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Cloning and Characterization of E2F-2, a Novel Protein with the Biochemical Properties of Transcription Factor E2F

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E2F is a mammalian transcription factor that appears to play an important role in cell cycle regulation. While at least two proteins (E2F-1 and DP-1) with E2F-like activity have been cloned, studies from several laboratories suggest that additional homologs may exist. A novel protein with E2F-like properties, designated E2F-2, was cloned by screening a HeLa cDNA library with a DNA probe derived from the DNA binding domain of E2F-1 (K. Helin, J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey, Cell 70:337–350, 1992). E2F-2 exhibits overall 46% amino acid identity to E2F-1. Both the sequence and the function of the DNA and retinoblastoma gene product binding domains of E2F-1 are conserved in E2F-2. The DNA binding activity of E2F-2 is dramatically enhanced by complementation with particular sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified components of HeLa cell E2F, and anti-E2F-2 antibodies cross-react with components of purified HeLa cell E2F. These observations are consistent with a model in which E2F binds DNA as a heterodimer of two distinct proteins, and E2F-2 is functionally and immunologically related to one of these proteins.

The mammalian transcription factor E2F was originally identified as a cellular factor recruited by adenovirus type 5 to initiate transcription from the viral E2 promoter (29, 30, 36). It has subsequently been suggested that E2F may play a critical role in cell cycle regulation of uninfected mammalian cells as well (for a recent review, see reference 33). E2F binding sites are found in the promoter regions of several cellular genes which are important for cell growth, including c-*myc*, *cdc2*, and *DHFR* (4, 8, 22, 33, 42). E2F activity appears to be regulated via complex formation with other cellular proteins in a cell cycle-dependent fashion. Binding of E2F to the retinoblastoma gene product (pRb) yields a complex which suppresses transcription of genes containing the E2F binding site (21, 43). Only the underphosphorylated form of pRb is found in the E2F-pRb complex (6), and the complex is present in the G_1 stage of the cell cycle and persists into the S phase (38, 40). Interactions of E2F with other cellular proteins, including the pRb homolog p107 and cyclins, suggest that a complicated set of growth regulatory functions are mediated by these complexes (3, 5, 11, 32, 40).

Clones of two distinct proteins with E2F-like activity have recently been identified. Human E2F-1 was identified by probing expression libraries with recombinant pRb (18, 28, 39). This protein displays many of the properties of authentic E2F, including binding to the E2F recognition element in a sequence-specific fashion and binding to pRb. A distinct protein (DP-1) was more recently cloned on the basis of amino acid sequence data obtained from E2F purified from mouse F9 cells (17). This cloned protein also displays the key biochemical properties of cellular E2F but displays little sequence homology to E2F-1 outside of the DNA binding domain.

Several lines of evidence suggest the existence of multiple forms or components of E2F. First, antibodies to E2F-1 alter the migration of only a subset of the HeLa E2F bands in a DNA gel shift assay (28). Second, we have isolated five individual proteins from highly purified HeLa cell E2F which bind to the E2F recognition element in a gel shift assay (25). These proteins can be functionally divided into two distinct complementation groups, and optimal reconstitution of E2F activity requires mixing of one protein from each group. This result strongly suggests that cellular E2F binds DNA as a heterodimer of two distinct proteins. Recombinant E2F-1, which contains both helix-loop-helix and hydrophobic zipper dimerization motifs (18, 26, 28, 39), can substitute for proteins in one of the two HeLa E2F complementation groups (25).

These observations suggest the presence of multiple forms and/or components of cellular E2F. We therefore attempted to identify homologs of E2F-1 by screening a cDNA library with a DNA fragment corresponding to the nucleotide sequence of the DNA binding domain of E2F-1. These studies resulted in the identification of E2F-2, a novel protein with the functional properties of E2F.

MATERIALS AND METHODS

Nucleic acid reagents. All oligonucleotides were from Midland Certified Reagent Co. Dideoxy sequencing was performed with Sequenase (U.S. Biochemical Corp.) or a Cyclist DNA sequencing kit (Stratagene). All cloned DNAs that were generated by polymerase chain reaction (PCR) were sequenced in their entirety to rule out the possibility of polymerase-introduced errors. PCR was performed with *Taq* polymerase and reagents from Perkin Elmer Cetus.

Construction of glutathione *S*-transferase (GST) fusion proteins of E2F-1. DNA fragments of E2F-1 sequence were amplified by PCR from plasmid pBSK-BP3-B (E. Harlow, Massachusetts General Hospital), using the following primers: E2F-1 (amino acids [aa] 90 to 191) with e20 and e24; E2F-1 (90 to 170) with e20 and e25; E2F-1 (90 to 150) with e20 and e26; E2F-1 (90 to 130) with e20 and e27; E2F-1 (110 to 191) with e21 and e24; E2F-1 (120 to 191) with f120 and e24; E2F-1 (130 to 191) with e22 and e24; E2F-1 (150 to 191) with e23 and e24; and E2F-1 (110 to 170) with e21 and e25.

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The sequences of the primers were as follows: e20, 5′-CCAGGATCCCGGAGGCTGGACCTGGAAACTG-3′; e21, 5′-CCAGGATCCGGCAGAGGCCGCCATCCAGG-3′; e22, 5′-CCAGGATCCACCTCACTGAATCTGACCACC-3′; e23, 5′-CCAGGATCCGTCGTCGACCTGAACTGGGC-3′; e24, 5′-GCGGAATTCTACAGCCACTGGATGTGGTTCTT GG-3′; e25, 5′-GCGGAATTCTAGATGTCATAGATGCGC CGCTTC-3′; e26, 5′-GCGGAATTCTAGACACCGTCAGC CGAGTGGC-3′; e27, 5′-GCGGAATTCTAGGTCTCATAG CGTGACTTCTC-3′; and f120, 5′-CCAGGATCCAAATCC CCCGGGGAGAAG-3′.

The amplified fragments were digested with *Bam*HI and *Eco*RI, cloned into pGEX-2T (Pharmacia) digested with *Bam*HI and *Eco*RI, and sequenced.

Isolation of E2F-2 cDNA by library screening. Nitrocellulose plaque lifts from a human HeLa S3 cDNA λgt11 library (Clontech) were hybridized under low-stringency conditions with a ^{32}P-labeled DNA fragment of E2F-1 corresponding to aa 110 to 191. Hybridization was performed in 30% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml at 42°C for 16 h or in the same buffer with 25% formamide at 37°C. Filters were washed in 2× SSC–0.1% SDS at 37°C twice for 30 min and then in 1× SSC–0.1% SDS at 37°C once for 1 h. Positive clones were identified by autoradiography and were subjected to two further rounds of plaque purification and hybridization. The cDNA inserts from 21 positive clones were partially sequenced, and two clones (9 and 10) that were clearly distinct from E2F-1 were further characterized. Phage DNA was prepared from the plaque-purified phage stocks of each, and the *Eco*RI cDNA inserts were subcloned from the phage DNA into pGEX-2T to generate pGEX-9 and pGEX-10. Both strands of each cDNA were sequenced. DNA and the amino acid homologies were determined by the Gap subroutine of GCG (version 7.2; Genetics Computer Group, Inc., Madison, Wis.).

Plasmid constructions. The pT5T expression system (15) was used to express full-length E2F-2 (aa 1 to 437) from its natural start codon in *Escherichia coli* BL21 (DE3) (Novagen). PCR was used to amplify the E2F-2 coding region from pGEX-9. The 5′ primer used (5′-CCAAGGATCCATTGG AGGATGATTAAATGCTGCAAGGGCCCC-3′) included a *Bam*HI site and optimal translation signals upstream of the initiating methionine (underlined) as prescribed for use in the pT5T vector. The 3′ primer used (5′-GAGAGCAAGCTTAG AACTCCTCATTAATCAACAGGTCCCCAAGG-3′) added three amino acids (Glu-Glu-Phe) to the natural C terminus of the protein for antibody recognition by the YL1/2 antitubulin antibody (Harlan Bioproducts, Indianapolis, Ind.) (41) and also included a *Hin*dIII site. The DNA was cloned as a *Bam*HI-*Hin*dIII fragment into pT5T digested with *Bam*HI and *Hin*dIII to generate pE2F-2-PCR. A large internal fragment of the PCR-generated sequence (1,185-bp partial *Apa*I-*Bcl*I fragment) was then replaced with the corresponding DNA fragment from pGEX-9 to reduce the possibility of errors. The PCR-generated sequences 5′ and 3′ to the exchanged fragment were sequenced in their entirety. The final product was designated pE2F-2.

A pT5T plasmid expressing full-length E2F-1 (aa 1 to 437) with the C-terminal Glu-Glu-Phe added was constructed by the same approach. The sequence of the 5′ primer was 5′-CCAGAGGATCCATTGGAGGATGATTAAATGGCCT TGGCCGGGGCCCC-3′, and the sequence of the 3′ primer was 5′-GAGAGCAAGCTTCTAGAACTCCTCGAAATCC AGGGGGGTGAGGTCC-3′. A 1,230 bp *Apa*I-*Xho*I fragment from the PCR clone was replaced with the corresponding E2F-1 fragment from pBSK-BP3-B to generate pE2F-1.

To construct a vector which would express full-length E2F-2 (aa 1 to 437) as a GST fusion, pGEX-9 was digested to completion with *Bam*HI and *Ecl*XI to remove the 5′ untranslated region and the sequence encoding the first 10 amino acids of the open reading frame. A synthetic oligonucleotide *Bam*HI-*Ecl*XI cassette was inserted which restored the coding sequence of the first 10 amino acids and fused them in frame with the upstream GST sequences. The sequences of the complementary oligonucleotides comprising the cassette were 5′-GATCCATGCTGCAAGGGCCCCGGGCCTTGGC TTC-3′ and 5′-GGCCGAAGCCAAGGCCCGGGGCCCTTG CAGCATG-3′. The resulting plasmid was designated pGEX-E2F-2.

GST fusion protein constructs for deletion variants of E2F-2 were prepared exactly as described above for E2F-1, using pGEX-9 as the template and the following primers: for E2F-2 (aa 87 to 244), f-90 and 9-21; for E2F-2 (87 to 193), f-90 and f-191; for E2F-2 (112 to 193), f-110 and f-191; for E2F-2 (122 to 193), f-120 and f-191; for E2F-2 (132 to 193), f-130 and f-191; for E2F-2 (152 to 193), f-150 and f-191; and for E2F-2 (410 to 427), 9R and 9B. The sequences of the primers were as follows: f-90; 5′-CCAGGATCCAAAAGGAAGCTGGAT CTGGAGG-3′; f-110, 5′-CCAGGATCCGTGGATGGCCTC CCCAGC-3′; f-120, 5′-CCAGGATCCAAATCCCCCGGGG AGAAG-3′; f-130, 5′-CCAGGATCCACTTCGCTGGGGC TGCTCAC-3′; f-150, 5′-CCAGGATCCGTCCTGGACCTG AACTGGG-3′; f-191, 5′-GCGGAATTCTATACCCACTGG ATGTTGTTCTTGGC-3′; 9-21, 5′-GCGGAATTCAGTTGG CCTTGTCCTCAGTC-3′; 9R, 5′-GGACGGATCCGACGA CTACCTGTGGGGCTTG-3′; and 9B, 5′-CCTCGAATTCA GTCGAAGAGATCGCTGATGCC-3′.

Plasmid pGEX BP3-117 expressing GST–E2F-1 (GST-RBP3 aa 89 to 437 [18]) was a kind gift of E. Harlow (Massachusetts General Hospital). Constructs, expression, and purification of GST-E7 and GST-E1A have been described previously (10).

Northern (RNA) blot analyses. A human multiple tissue Northern blot (Clontech) was hybridized with a ^{32}P-random-labeled DNA fragment corresponding to the E2F-2 clone 9 cDNA by using QuikHyb hybridization solution (Stratagene) and washed as recommended by the manufacturer. MRC5 human fibroblast cells were a kind gift from Frank Banker (Merck Research Laboratories). Tumor cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and grown in the recommended medium at 37°C in a 6% CO_2-containing atmosphere. Poly(A)$^+$ RNA was isolated by using the Fast Track mRNA isolation kit (Invitrogen) according to the manufacturer's procedure. The poly(A)$^+$ RNA (2 μg) was denatured and separated on a 1% agarose formaldehyde-containing gel as described elsewhere (37). The RNA was then transferred to a Zeta-Probe membrane (Bio-Rad) and hybridized with an E2F-2 probe as described above. Blots were hybridized a second time with a ^{32}P-random-labeled 2-kb fragment of human actin DNA as a control probe.

Expression and gel mobility shift assays of GST-E2F fusion proteins. Expression of the GST fusion proteins was induced as previously described (10). Cells were collected by centrifugation; lysates were prepared by one round of freezing and thawing, suspension in phosphate-buffered saline (0.2 ml/ml of cells at 1 A_{600} unit), and sonication. Lysates were clarified by centrifugation, and the relative amounts of GST fusion proteins expressed were determined by immunoblot analysis using a rabbit anti-GST antibody. Crude lysates containing

approximately equal amounts of GST fusion proteins were incubated with ^{32}P-end-labeled, double-stranded DNA probe containing a single palindromic E2F site (underlined): 5′-TAG TTTTCGATATTAAATTTGAGTTTTCGCGCGAAACTAG-3′. Cold competitor DNA contained either a wild-type E2F site or a mutated site (TTTCGATCCAAA). The assay and buffer conditions were previously described (25).

Purification and gel mobility shift assays of recombinant proteins and HeLa E2F. Full-length E2F-2 protein with the C-terminal tubulin tripeptide epitope was affinity purified essentially as described previously (41) and electroeluted from an SDS-polyacrylamide gel as previously described (25). The conditions for the gel shift assay, the affinity purification of HeLa E2F (25), and the purification of recombinant pRb60 (14) have been previously described. Denatured salmon sperm DNA was included in the assay at 2 μg/ml.

In vitro binding assays. Bacterial lysates containing GST fusion proteins were made as described above. The relative amounts of the GST fusion proteins in the lysates (as determined by immunoblot analysis using anti-GST antibody) were normalized by dilution with lysate lacking without GST fusion protein. For pRb60 binding assays, 50 μl of normalized lysates was incubated with or without 0.6 μg of purified pRb60 in a final volume of 100 μl of phosphate-buffered saline–0.1% Nonidet P-40 for 1.5 h at 4°C. Where indicated, partially purified GST-E1A was also included in the binding reaction as a competitor. The pRb60 and any associated proteins were then recovered by using monoclonal anti-pRb antibody XZ55 (E. Harlow, Massachusetts General Hospital) (23), followed by rabbit anti-mouse immunoglobulin G (Cappel) and protein A-Sepharose beads (Pharmacia). The beads were washed three times in cold phosphate-buffered saline, and bound proteins were eluted by boiling in SDS-gel loading buffer. Proteins were resolved on an SDS–12% polyacrylamide gel (Novex), and GST fusion proteins were detected by immunoblot analysis using anti-GST antibody.

For protein dimerization assays, 50 μl of bacterial lysates containing equivalent amounts of GST fusion proteins of E2F-1 or E2F-2 was incubated with purified full-length, tubulin tripeptide-tagged E2F-1 or E2F-2. The binding assay was performed as described above except that rabbit anti-GST antibody and protein A-Sepharose beads were used to capture protein complexes. The binding of tubulin epitope-tagged E2F-1 or E2F-2 to the GST fusion proteins was detected by immunoblot analysis using the YL1/2 antitubulin antibody.

Complementation experiments. Purified tubulin epitope-tagged E2F-1 and E2F-2 were used at 5 and 2 μg/ml, respectively, in the complementation study. Individually purified HeLa E2F components were separated and gel eluted as described previously (25). Denaturation and refolding of proteins individually or in combination and testing for E2F DNA binding activity have also been described previously (25). For the gel shift assay, an excess of unlabeled competitor DNA containing a mutated E2F site was added to eliminate nonspecific binding as previously shown (25).

Antibodies. A peptide corresponding to amino acids 196 to 210 of E2F-2 (GMFEDPTRPGKQQQL) was synthesized as a mitogen-associated protein fusion (35), and polyclonal antibodies were raised in rabbits (Research Genetics, Huntsville, Ala.). Immunoglobulin G was purified by protein A-Sepharose chromatography and used in immunoblot analysis at a final concentration of 2.5 μg/ml.

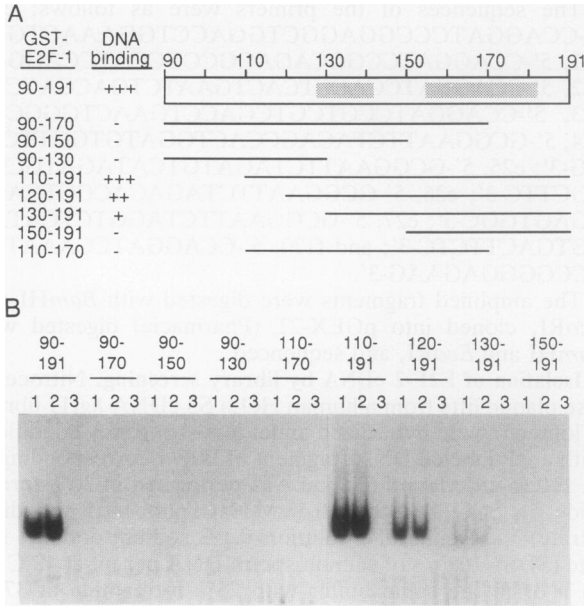

FIG. 1. Identification of the minimal DNA binding domain of E2F-1. GST–E2F-1 fusion proteins containing the indicated E2F-1 amino acids were tested for the ability to bind a radiolabeled DNA sequence containing a single palindromic E2F binding site in the gel shift assay. Fusion protein concentrations were normalized by Western blotting with an anti-GST antibody. (A) Summary of E2F-1 deletion constructs. The open bar represents the previously identified E2F-1 DNA binding domain (E2F-1 [aa 90 to 191] [18]), and the shaded regions represent the helices of the helix-loop-helix domain. The solid lines represent regions included in each construct. (B) Gel shift assay results. Lanes: 1, no competitor; 2, competition with excess unlabeled mutant DNA; 3, competition with excess unlabeled wild-type DNA.

Silver staining. Silver staining of SDS-polyacrylamide gels was performed with the Quick-Silver kit from Amersham.

Nucleotide sequence accession number. The sequence of E2F.2 clone 9 has been assigned GenBank accession number L22846.

RESULTS

Identification of the minimal DNA binding domain of E2F-1. The primary functional property of an E2F-related protein is its ability to bind the E2F DNA binding site. The DNA binding domain of different E2F proteins is therefore likely to be highly conserved, and the DNA encoding this domain represents a highly specific screening probe to identify new homologs of E2F-1. Elimination of extraneous sequences not involved in DNA binding should provide optimal specificity. Previous deletion analyses by other groups have shown that aa 90 to 191 (18) or 123 to 224 (39) retain site-specific DNA binding activity when expressed as GST fusion proteins. Taken together, these observations suggest that a smaller segment corresponding to aa 123 to 191 may encompass the minimal DNA binding domain of E2F-1. We attempted to verify this hypothesis by performing a deletion analysis of the region encoding aa 90 to 191 of E2F-1 (Fig. 1A).

Both N- and C-terminal deletions were generated within the E2F-1 (aa 90 to 191) domain, and the resulting fragments were expressed as GST fusion proteins in *E. coli*. The ability of these fusion proteins to bind specifically to an E2F DNA

binding site was determined in a gel shift assay (Fig. 1B). Amino acids 90 to 109 appeared to be dispensible for DNA binding, since GST–E2F-1 (110 to 191) exhibits wild-type activity. However, any additional deletions resulted in proteins with reduced DNA binding activity. GST–E2F-1 (aa 120 to 191) showed reduced activity, while deletion of aa 171 to 191 from the C terminus of the DNA binding domain resulted in a complete loss of DNA binding activity. These results suggest that aa 110 to 191 encode the fully active DNA binding domain of E2F-1. Significant structural features of this region include a cluster of basic residues (aa 109 to 127) followed by overlapping helix-loop-helix (aa 128 to 181) (28) and leucine zipper-like (aa 153 to 174) (39) dimerization domains. A second zipper-like structure in E2F-1 (aa 199 to 234) (28) is not necessary for DNA binding activity.

Isolation of cDNAs encoding E2F-2. A radiolabeled DNA fragment corresponding to amino acids 110 to 191 of E2F-1 was used to screen a HeLa cDNA λgt11 library under low-stringency hybridization conditions. After plaque purification, the 21 positive clones obtained were initially characterized by PCR amplification of the cDNA inserts and direct sequencing from both ends of the amplified products. We obtained 14 unique cDNAs from the 21 original isolates, 12 of which possessed sequences identical to E2F-1 and two of which were distinct from E2F-1. These two distinct clones, designated 9 and 10, were further analyzed by preparing phage DNA from phage stocks of the two clones and subcloning the cDNA inserts directly into a pGEX vector. The cDNAs were sequenced and shown to contain overlapping regions of the same gene. The DNA sequence is shown in Fig. 2A. Clone 9 contained a 1,766-bp cDNA (nucleotides 1 to 1766 of Fig. 2A). Clone 10 contained a 1,963-bp cDNA (nucleotides 685 to 2647 of Fig. 2A). The largest open reading frame contained within these overlapping cDNA fragments is 1,311 nucleotides long (437 aa), with in-frame stop codons both 5′ (underlined) and 3′ (boxed) to the open reading frame (Fig. 2A). The protein encoded by this open reading frame (which is completely contained in clone 9) was designated E2F-2 (predicted molecular size = 47.5 kDa).

When compared with sequences in the GenBank data base, E2F-2 showed no significant homology to any known genes other than E2F-1. An amino acid alignment between E2F-2 and E2F-1 is shown in Fig. 2B (overall amino acid identity = 46%). As expected, the region corresponding to the DNA binding domain of E2F-1 (aa 110 to 191) is highly conserved in E2F-2 (72% amino acid identity). The high degree of conservation in this region includes several basic amino acids (# in Fig. 2B) and hydrophobic residues (* in Fig. 2B) that comprise the amphipathic helices (boxed) of a potential helix-loop-helix structure (28). Both of the zipper-like structures identified in E2F-1 (28, 39) are conserved in E2F-2 (hydrophobic residues denoted with + and ‡, respectively). Thus, the entire basic-helix-loop-helix-zipper region predicted for E2F-1 is conserved in E2F-2. Finally, the pRb binding domain of E2F-1 (aa 409 to 426) (18) is also highly conserved (72% identity) in E2F-2 (aa 410 to 427). Homology between E2F-2 and the recently isolated E2F-like DP-1 protein is more limited and is similar to that reported between DP-1 and E2F-1 (17).

Northern blot analysis of E2F-2 mRNA. The relative levels of E2F-2 mRNA in various human tissues were examined in a Northern blot by probing poly(A)$^+$ RNA with a DNA fragment of the E2F-2 cDNA. As shown in Fig. 3A, among eight tissues examined, only placenta showed a readily detectable level of E2F-2 mRNA. The size of the message (approximately 6 kb) cannot be accurately determined because of the poor resolution of larger RNAs. We also examined poly(A)$^+$ RNA from 32 cell lines corresponding to 13 different tumor types, of which a representative set is shown in Fig. 3B. The approximately 6-kb E2F-2 mRNA was present in all cell lines, although at different levels. Other species of approximately 2.5, 3.0, and 8 kb were also detected in some cell lines. The mRNA represented by the band at approximately 3.0 kb is likely to be the homologous E2F-1 message, since a band in precisely the same position appears when the blot is probed with E2F-1 cDNA, and it corresponds to the size expected for E2F-1 as indicated by previous results (18, 28). The MRC5 primary lung fibroblasts exhibited an extremely low level of E2F-2 mRNA. While the cancer cell lines appear to display higher levels of E2F-2 mRNA than the primary cell culture does, no obvious correlation between tumor origin and E2F-2 message levels was observed (data not shown).

E2F-2 binds to an E2F DNA binding site and to pRb. To test the ability of E2F-2 to bind specifically to an E2F consensus DNA binding site, full-length E2F-2 protein having a C-terminal tubulin tripeptide tag was expressed in *E. coli*, purified, and compared with purified HeLa cell E2F in a gel mobility shift assay (Fig. 4A). As seen with HeLa cell E2F, E2F-2 protein bound to a DNA probe containing an E2F consensus sequence, and the binding was specific since it was competed for with excess unlabeled wild-type, but not mutant, E2F oligonucleotide.

Another important characteristic of E2F is its ability to bind to pRb. A recombinant 60-kDa fragment of this protein, pRb60, which has previously been shown to bind HeLa E2F (24), was tested for its ability to bind E2F-2 in the gel shift assay. The mobility of the E2F-2 DNA complex was reduced by the addition of purified pRb60 protein, as was also the case for HeLa E2F (Fig. 4A). Thus, recombinant E2F-2 exhibits two important properties of cellular E2F, sequence-specific DNA binding and association with pRb.

Identification of the DNA binding domain of E2F-2. To identify the DNA binding domain of E2F-2, deletion fragments were expressed as GST fusion proteins and tested in the gel mobility shift assay. Comparison of the amino acid sequences of E2F-2 and E2F-1 (Fig. 2B) suggests that a construct including E2F-2 aa 87 to 193 should possess DNA binding activity. On the basis of the deletion analysis of E2F-1 shown in Fig. 1, analogous deletions were made in GST–E2F-2. In addition, GST–E2F-2 (aa 87 to 244) was generated to test the contribution of the C-terminal putative zipper region to DNA binding activity. The concentrations of the GST–E2F-2 fusion proteins were normalized by Western blotting (immunoblotting) (Fig. 4C), and the proteins were tested for specific E2F DNA binding in the gel shift assay (Fig. 4B). The C-terminal leucine zipper motif of E2F-2 is not necessary for DNA binding activity (compare the 87–244 construct with the 87–193 construct), as was also observed in the E2F-1 deletion studies (18, 28). However, removal of aa 87 to 111 had a pronounced effect on the E2F-2 construct, whereas removal of the homologous region from GST-E2F-1 (aa 90 to 109) had no effect (Fig. 1B). Additional N-terminal deletions of E2F-2 to aa 122 and 132 reduced binding even further (as seen on a longer exposure), and the 152–193 construct was completely inactive, as for E2F-1. Thus, the fusion protein containing E2F-2 aa 87 to 193 was the smallest construct tested which retained essentially complete DNA binding activity. This region corresponds to the DNA binding region identified for E2F-1 (aa 110 to 191) except that it includes 25 additional N-terminal amino acids.

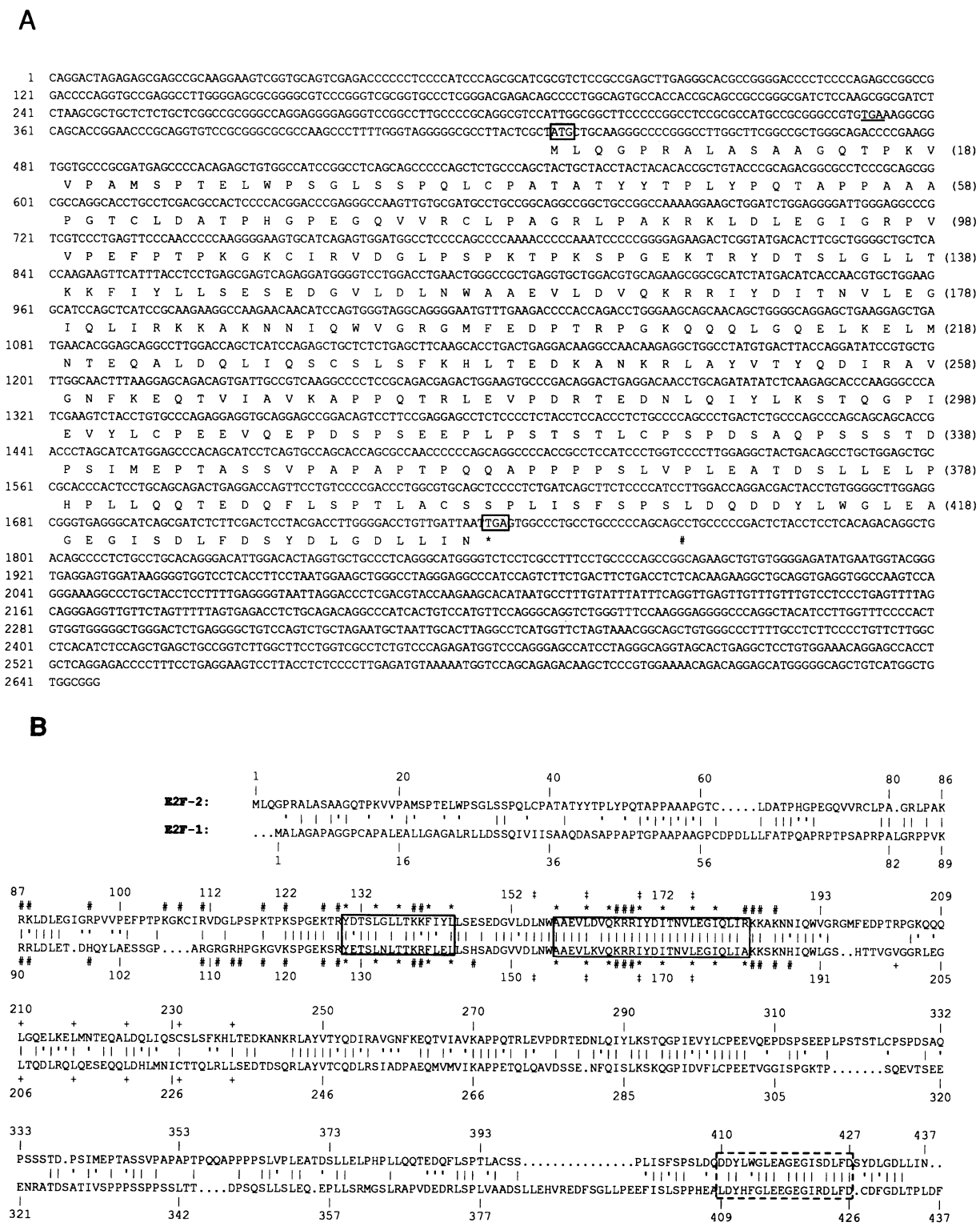

FIG. 2. Sequence of E2F-2. (A) Nucleotide sequence obtained from clones 9 (positions 1 to 1766; end indicated by #) and 10 (685 to 2647). The amino acid sequence of the longest open reading frame (437 aa) is provided underneath the corresponding nucleotides. The initiating Met codon and the stop codon are boxed. The 5′ in-frame stop codon is underlined. (B) Amino acid sequence comparison of E2F-2 and E2F-1, generated by the Gap subroutine of GCG (see Materials and Methods). Several conserved features are marked as follows: helices of the helix-loop-helix domain (aa 130 to 144 and 158 to 183) are boxed with a solid line; conserved hydrophobic residues within these helices are denoted by *; basic residues within the DNA binding region are denoted by #; conserved hydrophobic residues of the N-terminal and C-terminal zipper-like motifs are denoted by ‡ and a +, respectively; and the pRb binding domain (aa 410 to 427) is boxed with a dashed line.

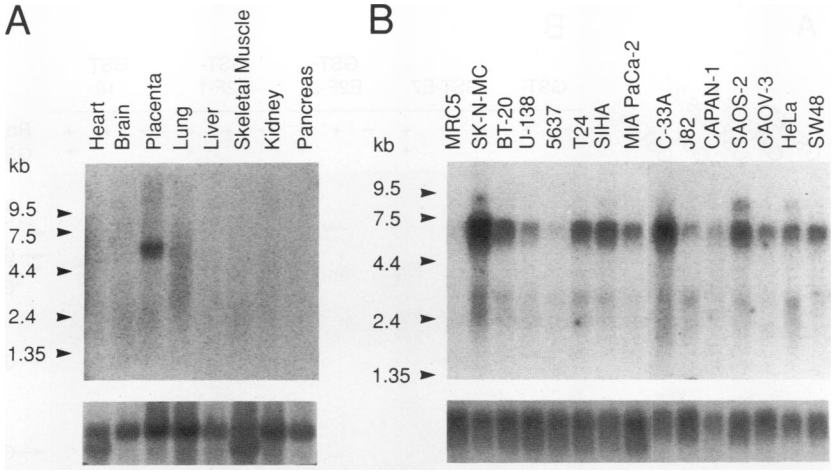

FIG. 3. Northern blot analysis of E2F-2 mRNA. Poly(A)$^+$ RNA extracted from normal human tissue (A) or various cell lines (B) was probed with E2F-2 clone 9 cDNA (upper sets). As a control, the same blots were probed with an actin probe (lower sets). Size markers are shown to the left of each panel.

This additional amino acid sequence requirement for E2F-2 may be due to differences in the arrangement of basic amino acids, which are clustered C terminal to position 110 in E2F-1 but are more N terminal in E2F-2 (marked with # in Fig. 2B).

E2F-2 binds to pRb60 through a conserved 18-aa domain. Since the gel shift analysis indicated that full-length E2F-2 was able to associate with pRb60 (Fig. 4A), we wished to map the region of E2F-2 responsible for pRb binding and to determine whether this binding could be competed for by a viral oncoprotein, E1A, as has been shown for E2F obtained from cell lysates (2, 6, 7). For these experiments, we tested the ability of GST–E2F-2 fusion proteins to bind to pRb60 by coimmunoprecipitation with an anti-pRb antibody.

The GST fusion proteins tested are shown in Fig. 5A. GST-E7 and GST–E2F-1 (aa 89 to 437), which have both been previously shown to bind to pRb60 (13, 18, 27), were included as positive controls. GST–E2F-2 is a fusion protein containing the complete coding sequence of E2F-2, and GST-18 is a fusion protein containing the 18-aa region of E2F-2 (aa 410 to 427) which is homologous to the 18-aa pRb-binding region of E2F-1 (aa 409 to 426) (18). As shown in Fig. 5B, GST alone did not bind to pRb60. GST-E7, GST–E2F-2, GST–E2F-1 and GST-18 were immunoprecipitated by anti-pRb antibodies in the presence (lanes 4, 7, 10, and 13) but not in the absence (lanes 3, 6, 9, and 12) of pRb60. In addition, the binding of all of the GST fusion proteins to pRb60 was inhibited by the addition of GST-E1A (lanes 5, 8, 11, and 14). Thus, the 18-aa region at the C terminus of E2F-2 is sufficient for specific association with the pocket region of pRb.

E2F-2 DNA binding activity is enhanced by heterodimerization. We have previously shown that affinity-purified HeLa cell E2F contains five prominent protein bands in the molecular size range of 50 to 60 kDa (numbered 1 to 5 from smallest to largest) which can be physically separated and shown to possess E2F activity in a gel shift assay (25). The five proteins can be divided into two functional groups (lower-molecular-weight bands 1 to 3 and higher-molecular-weight bands 4 and 5) on the basis of the observation that the DNA and pRb binding activities of the purified components are greatly enhanced by mixing one component from each

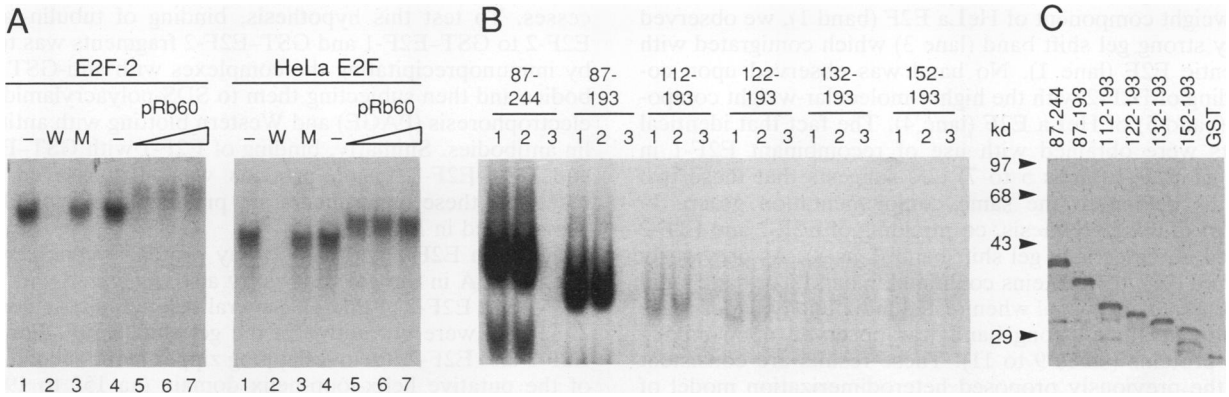

FIG. 4. Gel shift analysis of E2F-2. (A) Full-length tubulin epitope-tagged E2F-2 (left) and affinity-purified HeLa E2F (right) were analyzed in the gel shift assay. Lanes: 1 and 4 to 7, no competitor; 2, competition with excess wild-type DNA; 3, competition with excess mutant DNA; 5 to 7, samples containing increasing concentrations of recombinant pRb60. (B) GST–E2F-2 fusion proteins containing the indicated E2F-2 amino acids were assayed as soluble *E. coli* lysates in the gel shift assay. Lanes: 1, no competitor; 2, competition with excess mutant DNA; 3, competition with excess wild-type DNA. (C) Western blot analysis of the lysates tested in panel B, using an anti-GST polyclonal antibody.

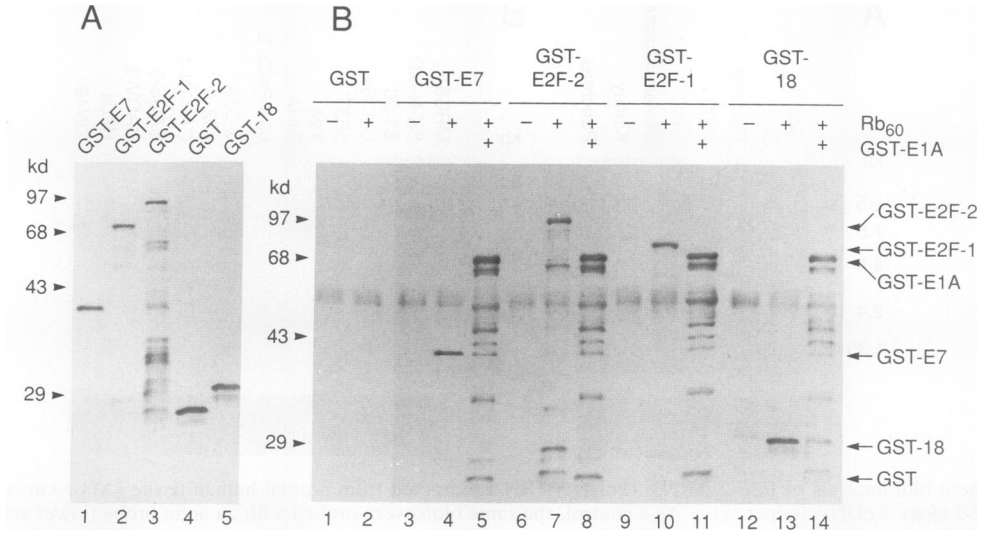

FIG. 5. Binding of E2F-2 protein to pRb60. (A) Western blot analysis of the *E. coli* lysates tested in panel B, using an anti-GST polyclonal antibody. (B) Soluble *E. coli* lysates containing the indicated GST fusion protein were incubated in the presence (lanes 2, 4, 5, 7, 8, 10, 11, 13, and 14) or absence (lanes 1, 3, 6, 9, and 12) of 100 nM recombinant pRb60 and immunoprecipitated with an anti-pRb monoclonal antibody. The immunoprecipitate was analyzed by SDS-PAGE and Western blotting with an anti-GST polyclonal antibody. The position of each GST fusion protein in the Western blot is indicated at the right. Affinity-purified GST-E1A was used as a competitor for pRb60 binding in lanes 5, 8, 11, and 14.

group. Of all possible combinations, heterodimerization of bands 1 and 5 consistently yielded the highest recovery of activity, and E2F-1 was shown to be functionally related to band 5 in these assays. We have also demonstrated that recombinant E2F-1 forms homodimers and have proposed that these homodimers may be responsible for the observed DNA and pRb binding activity of E2F-1 in the absence of its normal partner (25). To determine whether the biochemical behavior of E2F-2 is consistent with this model and to assess whether E2F-2 acts as the normal binding partner of E2F-1, we performed mixing experiments between E2F-2, gel-purified components of HeLa cell E2F, and recombinant E2F-1.

E2F DNA binding activity was monitored in the gel shift assay (Fig. 6A). Recombinant purified E2F-1 and E2F-2 and HeLa cell E2F components were titrated to yield no detectable signal under these assay conditions. Upon denaturing and refolding of E2F-2 in the presence of the lower-molecular-weight component of HeLa E2F (band 1), we observed a very strong gel shift band (lane 3) which comigrated with authentic E2F (lane 1). No band was observed upon co-refolding of E2F-2 with the higher-molecular-weight component (band 5) of HeLa E2F (lane 4). The fact that identical results were obtained with use of recombinant E2F-1 in place of E2F-2 (lanes 5 to 7) (25) suggests that these two proteins belong to the same complementation group. In support of this hypothesis, co-refolding of E2F-1 and E2F-2 yielded no detectable gel shift band (lane 8). As previously reported (25), the proteins contained in band 1 or band 5 did not generate any signal when tested individually under these conditions, but a strong band was observed upon mixing these proteins (lanes 9 to 11). These results are consistent with the previously proposed heterodimerization model of E2F, with E2F-1 and E2F-2 belonging to the same complementation group as the higher-molecular-weight component of HeLa cell E2F (band 5) (25).

Additional data in support of this model are provided by analysis of HeLa E2F by a Western blot with anti-E2F-2 antibodies. Rabbit polyclonal antisera generated against a nonconserved region of E2F-2 (aa 196 to 210) was tested for cross-reactivity against HeLa cell E2F and purified E2F-1 (Fig. 6B and C). As anticipated, this antiserum detects E2F-2 but not E2F-1 (Fig. 6C, lanes 2 and 3). In addition, the anti-E2F-2 antiserum detects bands 4 and 5 of HeLa E2F (Fig. 6C, lane 1), as judged by overlaying the Western blot autoradiogram (Fig. 6C) on the silver-stained gel of the same samples (Fig. 6B). E2F-2 is therefore both functionally and immunologically related to the higher-molecular-weight components of authentic HeLa E2F.

Identification of E2F-2 dimerization domains. If E2F-2 dimerization is necessary for DNA binding, it would be predicted that the regions of E2F-2 sufficient for physical dimerization would be contained within the regions required for DNA binding activity (Fig. 4). Alternatively, if DNA binding and dimerization are unrelated, then entirely different regions of E2F-2 might be involved in these two processes. To test this hypothesis, binding of tubulin-tagged E2F-2 to GST–E2F-1 and GST–E2F-2 fragments was tested by immunoprecipitating the complexes with anti-GST antibodies and then subjecting them to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting with antitubulin antibodies. Similarly, binding of E2F-1 with GST–E2F-1 and GST–E2F-2 fusion proteins was also assayed. The results of these experiments are presented in Table 1 and summarized in Fig. 7.

For both E2F-1 and E2F-2, any protein fragment which bound DNA in the gel shift assay also dimerized with both E2F-1 and E2F-2 (Table 1). Several deletion mutants which dimerized were not active in the gel shift assay. For both E2F-1 and E2F-2, the overlapping zipper motif-second helix of the putative helix-loop-helix domain (aa 152 to 193 for E2F-2) was sufficient to allow dimerization, although these fragments were not capable of binding DNA under our assay conditions (Fig. 1 and 4). Using the more extensive E2F-1 deletion library, however, we observed that this fentire helix was not absolutely necessary for dimerization since GST–

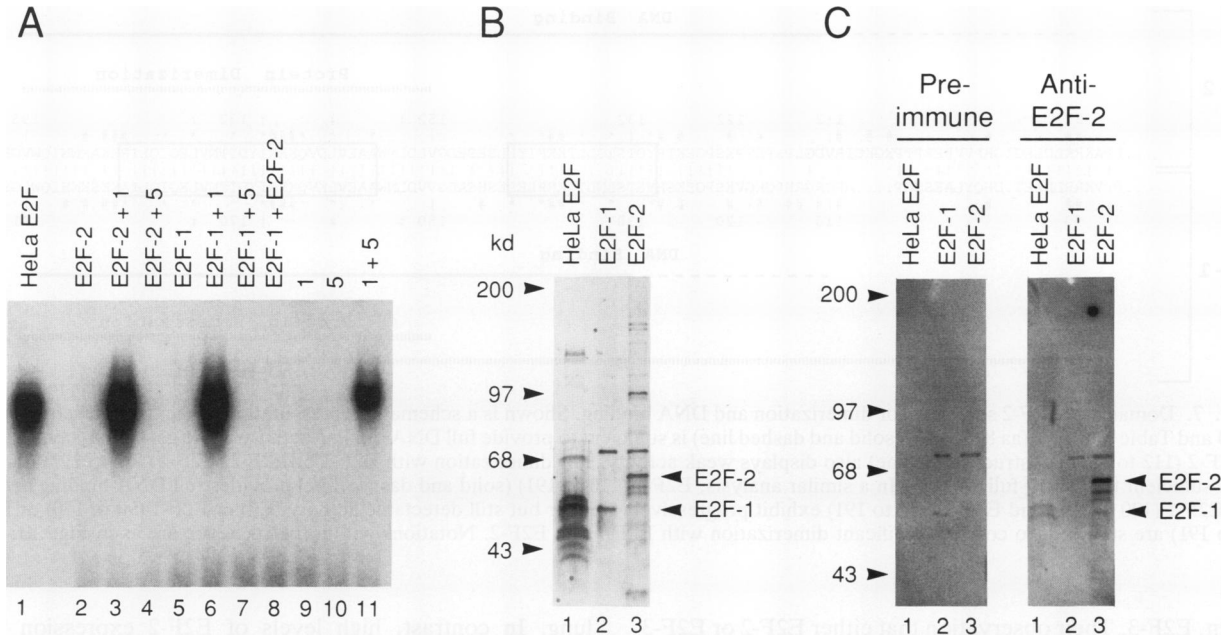

FIG. 6. E2F-2 dimerization studies. (A) Affinity-purified E2F-2 (2 μg/ml) and E2F-1 (5 μg/ml) were refolded with protein eluted from band 1 (lanes 3 and 6) or band 5 (lanes 4 and 7) of SDS-PAGE-purified HeLa cell E2F as described previously (25) and subjected to a gel shift assay. An excess of unlabeled DNA containing a mutant E2F binding site was added to each sample to eliminate nonspecific binding. Lane 1 contains affinity-purified HeLa cell E2F. Bands 1 and 5 were also assayed individually (lanes 9 and 10, respectively) or after mixing and co-refolding (lane 11). (B) Silver stain of HeLa cell E2F and affinity-purified E2F-1 and E2F-2 following SDS-PAGE. The prominent HeLa E2F bands at approximately 50 kDa are bands 1 to 3, and the darker bands at approximately 55 kDa (comigrating with *E. coli* E2F-1) are bands 4 and 5 (25). (C) Western blot of the samples in panel B, using rabbit pre-immune serum (left) or a rabbit anti-E2F-2 (aa 196 to 210) antiserum (right).

E2F-1 (aa 90 to 170) (containing the N-terminal helix, the loop, and half of the C-terminal helix) fragment also displayed dimerization activity. Overall, the observation that the regions of E2F-1 and E2F-2 sufficient for dimerization with full-length protein are contained within the domains sufficient for DNA binding supports the hypothesis that dimerization activity is required for DNA binding activity.

TABLE 1. Mapping of the E2F-1 and E2F-2 dimerization domains

GST fusion protein	Binding to:		
	E2F-1	E2F-2	DNA[a]
GST–E2F-1			
88–437	+	+	+++[b]
90–191	+	+	+++
90–170	+	+	–
90–150	±	±	–
90–130	–	–	–
110–191	ND[c]	ND	+++
120–191	ND	ND	++
130–191	ND	ND	+
150–191	+	+	–
GST–E2F-2			
1–437	+	+	+++[b]
87–244	+	+	+++
87–193	+	+	+++
112–193	+	+	+
122–193	+	+	±
132–193	+	+	±
152–193	+	+	–

[a] Data from Fig. 1 and 4.
[b] Data not shown.
[c] ND, not determined.

DISCUSSION

Historically, the term "E2F" refers to factors exhibiting several defined biochemical activities rather than to a specific protein. These activities include sequence-specific binding to and activation of the adenovirus E2 promoter and binding to pRb. Results from our laboratory (25) and others (17, 28) provide compelling evidence for the existence of multiple E2F's and/or multiple components of E2F. E2F-1, the first protein to be cloned with the properties of E2F, contains a well-defined DNA binding domain. In the current study, a DNA probe derived from regions of E2F-1 which provide optimal DNA binding activity, including the basic region and the overlapping helix-loop-helix and zipper domains, detected a novel clone in a human HeLa cDNA library. This clone contains a 1,311-bp open reading frame, complete with both 3′ and 5′ stop codons (Fig. 2). The protein encoded by this open reading frame, E2F-2, shows significant sequence homology with E2F-1. The DNA binding domain is highly conserved. E2F-1's overlapping putative helix-loop-helix and zipper dimerization regions are also observed in E2F-2, although the basic region is somewhat more diffuse. As with E2F-1, these domains are necessary for optimal DNA binding activity in E2F-2 (Fig. 4). The conservation of these sequences between E2F-1 and E2F-2 is not surprising, since the cloning strategy for E2F-2 was based on the presumed conservation of the DNA binding domain. Using a similar cloning strategy, Lees et al. (31) independently isolated cDNAs for E2F-2 and a related

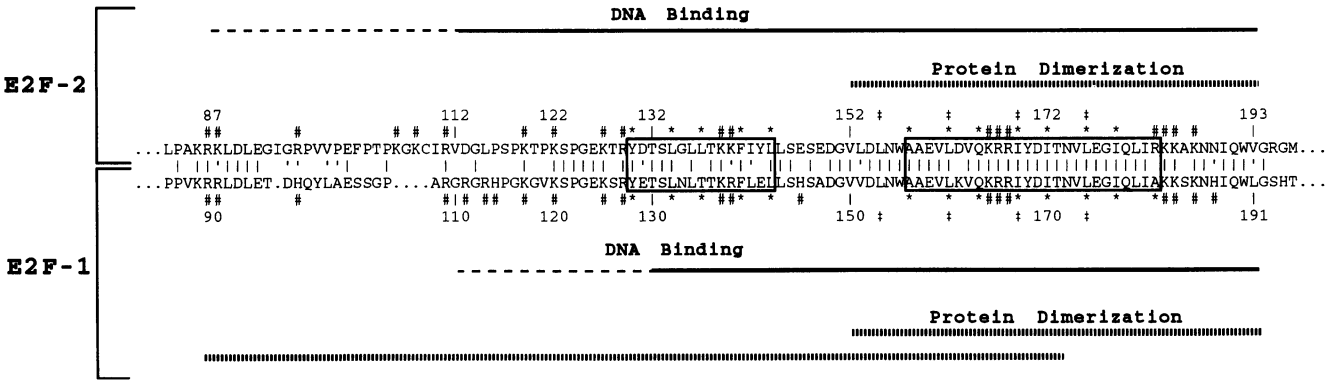

FIG. 7. Domains of E2F-2 sufficient for dimerization and DNA binding. Shown is a schematic representation of the data presented in Fig. 1 and 4 and Table 1. E2F-2 (aa 87 to 193) (solid and dashed line) is sufficient to provide full DNA binding activity in the gel shift assay, although the E2F-2 (112 to 193) construct (solid line) also displays weak activity. For dimerization with E2F-1 or E2F-2, E2F-2 (152 to 193) (hatched line) is sufficient to convey full activity. In a similar analysis, E2F-1 (110 to 191) (solid and dashed line) provides full DNA binding activity, while E2F-1 (120 to 191) and E2F-1 (130 to 191) exhibit progressively weaker but still detectable activity. Either E2F-1 (90 to 170) or E2F-1 (150 to 191) are sufficient to convey significant dimerization with E2F-1 and E2F-2. Notations within the sequence are as in Fig. 2B.

protein, E2F-3. Their observation that either E2F-2 or E2F-3 can specifically activate transcription of an E2F-responsive reporter gene demonstrates that these proteins are transcription factors (31).

Several other significant sequences of E2F-1 outside of the DNA binding domain are also conserved in E2F-2. These conserved features include the C-terminal pRb binding domain (E2F-2 [aa 409 to 426]), a second zipper-like motif (E2F-2 [aa 210 to 238]) (28), and a potential Cdk kinase phosphorylation site (E2F-2 [aa 122 to 124]) (39). The conservation of the second zipper motif is especially striking, since there is relatively little overall sequence conservation between E2F-1 and E2F-2 in this region. Although this zipper motif is not necessary for E2F-2's dimerization, pRb binding, or DNA binding activities, it may play a role in interactions with other cellular proteins. The extensive similarity in regions outside of the DNA binding domain supports the notion that E2F-1 and E2F-2 play similar roles in regulating cell physiology. In contrast, DP-1, a protein with biochemical properties similar to those of E2F which was cloned by using protein sequence obtained from E2F isolated from mouse F9 cells, displays less homology to E2F-2 or E2F-1 outside of the DNA binding domain (17).

Like HeLa cell E2F, E2F-2 binds pRb, and E1A protein blocks this binding (1, 2, 6, 20). However, E2F-2 does not contain the LXCXE motif which is necessary and sufficient for high-affinity pRb binding activity by E1A and many other viral and cellular proteins (9, 12, 16, 27, 34). E2F-2 and E2F-1 each contain an 18-aa pRb binding domain which is not observed in the LXCXE-containing pRb-binding proteins but which is sufficient to confer pRb binding activity. These observations suggest that E2F-2 and, by extrapolation, cellular E2F bind to pRb at a location which is distinct from the LXCXE binding site. Consistent with this hypothesis is the observation that a human papillomavirus type 16 E7 LXCXE peptide which blocks E7 binding to pRb does not inhibit E2F binding to pRb (24).

Northern blot analysis of normal tissues and over two dozen cell lines demonstrates that E2F-2 is encoded by an mRNA whose expression is both tissue and cell type specific. In the tissue blot, placenta displayed the highest level of expression. E2F-2 message was undetectable in six of the seven other tissues tested, with low levels observed in the lung. In contrast, high levels of E2F-2 expression were observed in many immortalized cell lines derived from tumor samples. No apparent correlation was observed between E2F-2 mRNA levels and tumor origin. E2F-2 message was readily detectable in every cell line tested but not in MRC-5 primary lung fibroblasts, which was also the only nonimmortal cell culture tested. These results suggest that E2F-2 may play a significant role in immortalized or rapidly proliferating cells.

We have previously shown that the DNA and pRb binding activities of HeLa cell SDS-PAGE-purified E2F components are dramatically enhanced by refolding the proteins together. These results suggest that E2F functions as a heterodimer of two distinct proteins (25). We have also shown that in the absence of heterodimer formation, E2F-1 binds DNA as a homodimer. Like E2F-1, E2F-2 contains putative overlapping helix-loop-helix and zipper motifs, and we have shown in this study that the overlapping N-terminal zipper motif-second helix is sufficient to allow dimerization with full-length E2F-1 or E2F-2. The dimerization domain is also necessary for DNA binding, and all deletion mutants which did not dimerize also did not bind DNA. These results are consistent with a model, shared with many other transcription factors, in which dimerization is a necessary precondition for DNA binding activity (26). Like E2F-1, the DNA binding activity of E2F-2 is dramatically enhanced by corefolding with band 1, but not band 5, of SDS-PAGE-purified HeLa cell E2F (25). E2F-2 therefore appears to belong to the same heterodimerization complementation group as E2F-1 and band 5 of HeLa cell E2F. This hypothesis is reinforced by the observation that a highly selective E2F-2 peptide antiserum cross-reacts with bands 4 and 5 of HeLa cell E2F but not with band 1, 2, or 3. Thus, E2F-2 is both functionally and immunologically related to bands 4 and 5 of HeLa cell E2F.

E2F-2 does not appear to represent the dimerization partner of E2F-1, since mixing of these two recombinant proteins does not enhance DNA binding activity. However, recent results from Helin et al. (19) suggest that a dimerization partner of E2F-1 is DP-1. From our comparison of the heterodimerization properties of E2F-1 and E2F-2, it appears likely that DP-1 will also function as a dimerization partner for E2F-2. It now seems clear that E2F is composed

of a family of proteins which might form a variety of complexes. Additional experiments will be necessary to elucidate the functional roles of each complex in the regulation of promoters containing E2F binding sites.

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REFERENCES

1. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell **62**:659–669.
2. **Bagchi, S., R. Weinmann, and P. Raychaudhuri.** 1991. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell **65**:1063–1072.
3. **Bandara, L. R., J. P. Adamczewski, T. Hunt, and N. B. LaThangue.** 1991. Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature (London) **352**:249–251.
4. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9**:4994–5002.
5. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355**:176–179.
6. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65**:1053–1061.
7. **Chittenden, T., D. M. Livingston, and W. J. Kaelin.** 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell **65**:1073–1082.
8. **Dalton, S.** 1992. Cell cycle regulation of the human cdc2 gene. EMBO J. **11**:1797–1804.
9. **Defeo-Jones, D., P. S. Huang, R. E. Jones, K. M. Haskell, G. A. Vuocolo, M. G. Hanobik, H. E. Huber, and A. Oliff.** 1991. Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. Nature (London) **352**:251–254.
10. **Defeo-Jones, D., G. A. Vuocolo, K. M. Haskell, M. G. Hanobik, D. M. Kiefer, E. M. McAvoy, M. Ivey-Hoyle, J. L. Brandsma, A. Oliff, and R. E. Jones.** 1993. Papillomavirus E7 protein binding to the retinoblastoma protein is not required for viral induction of warts. J. Virol. **67**:716–725.
11. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell **68**:167–176.
12. **Dowdy, S. F., P. W. Hinds, K. Louie, S. I. Reed, A. Arnold, and R. A. Weinberg.** 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell **73**:499–511.
13. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–937.
14. **Edwards, G. M., H. E. Huber, J. D. DeFeo, G. Vuocolo, P. J. Goodhart, R. Z. Maigetter, G. Sanyal, A. Oliff, and D. C. Heimbrook.** 1992. Purification and characterization of a functionally homogeneous 60-kDa species of the retinoblastoma gene product. J. Biol. Chem. **267**:7971–7974.
15. **Eisenberg, S. P., R. J. Evans, W. P. Arend, E. Verderber, M. T. Brewer, C. H. Hannum, and R. C. Thompson.** 1990. Primary structure and functional expression from complementary DNA of a human interleukin-1 antagonist. Nature (London) **343**:341–346.
16. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J.-Y. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73**:487–497.
17. **Girling, R., J. F. Partridge, L. R. Bandara, N. Burden, N. F. Totty, J. J. Hsuan, and N. B. La Thangue.** 1993. A new component of the transcription factor DRTF1/E2F. Nature (London) **362**:83–87.
18. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70**:337–350.
19. **Helin, K., C. Wu, A. R. Fattaey, J. A. Lees, B. D. Dynlacht, C. Ngwu, and E. Harlow.** Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative transactivation. Genes Dev., in press.
20. **Hiebert, S. W., M. Blake, J. Azizkhan, and J. R. Nevins.** 1991. Role of E2F transcription factor in E1A-mediated *trans* activation of cellular genes. J. Virol. **65**:3547–3552.
21. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6**:177–185.
22. **Hiebert, S. W., M. Lipp, and J. R. Nevins.** 1989. E1A-dependent trans-activation of the human MYC promoter is mediated by the E2F factor. Proc. Natl. Acad. Sci. USA **86**:3594–3598.
23. **Hu, Q. J., C. Bautista, G. M. Edwards, J. D. Defeo, R. E. Jones, and E. Harlow.** 1991. Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. Mol. Cell. Biol. **11**:5792–5799.
24. **Huang, P. S., D. R. Patrick, G. Edwards, P. J. Goodhart, H. E. Huber, L. Miles, V. M. Garsky, A. Oliff, and D. C. Heimbrook.** 1993. Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. Mol. Cell. Biol. **13**:953–960.
25. **Huber, H. E., G. Edwards, P. J. Goodhart, D. R. Patrick, P. S. Huang, M. Ivey-Hoyle, S. F. Barnett, A. Oliff, and D. C. Heimbrook.** 1993. Transcription factor E2F binds DNA as a heterodimer. Proc. Natl. Acad. Sci. USA **90**:3525–3529.
26. **Jones, N.** 1990. Transcriptional regulation by dimerization: two sides to an incestuous relationship. Cell **61**:9–11.
27. **Jones, R. E., R. J. Wegrzyn, D. R. Patrick, N. L. Balishin, G. A. Vuocolo, M. W. Riemen, D. Defeo-Jones, V. M. Garsky, D. C. Heimbrook, and A. Oliff.** 1990. Identification of HPV-16 E7 peptides that are potent antagonists of E7 binding to the retinoblastoma suppressor protein. J. Biol. Chem. **265**:12782–12785.
28. **Kaelin, W. J., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70**:351–364.
29. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A transactivation. Cell **45**:219–228.
30. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc. Natl. Acad. Sci. USA **84**:2180–2184.
31. **Lees, J. A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13**:7813–7825.
32. **Mudryj, M., S. H. Devoto, S. W. Hiebert, T. Hunter, J. Pines, and J. R. Nevins.** 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell **65**:1243–1253.
33. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258**:424–429.
34. **Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53**:539–547.
35. **Posnett, D. N., H. McGrath, and J. P. Tam.** 1988. A novel method for producing anti-peptide antibodies. J. Biol. Chem. **263**:1719–1725.

36. **Raychaudhuri, P., R. Rooney, and J. R. Nevins.** 1987. Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. EMBO J. **6:**4073–4081.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 7.43–7.45. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Schwarz, J. K., S. H. Devoto, E. J. Smith, S. P. Chellappan, L. Jakoi, and J. R. Nevins.** 1993. Interactions of the p107 and Rb proteins with E2F during the cell-proliferation response. EMBO J. **12:**1013–1020.
39. **Shan, B., X. Zhu, P.-L. Chen, T. Durfee, Y. Yang, D. Sharp, and W.-H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. **12:**5620–5631.
40. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell **68:**157–166.
41. **Stammers, D. K., M. Tisdale, S. Court, V. Parmar, C. Bradley, and C. K. Ross.** 1991. Rapid purification and characterization of HIV-1 reverse transcriptase and RNase H engineered to incorporate a C-terminal tripeptide alpha-tubulin epitope. FEBS Lett. **283:**298–302.
42. **Thalmeier, K., H. Synovzik, R. Mertz, E. L. Winnacker, and M. Lipp.** 1989. Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. Genes Dev. **3:**527–536.
43. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) **358:**259–261.