165 of E2F-1. By using exonuclease III-S1 nuclease digestion to generate nested sets of deletions (24), the remaining novel clones were sequenced with use of Sequenase 2.0 (United States Biochemical Corp.).

Genomic DNA cloning. Bacteriophage and cosmid human genomic DNA libraries were hybridized with ^{32}P-labeled probes to isolate E2F-1-related genes. These probes included a 1.0-kb *Sac*II-*Xho*I fragment of the human E2F-1 cDNA, a 0.2-kb *Kpn*I-*Sma*I genomic DNA fragment of the human E2F-2 gene, a 2.2-kb *Sma*I-*Sac*I fragment of the E2F-2 cDNA, and 0.25-kb *Eco*RV-*Hin*cII and 1.8-kb *Eco*RV-*Sma*I fragments of the human E2F-3 cDNA.

Fluorescence in situ hybridization. Bromodeoxyuridine-synchronized, phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal donor were used as a source of metaphase chromosomes. Genomic DNA subcloned into phage or cosmid vectors was nick translated with digoxigenin-11-UTP and hybridized overnight at 37°C to fixed metaphase chromosomes according to the method of Pinkel et al. (42), except for the inclusion of 0.33 μg of highly reiterated human DNA self-annealed to C_0t = 1 (Bethesda Research Laboratories, Gaithersburg, Md.) per μl. Signals were detected by incubating the slides with fluorescein-conjugated sheep antidigoxigenin antibodies (Boehringer Mannheim, Indianapolis, Ind.) followed by counterstaining in propidium iodide solution containing antifade (1,4-diazabicyclo[2.2.2]octane; Sigma Chemical, St. Louis, Mo.). Fluorescence microscopy was performed with a Zeiss standard microscope equipped with fluorescein epifluorescence filters.

Somatic cell hybrids. A panel of 18 human × mouse somatic cell hybrids containing different human chromosomes was obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, N.J.); the characterization and human chromosome content of these hybrids are described in the repository catalog. We also derived seven human × hamster somatic cell lines by polyethylene glycol-mediated fusion of cryopreserved human leukemic bone marrow blasts with E36 hamster cells, as previously described (37). Ten micrograms of DNA from each hybrid line was digested with *Bgl*II and subjected to Southern blotting analysis using the 0.2-kb *Kpn*I-*Sma*I E2F-2 genomic DNA probe, which identified a 7.0-kb human genomic restriction fragment. The same blots were hybridized with a 0.25-kb *Eco*RV-*Hin*cII E2F-3 cDNA probe, which identifies a 9.5-kb restriction fragment of the human E2F-3 gene and 8.0- and 6.0-kb fragments of the two E2F-3 pseudogenes.

Northern (RNA) blot analysis. Total cellular RNA was isolated from either ML-1 (premyeloid leukemia), C-33A (cervical carcinoma cell line), U118 (glioblastoma), 293 (adenovirus-transformed kidney epithelial cell), or NGP (neuroblastoma) cells by the method of Chomczynski and Sacchi (6a). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography (1), and 5 μg was fractionated by electrophoresis on a 1.0% formaldehyde agarose gel and then transferred to Hybond-N$^+$ membranes (Amersham). The human tissue blot (Clontech Laboratories, Inc.) contains 2 μg of poly(A)$^+$ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas per lane. The blots were screened with fragments of either E2F-1 (encoding amino acids 259 to 437), E2F-2 (encoding amino acids 195 to 437), E2F-3 (encoding amino acids 224 to 425), or human β-actin (as an internal control) that had been labeled with [α-^{32}P]dCTP by random priming (14). Hybridization was carried out for 18 h at 42°C in 5× SSPE–10× Denhardt's solution–50% formamide–2% SDS–100 μg of sonicated salmon sperm DNA per ml. The filters were washed at room temperature three times for 20 min each time in 2× SSC–0.05% SDS and then at 60°C twice for 20 min each time in 0.1× SSC–0.1% SDS.

Construction of glutathione *S*-transferase (GST) fusion proteins. E2F-2 and E2F-3 sequences were prepared by polymerase chain reaction with Vent polymerase (New England BioLabs), using the following primers: for E2F-2 (amino acids 85 to 200), 10.2 and 10.3; for E2F-2 (amino acids 195 to 437), 10.11 and 10.17; for E2F-2 (amino acids 244 to 437), 10.14 and 10.17; for E2F-2 (amino acids 291 to 437), 10.15 and 10.17; for E2F-2 (amino acids 381 to 437), 10.16 and 10.17; for E2F-3 (amino acids 132 to 230), 31.2 and 31.3; and for E2F-3 (amino acids 132 to 270), 31.2 and 31.9.

Sequences of the primers were as follows: 10.2, 5′-GATCCCATGGGATCCCCGGCCAAAAGGAAGCT-3′; 10.3, 5′-CTAGGAATTCGTCTTCAAACATTCCCCT-3′; 10.11, 5′-CATCCATGGGATCCAGGGGAATGTTTGAAGACCC-3′; 10.14, 5′-GATGGATCCAACAAGAGGCTGGCCTATG-3′; 10.15, 5′-GATGGATCCCTCAAGAGCAGGGAAGG-3′; 10.16, 5′-GATGGATCCCTCCTGCAGCAGACTGAG-3′; 10.17, 5′-CTAGAATTCAAGCTTGGCTGTCAGCCTGTCT

GTGA-3'; 31.2, 5'-GATCGGATCCATGGCAAAGCGAAG GCTGGA-3'; 31.3, 5'-GATCGGATCCTCAGCCCATCCAT TGGACGTTG-3'; and 31.9, 5'-GTACGGATCCTCGGTTAA CAGTTTGAGGTCC-3'. The amplified fragments were subcloned into pGEX2T as *Bam*HI-*Eco*RI fragments. The resulting GST fusion proteins were expressed and purified from *Escherichia coli* as described previously (48). After binding to glutathione-agarose, the size, purity, and concentration of the GST fusion proteins were evaluated by Coomassie blue staining of SDS-polyacrylamide gels.

Gel retardation assays. Gel retardation assays were performed with 7 to 200 ng of GST fusion in a binding buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Nonidet P-40 to which 0.1 to 0.5 ng of ^{32}P-labeled oligonucleotide probe, corresponding to either the wild-type (5'-ATTTAAGTTTCGCGCCCTTTCTCAA-3') or a mutant (5'-ATTTAAGTTTCGATCCCTTTCTCAA-3') E2F site, was added. The reaction products were separated on a 4% polyacrylamide gel run at 180 V in 0.25 Tris-borate-EDTA at 4°C for 1 h. The gel was then dried and subjected to autoradiography.

In vitro binding experiments. cRNA was synthesized by in vitro transcription using either T7 or T3 RNA polymerase (Promega). The resulting cRNA was translated in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (New England Nuclear). The synthesized polypeptides were then incubated for 20 min with 2 μg of glutathione-agarose-bound GST fusion protein in E1A lysis buffer (ELB$^+$; 250 mM NaCl, 50 mM HEPES [pH 7.0], 0.1% Nonidet P-40) containing 50 mM NaF, 5 mM EDTA, 0.1 mM sodium orthovanadate, 50 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, 1 mM dithiothreitol. The supernatant was then reincubated for 20 min with 2 μg of either GST or GST fusion protein that had been prebound to glutathione-agarose (Sigma). The recovered complexes were washed four times with ELB$^+$ and then resolved on SDS–10% polyacrylamide gels.

Cell culture and labeling. All cells were cultured at 37°C on 100-mm-diameter tissue culture plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were labeled for 4 h at 37°C with, per plate, 1 mCi of Trans[^{35}S]-label (ICN) in 2 ml of methionine-free medium or 5 mCi of $^{32}P_i$ (carrier free; ICN) in 1 ml of phosphate-free medium.

Antisera. Polyclonal antisera to E2F-2 and E2F-3 were raised by injecting mice with GST fusion proteins containing amino acids 85 to 200 of E2F-2 or amino acids 132 to 230 of E2F-3 as described by Harlow and Lane (20a). The antibodies against pRB (C36 and XZ77), p107 (SD2, SD4, SD6, SD9, and SD15), simian virus 40 large T antigen (PAb419), and E2F-1 have been described previously (11, 20, 22, 27).

Immunoprecipitation-reimmunoprecipitation experiments. Cells were collected by centrifugation and lysed on ice for 30 min in 0.5 ml of ELB$^+$. The lysate was precleared with 50 μl of normal rabbit serum and 100 μl of fixed and killed *Staphylococcus aureus* (Zymed), and the supernatant was used in immunoprecipitation with either 2 μl of the mouse polyclonal antisera or 100 μl of tissue culture supernatant from monoclonal antibody hybridoma cells. The immunocomplexes were recovered on protein A-Sepharose beads, washed four times with ELB$^+$, and then resuspended in 50 μl of 2% SDS–0.1 M dithiothreitol. For reimmunoprecipitation experiments, the sample was boiled for 5 min. The supernatant was then diluted to 1 ml with ELB$^+$ and precleared with protein A-Sepharose beads prior to incubation with the second antibody. The proteins were resolved on SDS–8% polyacrylamide gels and detected by either fluorography (^{35}S-labeled proteins) or autoradiography (^{32}P-labeled proteins).

Partial proteolytic mapping. Immune complexes were separated on an SDS–8% polyacrylamide gel. The gel was dried, and the proteins were located by autoradiography. The bands were excised and digested with various concentrations of *S. aureus* V8 protease as described previously (8). The digestion products were then separated on SDS–20% polyacrylamide gels and visualized by autoradiography.

Transient transfections. The E2F-1 expression construct E2F-1(89-437) has been described previously (22). Expression constructs E2F-2(85-437) and E2F-3(132-425) were prepared by polymerase chain reaction with Vent polymerase by BioLabs using the following primers: for E2F-2(85-437), 10.8 (5'-GATCCCATGGGATCCCCGGCCAAAAGGAAG CT-3') and 10.7 (5'-CTAGTCTAGAGGATCCGGCTGTCA GCCTGTCTGTGA-3'); and for E2F-3(132-425), 31.2 (5'-GATCGGATCCATGGCAAAGCGAAGGCTGGA-3') and 31.15 (5'-CTAGGATCCGGATCGAAGGAGAGTTCACAC GAAGC-3').

The amplified fragments were subcloned into pCMV-neoBam as *Bam*HI fragments, and the coding sequences of the resultant expression constructs, pCMVE2F-2(85-437) and pCMVE2F-3(132-425), were confirmed by sequencing. The reporter constructs pE2 wt CAT and pE2 (−64/−60,−45/−36) CAT have been described previously (36). Transfections were performed essentially as described previously (18). C-33A cells were grown in 9-cm-diameter tissue culture dishes and transiently transfected with 10 μg of carrier DNA, 4 μg of reporter construct, 3 μg of pRSVLUC (54), 0, 0.1, 0.3, 1, or 3 μg of expression plasmid, and pCMV to give a total of 20 μg of DNA. Cells were harvested after 24 to 36 h and lysed by freeze-thaw in 200 μl of 25 mM Tris-HCl (pH 8.0), and the extracts assayed for chloramphenicol acetyltransferase and luciferase activities according to standard procedures (10, 47).

RESULTS

Isolation of cDNA clones encoding E2F-2 and E2F-3. To date, all known E2F-responsive genes contain one or more sequences that are closely related to the consensus E2F binding site. Therefore, it seemed likely that if other E2F-like proteins existed, they would share considerable homology with the DNA binding domain of E2F-1. As an initial test, 1 million recombinant phage from a human NALM-6 cDNA library were screened under low-stringency conditions with a radiolabeled probe that encompassed the known minimal DNA binding domain of E2F-1 (22). Of the 10 independent positives identified, 9 continued to hybridize to the probe under high-stringency conditions, and sequencing confirmed that they all contained at least some portion of the DNA binding domain of E2F-1. The remaining positive clone, clone 10, was no longer detected when the filters were washed under high-stringency conditions. Consistent with this observation, most of our E2F-1-specific primers failed to hybridize to this clone. However, a single primer (pC15.1), derived from sequences at the C-terminal portion of the DNA binding domain, gave rise to sequence that was related but clearly distinct from E2F-1.

Further sequencing showed that clone 10 contained a continuous open reading frame of 414 amino acids (Fig. 1A) followed by approximately 3.5 kb of 3' untranslated region prior to the poly(A)$^+$ tail. This coding sequence is highly homologous to that of E2F-1, suggesting that these proteins

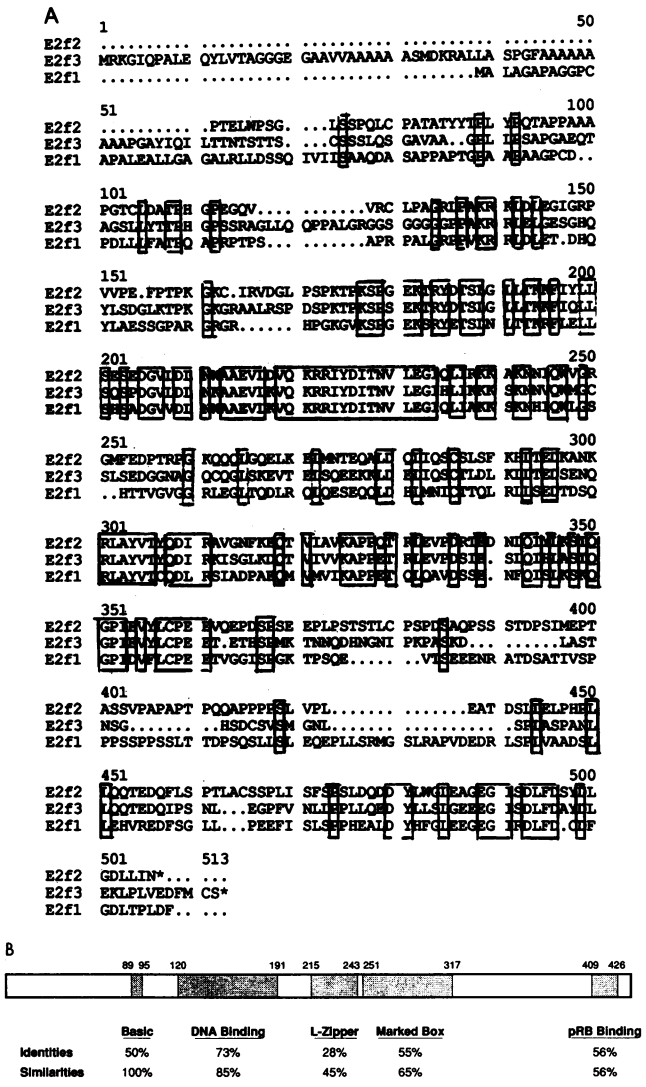

FIG. 1. Sequence comparisons of E2F-1, E2F-2, and E2F-3. (A) Amino acid sequences of E2F-1, E2F-2, and E2F-3. Boxes indicate residues that are identical to all three proteins. (B) Locations of conserved motifs in relation to the sequence of E2F-1.

are closely related. Furthermore, the highest degree of homology mapped to the region of E2F-1 that is known to be both necessary and sufficient for DNA binding. In light of the close homology, we have designated this novel protein E2F-2.

Despite its large size, clone 10 does not encode an initiating methionine and must therefore be a partial cDNA. Although we have tried several different strategies, we have been unable to extend this clone further. However, during the course of this work, we learned that Ivey-Hoyle and colleagues had also isolated cDNA clones encoding an E2F-1-related protein (30). After sequence comparisons, it was clear that these proteins were identical and that we lacked 29 amino acids of coding sequence. To avoid confusion, we have therefore numbered our mutants from the initiating ATG designated by Ivey-Hoyle et al. (30).

In an attempt to isolate additional E2F family members, we screened 2 million clones from both the NALM-6 library and a human fetal brain library (Stratagene) with a mixture of probes derived for the known DNA binding domain of E2F-1 and the putative DNA binding domain of E2F-2. This screen gave rise to an additional 52 positive clones, 46 of which contained sequences that were identical to E2F-1. Of the remaining clones, five corresponded to the E2F-2 sequence, but all were shorter than the original cDNA clone. In addition, this screen identified one additional positive, clone 31. This clone contains a 1,275-bp open reading frame that is almost immediately preceded by an in-frame termination codon. By virtue of its homology to E2F-1, the 425-amino-acid protein that is encoded by this clone has been designated E2F-3.

To complete this screen, we rehybridized the cDNA libraries at low stringency with the putative DNA binding domain of E2F-3. However, all 36 of the identified positive clones corresponded to either E2F-1, E2F-2, or E2F-3 itself. It therefore seemed unlikely that this strategy would lead to the identification of any additional family members.

E2F amino acid comparisons. Comparison of the predicted amino acid sequences of E2F-1, E2F-2, and E2F-3 revealed four regions of homology (Fig. 1B). Helin et al. (22) have previously shown that the DNA binding domain of E2F-1 is contained within amino acids 89 to 191 of this protein. Comparison with the equivalent region of E2F-2 (amino acids 85 to 200) and E2F-3 (amino acids 132 to 248) indicates that this region contains two highly conserved domains that are separated by a linker that varies in size from 25 to 32 amino acids and maintains little sequence conservation. The first conserved domain corresponds to a small six-amino-acid motif (basic-basic-basic-L-acidic-L) that is highly charged. The second corresponds to the region of highest sequence identity (73% between all three clones), and it maps to the carboxy-terminal 71 amino acids of this region. Mapping studies indicate that this carboxy-terminal region is contained within a single exon (see below and reference 57), and it provides the primary residues required for E2F-1 to contact DNA (30).

The second region of homology is a putative leucine zipper that spans residues 215 to 243 of E2F-1 (31). Interestingly, there is little sequence conservation in this region apart from the hydrophobic residues at the 7 position. Although there is no evidence that this sequence forms an alpha helix, the conservation of this heptad repeat of L-X_6-L-X_6-L-X_6-C-X_6-L suggests that this region might serve as a site for dimerization with other proteins or with E2F itself.

The third region of homology is in a previously unrecognized area just carboxy terminal to the putative leucine zipper. This region extends from residues 251 to 317 of E2F-1. Until a function can be ascribed to this region, we are referring to it as the marked region. The three E2F clones identified here show approximately 55% identity in this region. When the sequence in the marked region was compared with others in the protein data bases, no homologies were identified. However, this high degree of sequence conservation suggests that this region may correspond to an important functional domain.

The last segment of homology is in the extreme carboxy terminus. This region of homology corresponds almost precisely with the region of E2F-1 that was identified as being both necessary and sufficient for interaction with pRB. In this region, 10 of the 18 residues are identical among E2F-1, E2F-2, and E2F-3.

Genomic cloning. Human genomic libraries were screened with E2F cDNA and genomic probes, and the series of recombinant phages and cosmid clones obtained were sub-

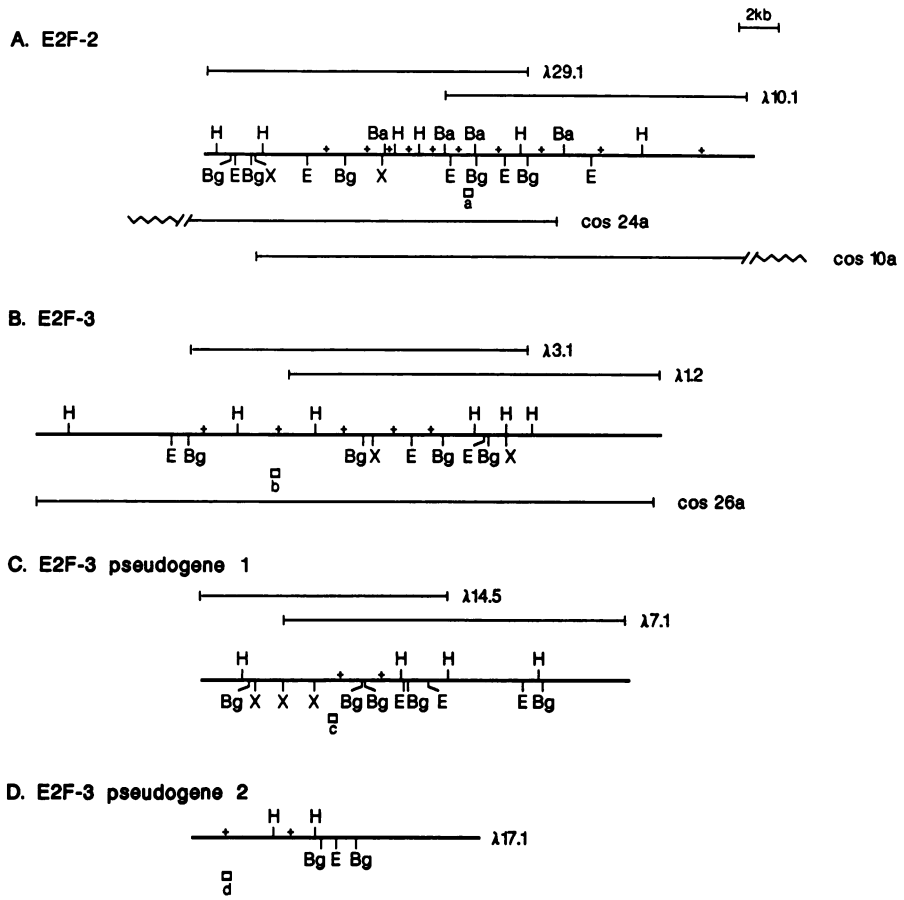

FIG. 2. Genomic restriction maps of the human E2F-2 and E2F-3 loci. Two sets of recombinant bacteriophages and cosmids representing the authentic E2F-2 (A) and E2F-3 (B) loci are shown, as well as clones from two additional E2F-3 pseudogenes (C and D). Restriction fragments that hybridized to the cDNA probes are indicated (+) above the maps, and fragments subjected to nucleotide sequencing analysis are demarcated by the open boxes below.

divided into four sets of nonoverlapping sequences, based on their restriction maps and preferential hybridization to the human E2F cDNAs (Fig. 2A). A fifth locus containing the E2F-1 gene was also isolated and will be reported separately. Two recombinant phages and two cosmids were obtained, and they contained overlapping sequences that hybridized to the E2F-2 cDNA over a genomic locus spanning approximately 20 kb (Fig. 2A). DNA sequence analysis of a 0.2-kb *Kpn*I-*Sma*I genomic DNA fragment from this locus (labeled "a" in Fig. 2A) showed that it matched the E2F-2 cDNA sequence within the most conserved, C-terminal portion of the DNA binding domain and that it was flanked by consensus splice acceptor and donor sequences, confirming that this locus encoded E2F-2.

Three independent sets of nonoverlapping phages and cosmids hybridized preferentially under high-stringency conditions to the human E2F-3 cDNA probes (Fig. 2B to D). Southern blotting analysis indicated that one of the three loci (Fig. 2B) included interrupted coding regions, while the other two loci contained only single hybridizing segments (Fig. 2C and D). Nucleotide sequencing of the fragment labeled "b" in Fig. 2B showed that it contained an exon matching the sequence of the E2F-3 cDNA flanked by consensus splice donor and acceptor sequences, indicating that it encoded the E2F-3 mRNA. By contrast, when hybridizing restriction fragments shown as "c" and "d" in Fig. 2C and D were sequenced, insertions, deletions, and in-frame termination codons were identified, indicating that these loci contain E2F-3 pseudogenes.

Chromosomal assignment of E2F-2 and E2F-3 genes. E2F is thought to play an important role in regulating the expression of genes required for cell proliferation. Therefore, it seems likely that deregulation of E2F expression or activity may lead to abnormal proliferation. We were interested in identifying the chromosomal localization of E2F-2 and E2F-3 to determine whether there might be any association with known diseases.

The chromosomal localization of the E2F-2 and E2F-3 genes and the two E2F-3 pseudogenes was determined by fluorescence in situ hybridization, using probes prepared from each of the genomic clones hybridized to normal metaphase human chromosomes (Fig. 3). Upon hybridization to the cosmid clones containing inserts from the E2F-2 gene, the only fluorescence signal emanated from the distal short arm of chromosome 1. The chromosomal arm was identified by cohybridization of the metaphase chromosomes with pUC1.77, a probe that is specific for chromosome 1 heterochromatin (7). On the basis of the distance of the hybridization signal from the centromere relative to the entire length of the short arm of chromosome 1p, E2F-2 was

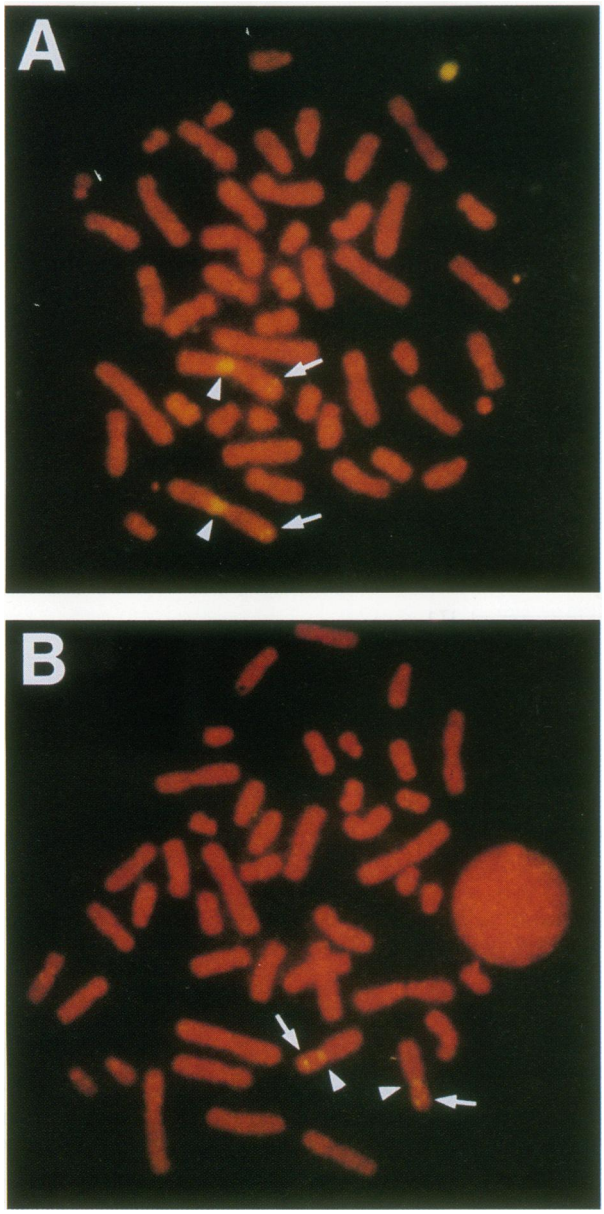

FIG. 3. Fluorescence in situ hybridization analysis using digoxigenin-labeled E2F-2 (A) and E2F-3 (B) genomic probes. Human cosmid clones for E2F-2 (cosmids 10a and 24a; Fig. 2A) and E2F-3 (cosmid 26a; Fig. 2B) were hybridized to human metaphase chromosomes from peripheral blood lymphocytes. Fluorescence signals resulting from hybridization of these probes are indicated by the white arrows at adjacent positions on sister chromatids. Centromere-specific probes (arrowheads) for chromosomes 1 (A) and 6 (B) were used to identify the chromosomes containing the E2F-2 and E2F-3 loci.

assigned to band p36. By using the same method, authentic E2F-3 was localized to chromosome band 6p22, based on cohybridization with the chromosome 6 centromere probe D6Z1 (Oncor, Gaithersburg, Md.). The E2F-3 pseudogenes shown in Fig. 2C and D were localized to chromosome bands 17q11-12 and 2q33-35, respectively.

These chromosomal assignments were confirmed by hybridizing the E2F-2 and E2F-3 cDNAs and genomic probes to Southern blots containing DNA extracted from a panel of rodent × human hybrid cell lines containing different combinations of human chromosomes (Table 1). A 7.0-kb *Bgl*II restriction fragment contained within the authentic human E2F-2 genomic locus was detected concordantly with human chromosome 1, consistent with the results obtained by fluorescence in situ hybridization. The E2F-3 cDNA probe identified a 9.5-kb restriction fragment within the human E2F-3 gene and 6.0- and 8.0-kb fragments within the two E2F-3 pseudogenes shown in Fig. 2C and D. These fragments were identified concordantly with human chromosomes 6, 17, and 2, again confirming the fluorescence in situ hybridization results.

Expression of E2F-2 and E2F-3. Previous studies detected E2F-1 expression in a wide variety of cell lines and tissues. Therefore, we were interested in comparing the expression patterns of E2F-2 and E2F-3 with that of E2F-1. Probes were prepared by using sequences carboxy terminal of the DNA binding domain, and their specificity was confirmed by DNA blotting. Northern blot analysis was performed on poly(A)$^+$ RNA from ML-1 (premyeloid leukemia), C-33A (a cervical carcinoma cell line), U118MG (glioblastoma), 293 (adenovirus-transformed kidney epithelial cell), and NGP (neuroblastoma) cells (Fig. 4A). Consistent with the sizes of the cDNA clones, both E2F-2 and E2F-3 give rise to large transcripts of 5.5 to 6 kb. These mRNAs are present at considerably lower levels than that of E2F-1 but have been detected in all of the cell lines examined to date. Interestingly, although the total level of expression varies considerably from one cell line to the next (even accounting for the differences in actin levels), the relative ratio of E2F-1 to E2F-2 to E2F-3 remains constant.

In independent tests, we also examined the expression of these transcripts in U2OS and SAOS-2 (two osteosarcoma cell lines), UOC-B1 (a pro-B-cell lymphoblastic leukemia cell line), HEL (an erythroleukemia cell line), NB1 and NB2 (two neuroblastoma cell lines), Rh18 (a rhabdomyosarcoma cell line), Colo320DM (a colonic adenocarcinoma cell line), and SW900 (a squamous cell lung carcinoma cell line). All showed similar-size transcripts, as shown in Fig. 4A.

Interestingly, when RNA gels were run for longer times prior to blotting, the E2F-3 band consistently resolved into a doublet while the E2F-1 and E2F-2 specific probes continued to detect a single band (data not shown). The significance of these two messages is unclear.

We have also examined the expression of these genes in normal human tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 4B). E2F-2 mRNA levels were highest in placenta and then lung and kidney. Lower levels were seen in liver and pancreas. In skeletal muscle, the 6.0-kb transcript was absent, but an approximately 2.5-kb mRNA was easily detected. We do not know the origin of this smaller RNA species. E2F-3 appears to be expressed in most of these tissues except brain at roughly equivalent levels (compared with the levels of actin controls). The E2F-3 doublet seen in tissue culture cell lines was not apparent in tissue Northern analysis.

DNA binding activity. If these novel genes are genuine members of the E2F family of transcription factors, we would expect them to bind to consensus E2F sites in a manner similar to that of E2F-1. The minimal DNA binding domain of E2F-1 has been mapped to amino acids 89 to 191 (22). Therefore we tested the ability of the equivalent regions of E2F-2 (amino acids 85 to 200) and E2F-3 (amino acids 132 to 230) to bind DNA as GST fusion proteins. In these experiments, E2F-2 was able to bind to DNA in a manner

TABLE 1. Assignment of E2F-2 and E2F-3 genes to human chromosome in rodent × human somatic cell hybrids

Human chromosome	Assignment of E2F-2					Assignment of E2F-3				
	No. of hybrids				% Discordancy	No. of hybrids				% Discordancy
	+/+	+/−	−/+	−/−		+/+	+/−	−/+	−/−	
1	3	0	0	22	0	1	6	2	16	32
2	1	2	2	20	16	3	4	0	18	16
3	1	2	7	15	36	5	2	3	15	20
4	1	2	0	23	8	1	6	0	18	24
5	0	3	3	19	24	2	5	1	17	24
6	1	2	6	16	28	7	0	0	18	0
7	1	2	3	19	20	4	3	0	18	12
8	2	1	3	19	16	4	3	1	17	16
9	0	3	3	19	24	0	7	3	15	40
10	1	2	1	21	12	2	5	0	18	20
11	0	3	3	19	24	2	5	2	16	28
12	0	3	7	15	40	5	2	2	16	16
13	2	1	3	19	16	3	4	2	16	24
14	1	2	2	20	16	1	6	2	16	32
15	2	1	2	20	12	3	4	1	17	20
16	1	2	2	20	16	3	4	0	18	16
17	1	2	2	20	16	2	5	1	17	24
18	1	2	5	17	28	5	2	1	17	12
19	1	2	2	20	16	2	5	1	17	24
20	2	1	3	19	16	3	4	2	16	24
21	1	2	6	16	32	5	2	2	16	16
22	0	3	6	16	36	4	3	2	16	20
X	2	1	10	12	44	5	2	7	11	36
Y	0	3	1	21	16	0	7	1	17	32

that was almost indistinguishable from that of E2F-1 (Fig. 5A). In contrast, this region of E2F-3 displayed little or no DNA binding activity in these experiments (data not shown). However, a slightly larger region of this protein, encompassing amino acids 132 to 289, did display sequence-specific DNA binding activity (Fig. 5A). However, when we tested a range of input fusion protein concentrations, it was clear that the GST–E2F-3 fusion protein bound to DNA with significantly reduced affinity or avidity relative to E2F-1 and E2F-2 (Fig. 5B). We are currently trying to determine whether this reduced binding reflects genuine differences in the affinity and/or sequence specificity of this protein or whether it merely reflects the ability of these GST-fusion proteins to adopt the correct conformation in vitro.

Recently, Huber and coworkers demonstrated that E2F binds to DNA as a heterodimer (29). However, mixing experiments failed to reveal any synergy between E2F-1, E2F-2, and E2F-3 in gel retardation assays (data not shown).

E2F-2 and E2F-3 bind to pRB in vivo. The pRB binding domain has been mapped to an 18-amino-acid motif at the carboxy terminus of E2F-1 (22). Since this motif is highly conserved in both E2F-2 and E2F-3, these proteins also seemed likely to bind either pRB or the related protein p107. To test the binding properties of these proteins, we raised polyclonal antisera that are specific for E2F-2 or E2F-3 and used them (alongside an E2F-1 antiserum [22]) in an immunoprecipitation-reimmunoprecipitation assay to determine whether E2F-2 or E2F-3 could be detected in association with pRB or p107 in vivo. ML-1 cells were labeled with [^{35}S]methionine and then immunoprecipitated with monoclonal antibodies specific for pRB (C36 and XZ77) or p107 (SD2, SD4, SD6, SD9, and SD15). The samples were then denatured by heating in 2% SDS and reimmunoprecipitated with either E2F-1-, E2F-2-, or E2F-3-specific polyclonal antisera or an antipeptide antibody that was raised against the 18-amino-acid pRB binding domain of E2F-1 (Fig. 6A). As previously described (11, 22), the E2F-1-specific polyclonal antiserum recognized a single protein species that is present in pRB but not p107 immunoprecipitations. In contrast, the anti-E2F peptide antibody recognized at least two proteins in the pRB immunoprecipitate, the lower one of which comigrates exactly with the E2F-1-specific band. In addition, this antiserum detects several bands within the p107 immunoprecipitate that appear to be distinct from the pRB-associated proteins recognized by this antiserum (11).

The anti-E2F-2 polyclonal antiserum failed to recognize any proteins from the p107 immunoprecipitation (Fig. 6A). However, this antiserum clearly detected a single, major band in the pRB immunoprecipitations. Interestingly, this E2F-2 species comigrated exactly with the larger protein species recognized by the anti-E2F peptide antibody. Similarly, the anti-E2F-3 antibodies immunoprecipitated two proteins that also appear to be specifically associated with pRB but not p107. We are currently trying to determine the exact identities of these two species.

The comigration observed in this experiment suggested that the pRB-associated proteins detected by the anti-E2F peptide antibody correspond to E2F-1 and E2F-2. After several attempts, it became clear that levels of these ^{35}S-labeled species were too low for us to be able to use V8 partial proteolytic mapping to confirm this. However, when $^{32}P_i$ was used to label cells, we continued to detect the same pattern of pRB- and p107-associated proteins (Fig. 6B), but the labeling was increased to sufficient levels to make V8 partial proteolytic mapping feasible. ML-1 cells were therefore labeled with $^{32}P_i$, and lysates were immunoprecipitated with monoclonal antibodies to pRB. The samples were then denatured by heating in 2% SDS and reimmunoprecipitated with either an E2F-1-, E2F-2-, or E2F-3-specific polyclonal antiserum or the anti-E2F peptide antibody and separated on

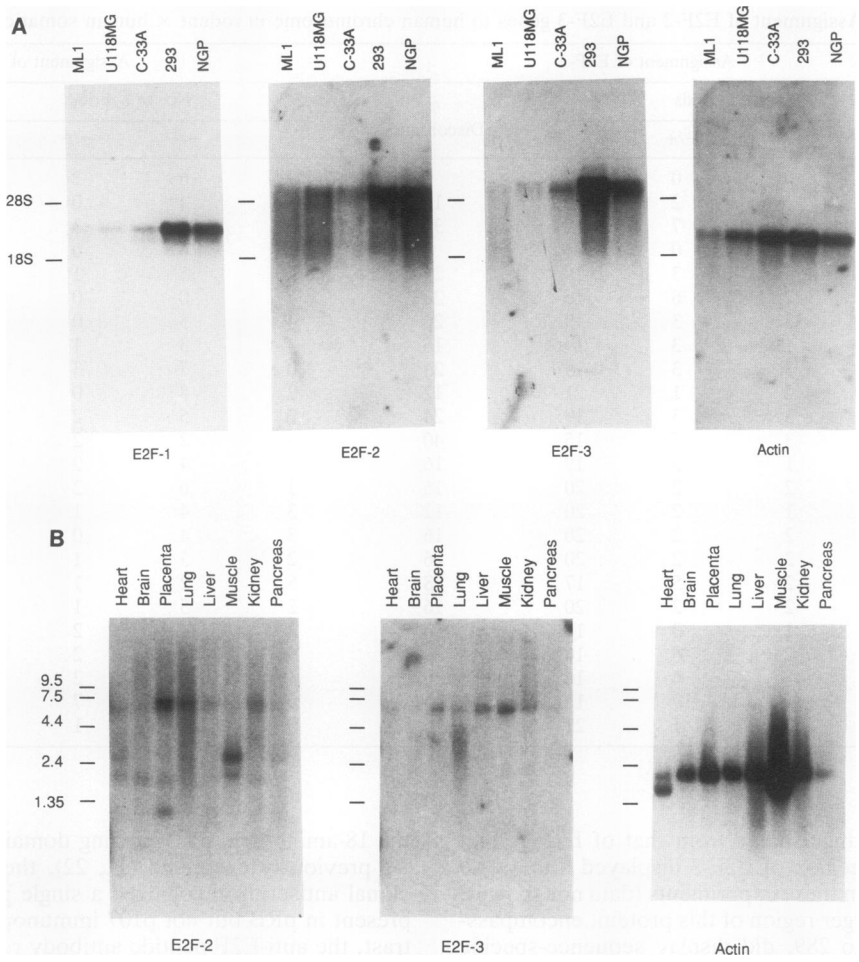

FIG. 4. Expression of E2F-2 and E2F-3 mRNAs, determined by Northern blot analysis of poly(A)$^+$ RNA from tissue culture cell lines (A) or normal human tissues (B) using probes specific for either E2F-1, E2F-2, or E2F-3. Dashes indicate the positions of 28S and 18S rRNA subunits (tissue culture cell lines) or RNA markers (human tissues). Exposure times for the cell lines/tissue blots were as follows: E2F-1, 2 days/0; E2F-2, 9 days/14 days; E2F-3, 9 days/14 days; and actin 1 h/3 h.

SDS–8% polyacrylamide gels (Fig. 6C). (The smaller species in the E2F-3 immunoprecipitate is absent in this experiment because it has been eluted from the bottom of the gel.) Each of the bands was then subjected to V8 partial proteolytic mapping (Fig. 6D). E2F-1, E2F-2, and E2F-3 each gave rise to a distinct pattern of partial digestion fragments. Moreover, the partial proteolytic maps of the two bands recognized by the anti-E2F peptide antibody were identical to those of E2F-1 (lower band) and E2F-2 (upper band), respectively. Despite the high degree of homology within the pRB binding domain of these three proteins, E2F-3 is not recognized by the antipeptide antibody, and its V8 partial proteolytic map is clearly distinct from those of the two species recognized by this antibody.

Because we were unable to detect any p107 binding in this cell line, we repeated this immunoprecipitation-reimmunoprecipitation experiment by using a cervical carcinoma cell line, C-33A, that lacks functional pRB protein. Even in the absence of pRB, we failed to detect any association between p107 and E2F-1, E2F-2, or E2F-3 in these assays (data not shown). Although we cannot rule out the possibility that these proteins show a low level of binding to p107, these data suggest that E2F-1, E2F-2, and E2F-3 bind preferentially to pRB in vivo.

The pRB binding domain maps to the C-terminal portion. To further map the pRB binding domain, the carboxy-terminal 243 [E2F-2(195-437)], 194 [E2F-2(244-437)], 146 [E2F-2(291-437)], or 56 [E2F-2(381-437)] amino acids of E2F-2 were expressed as GST fusion proteins and tested for the ability to bind to in vitro-translated pRB, p107, or luciferase as a control. Levels of binding were also compared with those for both GST alone and GST-E1A (Fig. 7). As expected, GST-E1A bound with approximately equal affinity to both pRB and p107, with binding limited only to the larger polypeptides that are known to maintain the integrity of the E1A-binding pocket. Consistent with the in vivo binding results, we were able to detect little or no binding of any of the E2F-2 mutants to p107. In contrast, all of the mutants displayed high-affinity binding to pRB, in a manner that was almost indistinguishable from that of E1A. Although such in vitro binding experiments do not prove the identity and/or specificity of potential binding partners, these data do support the strong preference for pRB binding observed in vivo. Furthermore, all of the E2F-2 deletion mutants bound specifically to pRB, indicating that the pRB binding domain lies at the C-terminal end of the protein, as previously described for E2F-1 (22).

We also tested a similar series of E2F-3 deletion mutants

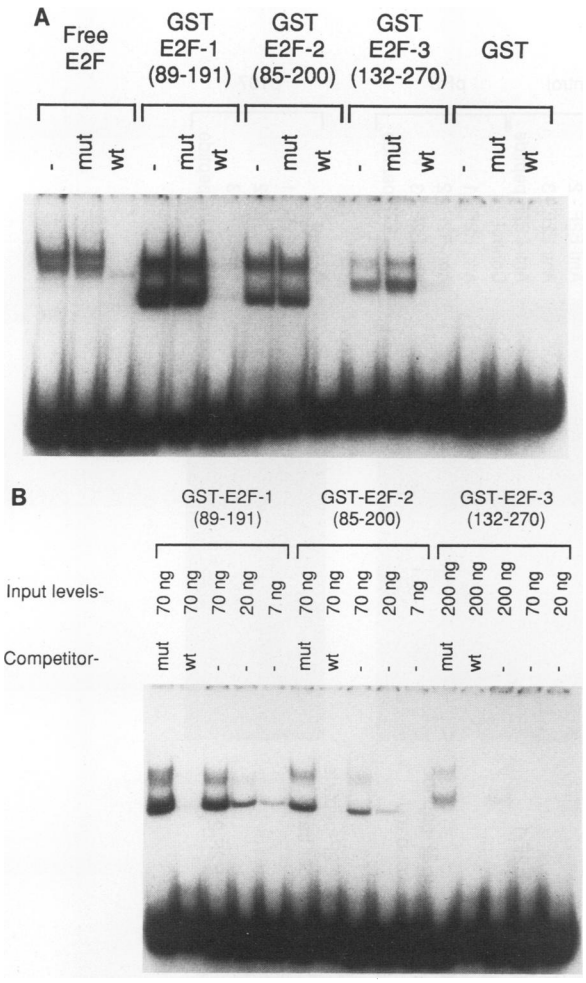

FIG. 5. E2F-2 and E2F-3 bind to E2F DNA recognition sequences. (A) Ten micrograms of 293 cell extract (free E2F), 70 ng of purified GST–E2F-1 (amino acids 89 to 191) or GST–E2F-2 (amino acids 85 to 200), or 200 ng of GST–E2F-3 (amino acids 132 to 270) or free GST was incubated with a labeled oligonucleotide containing a single E2F binding site. Wild-type (wt) or mutant (mut) competitor oligonucleotides were added at a 100-fold excess as indicated. (B) Different input levels of GST–E2F-1 (70 to 7 ng), GST–E2F-2 (70 to 7 ng), or GST–E2F-3 (200 to 20 ng) were incubated with the E2F oligonucleotide probe in the presence or absence of competitor oligonucleotides as indicated.

in this assay. Surprisingly, these mutants failed to bind either pRB or p107 (data not shown). Since E2F-3 is clearly associated with pRB and not p107 in vivo, we assume that this lack of binding reflects either aberrant folding or a strong requirement for posttranslational modifications.

Transactivation. Finally, we compared the ability of these E2F family members to activate transcription from either the wild-type E2 promoter ([+E2F]) or mutant E2 promoters in which either the ATF (−80 to −70) or the two E2F (−64 to −60 and −45 to −36) ([−E2F]) sites have been mutated (36). These reporters were transiently transfected into C-33A, a human cervical carcinomas cell line that expresses a functionally inactive form of the pRB protein, along with increasing levels of an E2F-1 [pCMVE2F-1(89-437)], E2F-2 [pCMVE2F-2(85-437)], or E2F-3 [pCMVE2F-3(132-425)] expression plasmid. In these experiments, expression of E2F-2 (Fig. 8A) or E2F-3 (Fig. 8B) reproducibly resulted in a 5- to 15-fold activation of the wild-type E2 promoter. This activation was independent of the presence of a functional ATF site (data not shown) but was completely abolished in the absence of the two E2F sites (Fig. 8). Although these inductions appear relatively small, they are increased by up to 50-fold when the reporter contains multiple copies of the consensus E2F site (data not shown). Together, these experiments demonstrate that E2F-2 and E2F-3 are both capable of activating transcription in a manner that is dependent upon the presence of at least one functional E2F site.

DISCUSSION

E2F is a cellular transcription factor that appears to play a major role in regulating the expression of genes that are essential for cell cycle progression. By virtue of its association with pRB, we and others have previously cloned a protein, called E2F-1, that displays properties of the endogenous E2F. Using low-stringency hybridization, we have isolated cDNA clones that encode two additional E2F-like proteins, E2F-2 and E2F-3. These three proteins appear to be closely related, with the highest degree of identity mapping to regions of the E2F-1 protein that are known to be required for either DNA binding, pRB binding, or transcriptional activation.

These novel proteins share many of the biochemical properties of E2F-1. First, E2F-1, E2F-2, and E2F-3 all bind to wild-type but not mutant E2F sites. Second, when transfected into tissue culture cells, E2F-1, E2F-2, and E2F-3 are all capable of activating transcription of known E2F-responsive genes in a manner that is dependent upon the presence of one or more intact E2F sites. Finally, using polyclonal antisera, we can detect association between pRB and each of these proteins in vivo. In contrast, we have been unable to detect any association between E2F-1, E2F-2, or E2F-3 with p107 in these assays. Although we cannot rule out the possibility that a fraction of these proteins associate with p107, we believe that these three proteins represent one distinct subclass of E2F, for which pRB is the major regulator.

Using peptide sequence from purified protein preparations, Girling and colleagues recently isolated a cDNA clone encoding one component of the murine equivalent of E2F (17). They designated this clone DP-1. DP-1 bears some homology to E2F-1, E2F-2, and E2F-3, limited primarily to a domain that corresponds to the carboxy-terminal portion of their minimal DNA binding domains. DP-1 has been shown to bind specifically to consensus E2F sites (17), indicating that this protein is also a member of this family of transcription factors. Recently, Helin et al. have demonstrated that E2F-1 and DP-1 can interact to generate stable heterodimers (23). Moreover, this interaction leads to an increase in the DNA binding, pRB binding, and activation of E2F-responsive transcription. Since E2F-2 and E2F-3 share many of the properties of E2F-1, it seems likely that these proteins will also function as heterodimers in vivo. Interestingly, Wu and Harlow have recently isolated at least one additional DP-1-like protein, suggesting that there is also a family of DP proteins (56).

Together, these data suggest that the endogenous E2F activity results from the concerted activity of multiple heterodimers that contain one E2F-like and one DP-like component. Similar multiplicity of heterodimeric subunits has been well documented for a number of other transcription

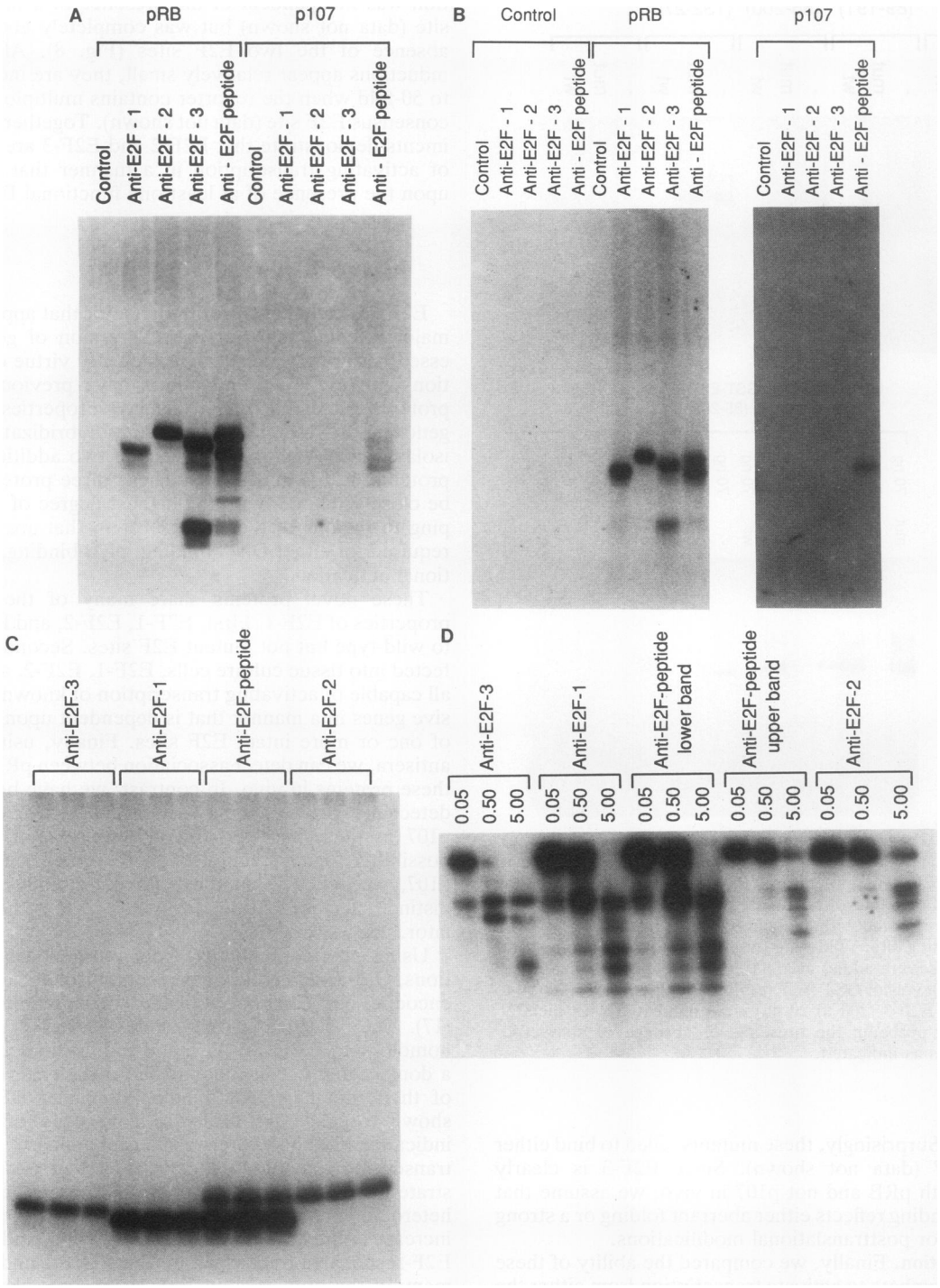

FIG. 6. E2F-2 and E2F-3 bind to pRB in vivo. (A to C) ML-1 cells were labeled with either [^{35}S]methionine (A) or $^{32}P_i$ (B and C). The lysates were immunoprecipitated with monoclonal antibodies specific for either pRB (XZ77 and C36) or p107 (SD2, SD4, SD6, SD9, and SD15) as indicated. These precipitates were then boiled in 2% SDS–0.1 M dithiothreitol, and the supernatant was diluted for immunoprecipitation with either a polyclonal antiserum raised against GST–E2F-1, GST–E2F-2, or GST–E2F-3 fusion protein or an antipeptide antibody (anti-E2F peptide) raised against the 18-amino-acid minimal pRB binding domain of E2F-1. PAb419 (first immunoprecipitation) and a mixture of normal mouse and normal rabbit sera (reimmunoprecipitation) were used as negative controls in these experiment. (D) The major proteins species were excised form the gel shown in panel C, subjected to partial V8 proteolytic digestion, and separated on SDS–20% polyacrylamide gels.

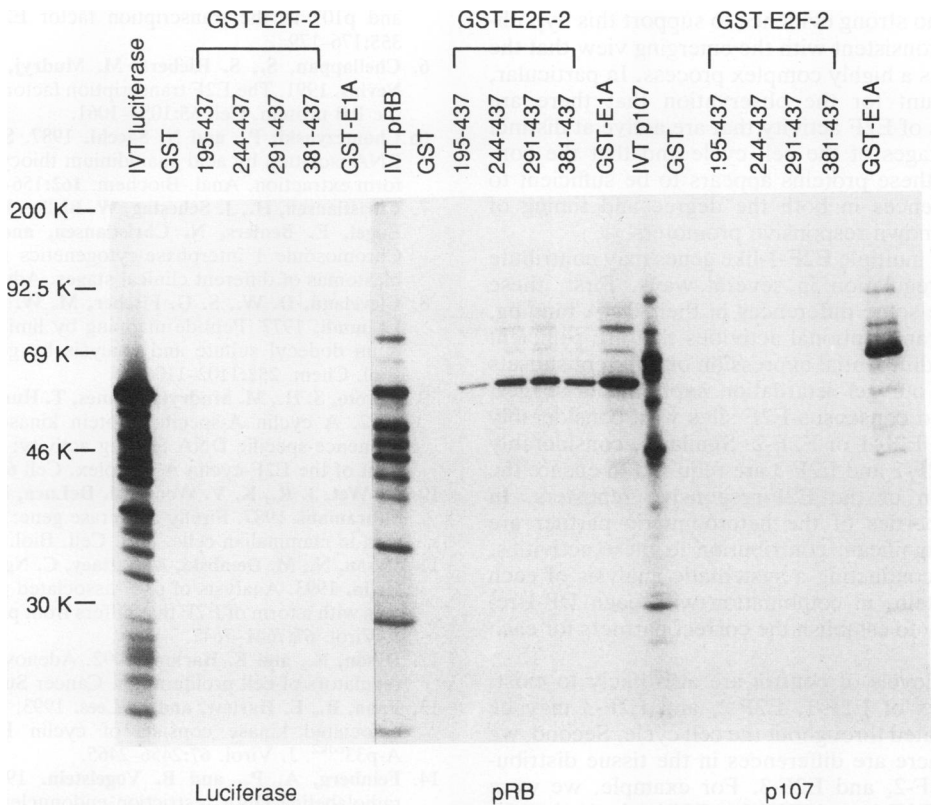

FIG. 7. The pRB binding domain maps to the C-terminal portion of E2F-2. Twenty microliters of in vitro-translated (IVT) luciferase, pRB (amino acids 1 to 928), or p107 (amino acids 190 to 1068) was incubated with 2 μg of GST, GST-E2F-2 (amino acids 195 to 437), E2F-2 (amino acids 244 to 437), E2F-2 (amino acids 291 to 437), E2F-2 (amino acids 381 to 437), or GST-13S E1A as indicated. Two microliters of each total in vitro translation reaction was included on the gel for comparison.

factors, including the families of Fos and Jun proteins (32, 40, 43) and the basic/helix-loop-helix proteins (39).

The majority of cell lines that we have tested appear to express E2F-1, E2F-2, and E2F-3. Why do cells require three independent pRB-regulated E2Fs? It is possible that these proteins are functionally redundant and that the cell maintains multiple genes to ensure that the regulation of this key cell cycle regulator cannot be easily abrogated. Alternatively, E2F-1, E2F-2, and E2F-3 may have subtly different functions that give rise to distinct elements of E2F activity.

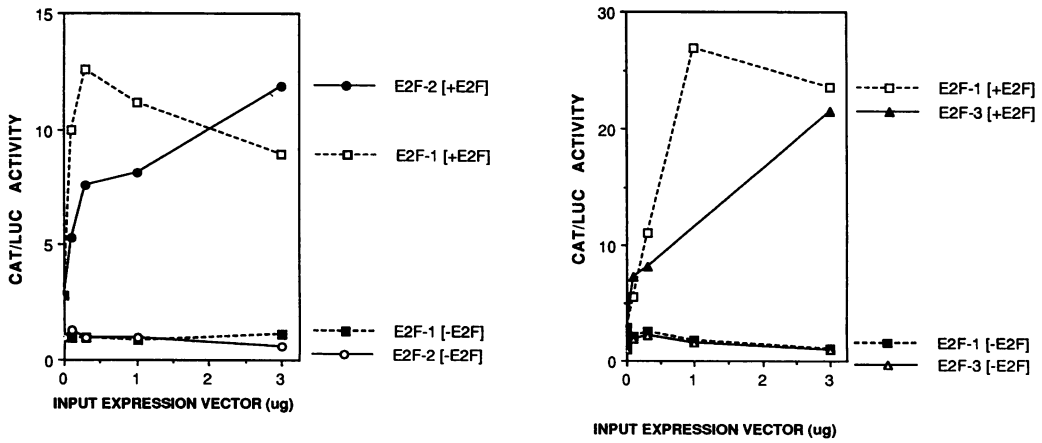

FIG. 8. E2F-2 and E2F-3 transactivate E2F reporter constructs. E2F-2 (amino acids 85 to 437) (A) and E2F-3 (amino acids 132 to 425) (B) were tested in transient transfection assays for the ability to activate transcription from reporter constructs containing either the wild-type ([+E2F]) or a mutant ([−E2F]) site. C-33A cells were transfected in duplicate with the indicated amounts of expression plasmids pCMVE2F-1(89-437), pCMVE2F-2(85-437), and pCMVE2F-3(132-425) along with 4 μg of either pE2 wt CAT [+E2F] or pE2 (−64/−60,−45/−36) CAT [−E2F], 3 μg of pRSVLUC, and carrier to give a total of 20 μg. CAT, chloramphenicol acetyltransferase; LUC, luciferase.

Although there is no strong evidence to support this hypothesis, it is entirely consistent with the emerging view that the regulation of E2F is a highly complex process. In particular, we have to account for the observation that there are multiple regulators of E2F activity that are active at distinct but overlapping stages of the cell cycle and that the concerted activity of these proteins appears to be sufficient to give rise to differences in both the degree and timing of activation of the known responsive promoters.

The existence of multiple E2F-1-like genes may contribute to this complex regulation in several ways. First, these proteins may have some differences in their DNA binding, pRB binding, or transcriptional activities that are sufficient to account for the differential expression of different subsets of genes. Indeed, our gel retardation experiments suggest that E2F-3 binds to consensus E2F sites with considerably lower affinity than E2F-1 or E2F-2. Similarly, considerably higher levels of E2F-2 and E2F-3 are required to ensure the maximal activation of the E2F-responsive reporters. In addition, the properties of the heterodimeric partner are likely to make a significant contribution to these activities. We are currently conducting a systematic analysis of each E2F-1-related protein, in combination with each DP-1-related protein, to try to establish the correct partners for each of these proteins.

Two additional levels of control are also likely to exist. First, the activities of E2F-1, E2F-2, and E2F-3 may be differentially regulated throughout the cell cycle. Second, we have noted that there are differences in the tissue distribution of E2F-1, E2F-2, and E2F-3. For example, we were unable to detect E2F-2 and E2F-3 mRNAs in brain, the tissue that contains the highest level of E2F-1 expression. A potential consequence of this observation is that different genes may be E2F responsive in different cells. In contrast, cells containing multiple E2Fs may use permutations of different heterodimers to give rise to graded responses to different combinations of input signals. Further investigation of this family of proteins will be essential if we are to understand fully the mechanism by which E2F-dependent transcription is regulated.

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Midori Saito and Marc Vidal contributed equally to this work.

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