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In Vivo Association of E2F and DP Family Proteins

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The mammalian transcription factor E2F plays an important role in regulating the expression of genes that are required for passage through the cell cycle. This transcriptional activity is inhibited by association with the retinoblastoma tumor suppressor protein (pRB) or its relatives p107 and p130. The first cDNA from the E2F family to be cloned was designated E2F-1, and multiple E2F family members have now been identified. They bind to DNA as heterodimers, interacting with proteins known as DP. Here we demonstrate that DP is also a family of polypeptides with at least two members (hDP-1 and hDP-2). Both hDP-1 and hDP-2 bind to all E2F family members in vivo, and each complex is capable of activating transcription. However, the various E2F/DP complexes display strong differences in the ability to bind to either pRB or p107 in vivo, and the specificity of pRB or p107 binding is mediated by the E2F subunit.

E2F was initially identified as a mammalian DNA-binding protein that was required for activation of the adenovirus E2 promoter (46). E2F-mediated transactivation is normally inhibited by association with negative regulators such as the retinoblastoma protein (pRB). During viral infection, E2F-dependent transcription is induced by expression of the viral E1A protein (8, 47). Recent studies have shown that E1A-mediated transactivation is dependent on the ability of E1A to bind to pRB, p107, or p130, releasing free E2F and stimulating transcription (2, 6, 11, 13, 17, 30, 32, 48).

The first E2F-associated protein identified was pRB, the product of a tumor suppressor gene that is commonly mutated in human tumors. The association of pRB and E2F does not appear to alter the affinity of E2F for DNA but is sufficient to inhibit its transcriptional activity (23, 27, 31, 52). pRB is phosphorylated by one or more of the cyclin-dependent kinases (26, 37, 40, 42–44). This phosphorylation is thought to be sufficient to change pRB such that it is no longer able to bind to E2F (10, 23, 28). This release of free E2F results in transcriptional activation. Thus, the loss of functional pRB through mutation, the expression of E1A, and phosphorylation of pRB all result in activation of E2F.

In addition to pRB, the activity of E2F is also regulated by its association with several other cellular proteins. These include the pRB-related proteins p107 and p130. The interaction of these proteins with E2F is more complicated, as the interactions also include stoichiometric levels of certain cyclin-dependent kinases. Independent complexes with cyclin A/cdk2 and cyclin E/cdk2 have previously been identified (6, 9, 13, 14, 39, 45, 51). The binding of these kinases to E2F is indirect and is mediated by virtue of their stable association with p130 and p107 (9, 13). As with pRB, the timing of these interactions is also tightly regulated. The p130/E2F complex appears predominately in cells that either are arrested in G_0 or are reentering the cell cycle from an arrested state (13). Initially this complex is formed in the absence of any kinase, but cyclin E/cdk2 appears to associate as soon as the cyclin E/cdk2 complex is formed (13). Similarly, formation of E2F/p107/cyclin E/cdk2 and E2F/p107/cyclin A/cdk2 appears to coincide with the formation and activation of each of these kinase complexes (14, 39, 45, 49, 51).

In normal cells, E2F acts to regulate the transcription of a large number of genes whose products appear to play an essential role in regulating passage through the cell cycle (46). Moreover, their expression appears to be linked to the stages of the cell cycle in which the products of these genes function. The proteins that interact with E2F are thought to function in concert to restrict the activation of E2F and therefore E2F-responsive genes to these distinct cell cycle stages.

In the last 2 years, six genes that encode components of E2F (E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and DP-1) have been cloned (7, 19, 21, 28, 35, 36, 41, 48a, 50, 51a, 53). The first, called E2F-1, was cloned by virtue of its ability to interact with pRB (28, 36, 50). Transfection of E2F-1 is able to activate transcription in a manner that is dependent on the presence of E2F binding sites (28, 36). Subsequently, four additional cDNA clones that are highly homologous to E2F-1 were identified (7, 19, 35, 41, 48a, 52a). The encoded proteins, E2F-2, E2F-3, E2F-4, and E2F-5, have properties similar to those of E2F-1, suggesting that E2F activity might result from the concerted activity of a family of closely related proteins (7, 19, 35, 41, 48a, 52a).

In independent studies, Girling and colleagues were able to purify and clone an E2F-like activity from mouse embryonal F9 cells (21). The cDNA isolated, DP-1, shares limited homology with E2F-1, E2F-2, or E2F-3 except for in a small region that corresponds to the DNA binding domain of each of these three proteins (21). DP-1 itself has little or no affinity for DNA (29). However, recently a number of groups have shown that E2F-1 and DP-1 can heterodimerize (4, 29, 38). Moreover, this dimerization appears to be essential for both high-affinity DNA binding and efficient transcriptional activation (4, 29, 38). This finding suggests that functional E2F might contain both E2F and DP subunits. To keep the nomenclature of these proteins clear in this report, we have called the individual polypeptides in the heterodimer by the name of the cloned cDNA, followed by the number that describes the order of

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their identification (E2F-1, E2F-2, etc., or hDP-1 or hDP-2). The general activity and the complex will be referred to as E2F without a number designation.

To characterize the nature of E2F, we have begun to examine how these proteins interact. We have been able to demonstrate that there is a family of DP proteins, both hDP-1 and hDP-2 bind to all the various E2F subunits, and each can serve as a potent transactivator. However, various E2F/DP complexes display strong differences in the ability to bind to either pRB or p107, and this specificity appears to be mediated by the E2F subunit.

MATERIALS AND METHODS

Cloning of hDP-2 cDNA. A cDNA probe, encoding codons 84 to 204 of mouse DP-1, was used to screen 1 million plaques from a cDNA library (in lambda ZAPII) prepared from the human pre-B-cell line Nalm 6. Hybridization was carried at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–30% formamide–5% dextran sulfate, 0.5% sodium dodecyl sulfate (SDS)–150 μg of denatured salmon sperm DNA per ml. Filters were washed three times at room temperature for 20 min in 2× SSC–0.1% SDS. Positive clones were detected by autoradiography and plaque purified. Novel clones were sequenced by using Sequenase 2.0 (U.S. Biochemical) as suggested by the manufacturer.

Plasmid constructions. All cytomegalovirus (CMV) expression plasmids were constructed in pCMV-Neo-Bam (3). pCMV-E2F-1 (1-437), pCMV-E2F-1 (89-437), pCMV-E2F-2 (85-437), pCMV-E2F-3 (132-425), pCMV-HA-hDP-1 (1-410), pCMV-RB, pCMV-RBΔ22, and pCMV-p107 have been described previously (29, 41, 54). pCMV-E2F-2 (1-437) was a kind gift of M. Ivey-Hoyle. pCMV-HA-hDP-2 (1-385) was generated by two steps of cloning. The complete open reading frame (385 codons) of hDP-2 was amplified by PCR and subcloned into the *Bam*HI site in pBSK-HA (29). This region was then isolated from pBSK-HA-hDP-2 as a *Hin*cII-*Xba*I fragment, blunt ended with DNA polymerase large fragment (Klenow), and then subcloned into the blunted *Bam*HI site of pCMV-Neo-Bam. The nucleotide sequence of the hDP-2 open reading frame was then confirmed. pQE-hDP-1 was constructed by isolating the *Bam*HI fragment containing hDP-1 (1-410) from pGST-hDP-1 (29) and inserted into pQE8 (Qiagen). pQE-hDP-2 (1-291) was constructed by isolating a fragment of hDP-2 cDNA encoding amino acids 1 to 291 from pBSK-HA-hDP-2 and inserted into pQE9 (Qiagen).

Northern (RNA) blots. Total cellular RNA were isolated from 293 (adenovirus-transformed primary embryonal kidney), HT230 (colon adenocarcinoma), IMR32 (neuroblastoma), CEM (T-lymphoblastoid leukemia), ML-1 (premyeloid leukemia), MCF7 (breast adenocarcinoma), SIHA (cervical squamous carcinoma), NGP (neuroblastoma), C33-A (cervical carcinoma), and HL60 (premyelocytic leukemia) cells by the method of Chomczynski and Sacchi (12). RNAs from the first seven listed cell lines were generously provided by K. Helin. Total cellular RNA (30 μg) was electrophoresed on 1% formamide agarose gels. The gels were stained with ethidium bromide, and approximately equal amounts of rRNA were observed (data not shown). The RNA was then transferred onto Hybond-N+ membranes (Amersham). Each lane of the human tissue blot (Clontech Laboratory, Inc.) contains 2 μg of poly(A)$^+$ RNA isolated from the indicated human tissues. For this blot, the levels of actin message have been quantitated in previous studies (41).

The blots were hybridized with ^{32}P-labeled PCR fragments containing either codons 342 to 410 of hDP-1 or codons 291 to 385 of hDP-2. In each case, the blots were then stripped and reprobed with PCR fragments encoding either the DNA binding domain of hDP-1 (codon 84 to 204) or the 5′ end of hDP-2 (codons 1 to 78). The specificity of these probes was confirmed by Southern blotting of the cDNA clones, and the hDP-1 and hDP-2 expression patterns were consistent, regardless of the region from which the probe was prepared.

Hybridization was performed at 42°C for 18 h in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2 PO_4, and 1 mM EDTA [pH 7.7])–10× Denhardt's solution–50% formamide–2% SDS–100 μg of denatured salmon sperm DNA per ml. The filters were washed twice at room temperature for 30 min in 2× SSC–0.1% SDS and then twice at 65°C in 0.1× SSC–0.1% SDS.

Antibodies. The recombinant polyhistidine–hDP-1 (1-410) protein encoded by pQE-hDP-1 and polyhistidine–hDP-2 (1-291) protein encoded by pQE-hDP-2 (1-291) were produced in *Escherichia coli* and purified through a nickel column under denaturing conditions as recommended by the manufacturer. The purified proteins were used to immunize BALB/c mice.

To prepare monoclonal antibodies against hDP-1, splenocytes from a mouse immunized with polyhistidine–hDP-1 (1-410) protein were fused to Sp2 myeloma cells 4 days after final boost. Positive tissue culture supernatants were identified by enzyme-linked immunosorbent assays against glutathione *S*-transferase (GST)–hDP-1 (1-410) protein. Positive clones were further tested for immunoprecipitation of [^{35}S]methionine-labeled hDP-1 protein synthesized in vitro, in E2F gel shift assays, and in Western blot (immunoblot) analyses. Eight cell lines (WTH1, -2, -3, -5, -6, -10, -16, and -24) were generated by single-cell cloning. All eight hDP-1 monoclonal antibodies are of the immunoglobulin G1 subclass and are specific in detecting hDP-1 in Western blot assays. Three of the eight monoclonal antibodies (WTH3, -16, and -24) recognize epitopes in the 1–204 region of hDP-1 and could specifically immunoprecipitate hDP-1 protein (data not shown). Four monoclonal antibodies (WTH1, -2, -5, and -10) recognize epitopes in the 204–410 region and are specific in supershifting hDP-1 in E2F gel shift assays (Fig. 6B).

Monoclonal antibodies against E2F-1 (KH20), pRB (XZ77), p107 (SD2, -4, -6, -9 and -15), simian virus 40 T antigen (Pab416 and Pab419), and hemagglutinin tag (12CA5) have been described previously (16, 18, 24, 29, 34). Mouse antisera raised against GST–hDP-1 (1-204), GST–E2F-2 (85-200), and GST–E2F-3 (132-230) fusion proteins have been described previously (29, 41).

Transient transfection. C33-A, a human cervical carcinoma cell line, was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Transient transfections were carried out as described previously (1, 22). For the gel shift assays, 90-mm-diameter dishes were transfected with 10 μg each of the E2F and DP expression plasmids and 10 μg of carrier DNA. For the chloramphenicol acetyltransferase (CAT) assays, cells were transfected with 4 μg of $E2F_4$-BCAT, 3 μg of pRSVluciferase, 13 μg of carrier DNA, the indicated amount of CMV expression plasmid, and pCMV-neo-Bam to give a total of 20.3 μg of DNA (29). Cells were harvested 24 to 36 h after transfection. CAT assays were performed as described previously (29, 41).

Gel shift assays. Whole-cell extracts were prepared as described previously (29). Five micrograms of protein was incubated for 10 min at room temperature in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–40 mM KCl–1 mM $MgCl_2$–0.5 mM EDTA–0.25% Nonidet P-40–5% glycerol–2 μg of denatured salmon sperm DNA. After addition of 0.1 ng of the ^{32}P-labeled E2F probe (ATTTAAGTTTCGCGCCCTTTCCAA), each reaction mixture was incubated for a further 15 min at room temperature. The reaction mixtures were then subjected to electrophoresis on a 4% native polyacrylamide gel at a constant 180 V at 4°C for 2.25 h in 0.25× Tris-borate-EDTA buffer. The gel was then dried and visualized by autoradiography.

To detect the presence of a specific protein, 1 μl of monoclonal tissue culture supernatant or polyclonal antiserum was added to the DNA binding reaction mixture prior to the addition of the proteins. To demonstrate the specificity of DNA binding, a 100-fold of excess of either wild-type (see above) or mutant (ATTTAAGTTTCGatCCCTTTCTCAA) E2F binding site was included in the binding reaction mixture.

To prepare for immunoprecipitation-deoxycholate-released proteins, 250 μg of ML-1 cell extract was incubated with 200 μl of monoclonal tissue culture supernatant in 1× DNA binding buffer (20 mM HEPES [pH 7.4], 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.25% Nonidet P-40, 5% glycerol). The antibody was precipitated by protein A-Sepharose and washed three times with 1× DNA binding buffer, and the associated proteins were released by the addition of 0.8% deoxycholate in 1× DNA binding buffer. Nonidet P-40 was added to give a final concentration of 1.5%, and the released proteins were tested in the DNA binding assay described above.

To prepare the DP-1-depleted extract, hDP-1 monoclonal antibodies (WTH16 and WTH24) and control monoclonal antibody Pab419 were coupled to protein G-Sepharose (25). ML-1 cell lysate (600 μg of proteins) was incubated for 1 h with either 200 μl of 10% protein G-Sepharose coupled with WTH16 and WTH24 or with same amount protein G-Sepharose coupled with Pab419. After a brief spin, the supernatants were used in gel shift assays.

Nucleotide sequence accession number. The accession number of the human DP-2 sequence is L40386.

RESULTS

Isolation of hDP-2 cDNA. The mouse DP-1 cDNA was isolated originally by Girling and colleagues (21). To isolate human homologs of DP-1, we screened a cDNA library prepared from the human pre-B-cell line Nalm 6 with a probe encoding the putative DNA binding domain of the mouse DP-1 protein. After hybridization and washing under low-stringency conditions, we identified five independent positive clones. The majority of these clones encoded human DP-1, as described previously (29). However, using this strategy, we also isolated an additional DP-1-related cDNA clone. This cDNA clone contained 1,266 nucleotides and encoded a 385-amino-acid protein with 67% identity with the human DP-1 protein (Fig. 1). This homology is highest in the region of DP-1 that contains the DNA binding and E2F dimerization domains. In addition, it extends downstream of these domains into a portion of hDP-1 whose function is unknown. Finally, although there is little sequence homology in this region, the C-terminal portions of hDP-1 and hDP-2 are unusually acidic in nature (13 and 11 acidic amino acids, respectively, out of the last 16

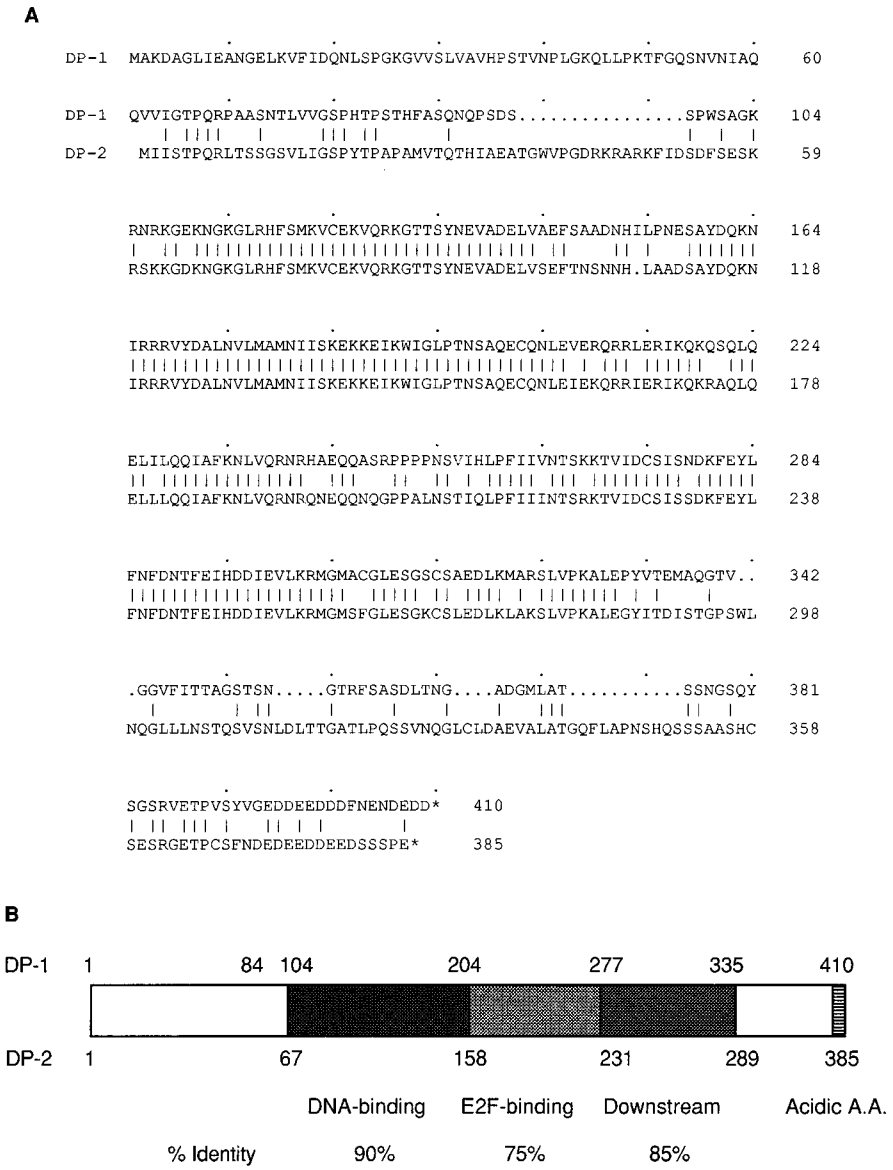

FIG. 1. Sequence comparison of hDP-1 and hDP-2. (A) Amino acid sequences of hDP-1 and hDP-2. Identical matches are indicated by vertical lines between the two sequences. (B) Schematic representation of hDP-1 and hDP-2 proteins. Percentages of amino acid (A.A.) identity between regions of the two proteins are indicated.

residues). Because of the high degree of sequence similarity, we have named this novel protein hDP-2.

We have compared the sequence of our hDP-2 cDNA clone with those contained in the Genome Sequence database. In addition to the mouse and human DP-1 clones, this search identified one other sequence that had been cloned previously as an mRNA expressed in human heart (accession number Z17843). Apart from one missing and one mismatch nucleotide, the sequence of this 300-bp fragment is identical to that of our hDP-2 clone.

Further attempts at low-stringency screening have yielded an additional 42 positive clones from different cDNA libraries. However, in each case these clones have been found to correspond to either hDP-1 or hDP-2. Since these clones were detected at relatively high frequency, we believe that hDP-1 and hDP-2 are both likely to make a significant contribution to the endogenous DP activity.

Our sequence begins 26 nucleotides upstream of the first methionine shown in Fig. 1 and does not contain a stop codon in this reading frame prior to the methionine. hDP-2 was independently isolated by Zhang and Chellappan (52a). Their sequence is identical to ours and extends a further 103 nucleotides upstream. This additional sequence includes an in-frame stop codon. Thus, the first methionine residue shown in Fig. 1 is thought to be the start site of translation.

Expression of hDP-1 and hDP-2 mRNAs. Initially, we sought to determine whether there was any variation in either the level or pattern of expression of hDP-1 and hDP-2. Specific DNA probes were prepared from the carboxy-terminal regions of hDP-1 and hDP-2 where there is little sequence homology and then used to probe Northern blots carrying either total RNA prepared from human cell lines or poly(A)$^+$ RNA from human tissues.

The hDP-1-specific probe detected a single 3-kb mRNA in

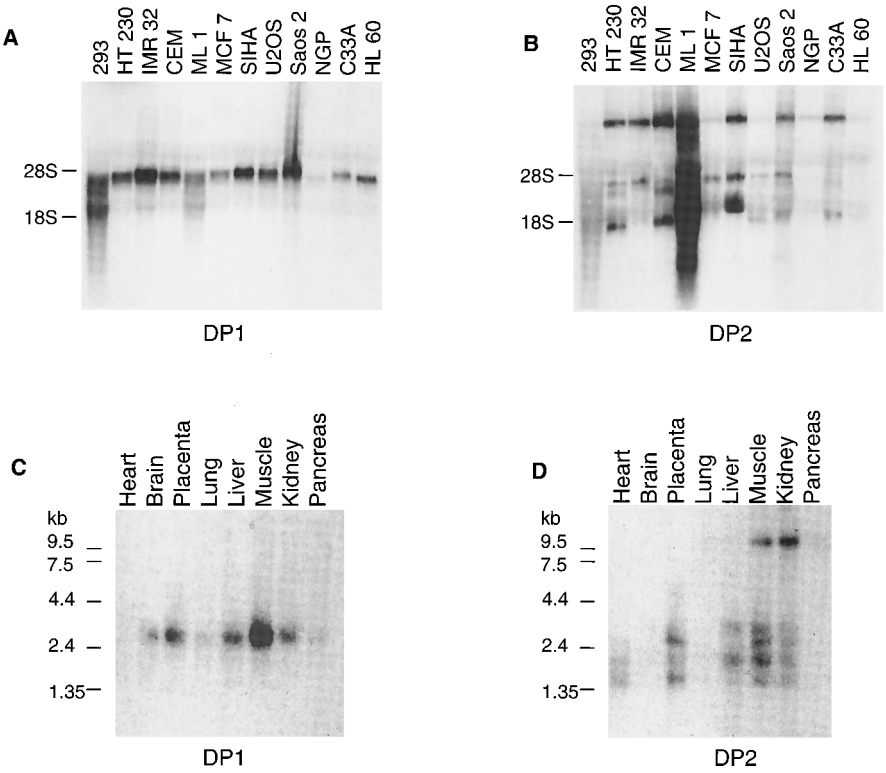

FIG. 2. Northern blot analysis of hDP-1 and hDP-2. Total cellular RNA from tissue culture cells (A and B) or poly(A)$^+$ RNA from normal human tissues (C and D) was hybridized with hDP-1 (A and C)- or hDP-2 (B and D)-specific DNA probes. Positions of 28S and 18S rRNAs and RNA markers are indicated. All films were exposed for 7 days with an intensifying screen at −80°C.

both the cell line and tissue Northern assays. hDP-1 expression was detected in all of the cell lines examined (Fig. 2A). Similarly, we were able to detect hDP-1 mRNA in the majority of human tissues tested (Fig. 2C). However, these showed considerable variation in the relative levels of expression. hDP-1 mRNA levels appeared to be considerably higher in muscle than in brain, placenta, and kidney. Expression was also detected in both lung and liver but at lower levels. Finally, hDP-1 mRNA was either absent or below the level of detection in human heart.

In contrast to the hDP-1-specific probe, the hDP-2-specific probe detected multiple transcripts (of approximately 1.5, 2, 2.5, 3, and 10 kb) in both cell lines and tissues (Fig. 2B and D). To verify this result, we prepared a second hDP-2-specific probe by using the 5′ end of the cDNA (encoding codons 1 to 78). This distinct probe recognized the identical pattern of mRNAs (data not shown). Moreover, since each probe hybridized to only one DNA band in Southern blots of genomic DNA cut with a variety of restriction enzymes (data not shown), the detection of multiple transcripts was unlikely to result from cross-hybridization to related genes. Therefore, we believe that these mRNAs are authentic hDP-2 transcripts that contain both the 5′ and 3′ coding sequences. To determine whether the hDP-2 coding region was alternatively spliced in any way, RNA from Saos-2 cells (kindly provided by J. Koh), which express at least four of the hDP-2 RNAs, was analyzed by reverse transcription-PCR. Using two primer sets (corresponding to codons 1 to 7 and 152 to 158 or codons 119 to 125 and nucleotides 38 to 48 downstream from the stop codon), we failed to generate any PCR products that were different in size from similar PCR products synthesized from our original cDNA clone. This suggests that the variation in transcript size is likely to arise from variations in the length of the 5′ and/or 3′ untranslated sequences. However, the exact origin of these multiple species of RNA has not been determined.

Unlike the case for hDP-1 mRNAs, the levels of hDP-2 mRNAs varied greatly between different cell lines (Fig. 2B). ML-1 cells expressed high levels of the hDP-2 mRNA, whereas 293, U20S, NGP, and HL60 cells had considerably lower levels of all of the transcripts. In human tissues, hDP-2 expression was detected in muscle, kidney, placenta, and liver, all of which also expressed hDP-1 at fairly high levels (Fig. 2D). hDP-2 transcripts were also detected in heart, a tissue in which we were unable to find any hDP-1 expression. In contrast, we failed to detect any expression of hDP-2 in brain, although this tissue expressed moderate levels of the hDP-1 mRNA. Finally, in addition to the apparent low level of hDP-1 expression, lung and pancreas did not give rise to any detectable hDP-2 mRNA. Together, these data suggest that there is considerable variation in the relative levels of hDP-1 and hDP-2 mRNA from different sources. hDP-2 transcripts were detected in a neuroblastoma cell line (IMR32) but not in brain tissue.

hDP-1 and hDP-2 cooperate with E2F-1, E2F-2, and E2F-3 in DNA binding. Heterodimerization between DP-1 and E2F-1 has been shown to potentiate their pRB binding, DNA binding, and transcriptional activation properties (4, 5, 29, 38). As a first step in comparing the biochemical properties of hDP-1 and hDP-2, we tested the abilities of these proteins to heterodimerize with E2F-1, E2F-2, or E2F-3. E2F and DP expression plasmids were transfected into C33-A cells in pairs, and the resulting cell lysates were tested for the ability to bind to the consensus E2F binding site in gel retardation assays.

As shown in Fig. 3, transfection with any of the individual expression plasmids failed to increase the DNA binding activ-

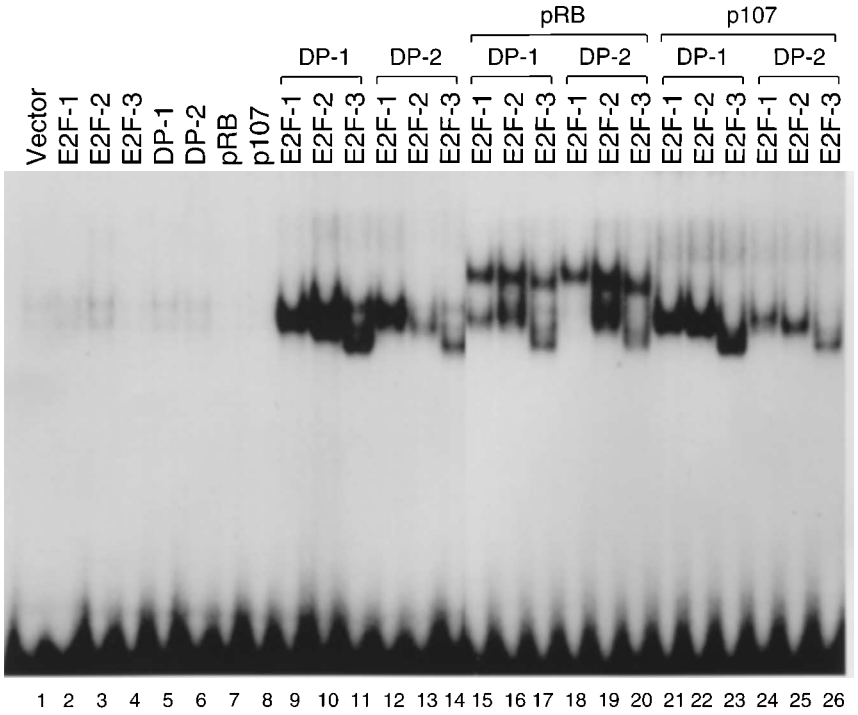

FIG. 3. DNA binding of E2F/DP heterodimers. Whole-cell extract (5 μg) of C33-A cells transfected with the indicated CMV expression plasmids were used in gel shift DNA binding assays with a ^{32}P-labeled oligonucleotide containing an E2F binding site. Lanes 1 to 14 and lanes 15 to 26 were taken from two gels that were run under identical conditions and exposed to the same film.

ity above that of the endogenous E2F (Fig. 3; compare lanes 2 to 8 with lane 1). Similarly, cotransfection of hDP-1 with hDP-2 or any two of the E2F molecules with each other did not form a functional DNA-binding complex (data not shown). However, when either hDP-1 or hDP-2 was cotransfected with any member of the E2F family of proteins, the DNA binding activity was significantly increased (Fig. 3, lanes 9 to 14). As expected, antibody supershifts confirm that these protein-DNA complexes contain both the E2F and DP subunits (see below).

hDP-1 and hDP-2 activate transcription with E2F-1, E2F-2, and E2F-3. Having determined that hDP-1 and hDP-2 can efficiently heterodimerize with all three E2Fs to increase their affinities for DNA, we wished to determine whether this dimerization also affected the abilities of these proteins to activate transcription. Different combinations of the E2F and DP expression vectors were transfected into C33-A cells along with a reporter construct ($E2F_4$-BCAT) in which transcription of the CAT gene is regulated by four E2F binding sites upstream of the TATA box of the adenovirus E1B gene (29).

As shown in Fig. 4, transfection of 100 ng of pCMV-hDP-1 or pCMV-hDP-2 generated 10- or 20-fold activation of transcription over the vector control plasmid. Similarly, 100 ng of either pCMV-E2F-1 (89-437), pCMV-E2F-2 (85-437), or pCMV-E2F-3 (132-425) alone generated 20-, 10-, and 11-fold activation, respectively. However, when 100 ng of the E2F expression plasmids was cotransfected with increasing amounts of the DP expression plasmid, synergistic activation was achieved, allowing a 70- to 400-fold induction (Fig. 4).

hDP-1 and hDP-2 associate with pRB but not p107 when complexed with E2F-1, E2F-2, or E2F-3. Previous studies have suggested that E2F-1, E2F-2, and E2F-3 all bind specifically to pRB and not p107 (28, 35, 36, 41, 50). Therefore, we wished to determine whether this specificity was maintained when these proteins were bound to either hDP-1 or hDP-2. As a first approach, we tested whether these DP/E2F heterodimers were able to associate with pRB or p107 when they were overexpressed by transient transfection (Fig. 3, lanes 15 to 26). The various combinations of E2F and DP expression plasmids were cotransfected into C33-A cells along with either the pCMV-pRB or pCMV-p107 expression construct, and the resulting cell extracts were then tested in gel retardation assays. Coexpression of pRB resulted in the appearance of a more slowly migrating E2F complex in band shift assays (Fig. 3, lanes 15 to 20), and antibody supershift confirmed the presence of pRB within this upper band (data not shown). In contrast, p107 did not appear to associate with any of the E2F/DP/DNA complexes (Fig. 3, lanes 21 to 26), although immunoblotting confirmed that pRB and p107 were expressed at similar levels in these experiments (53). This finding suggests that the specificity of E2F-1, E2F-2, and E2F-3 for pRB is maintained regardless of the nature of the DP subunit. The specificity of these interactions was supported by several of the experiments described below.

The transcriptional activity of the E2F/DP heterodimers is inhibited by pRB. The tumor suppression function of pRB is thought to arise, at least in part, from its ability to inhibit the transcriptional activity of E2F. Since pRB appears to bind to each of the six possible combinations of E2F-1, E2F-2, and E2F-3 with hDP-1 and hDP-2, we wished to determine whether this binding was sufficient to inhibit their transcriptional activities. Each combination of the E2F/DP expression vectors was transfected into the pRB-negative C33-A cells in the presence or absence of expression vectors containing either the wild-type *RB-1* gene (pCMV-pRB) or a naturally occurring mutant form of the *RB-1* gene (pCMV-pRBΔ22) that lacks exon 22 (33). In each case, the activities of these complexes were as-

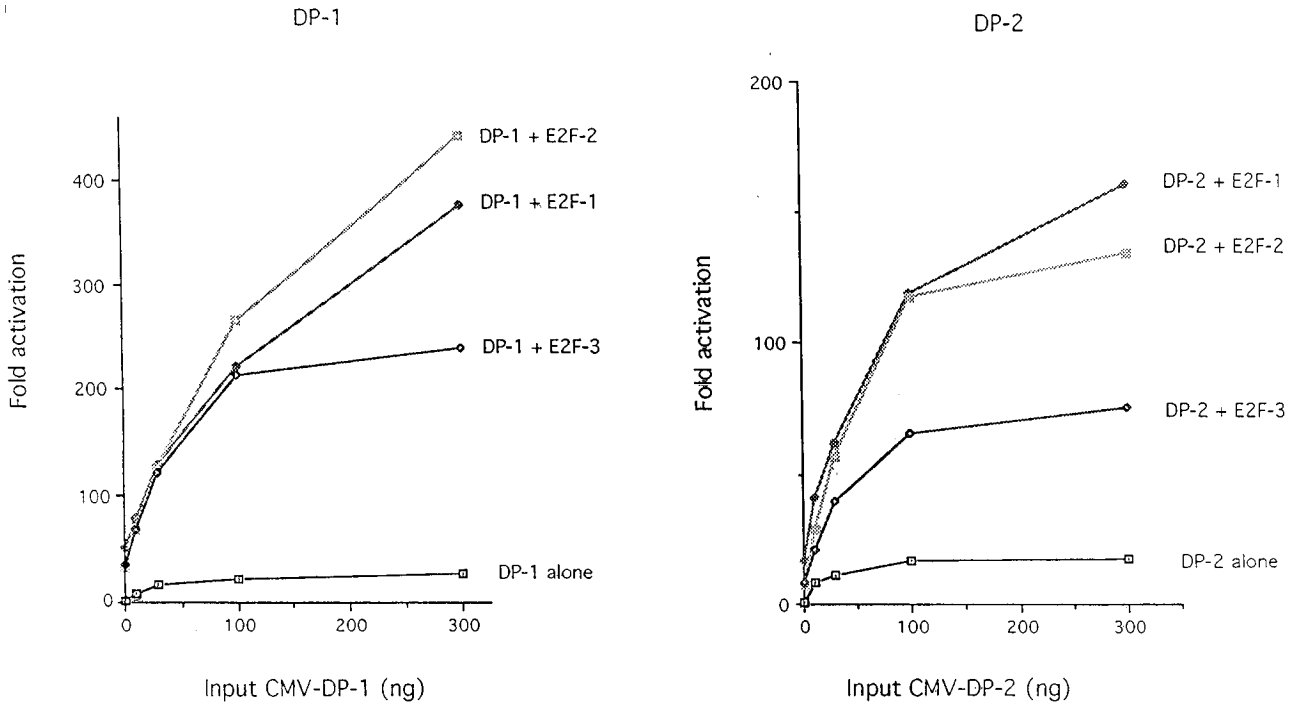

FIG. 4. Cooperation between E2Fs and DPs in transactivation. CAT assays were performed with C33-A cell lysates transfected with CMV expression plasmids in duplicate at 10, 30, 100, or 300 ng per dish. The reporter plasmid ($E2F_4$-BCAT) contains four E2F binding sites upstream of the E1B TATA box and CAT gene. Transfection efficiency was normalized by luciferase activity (15). CAT activity was calculated as fold of activation over control cells that were transfected with CMV vector alone. Each value is the average of the duplicate transfections.

sayed by their abilities to transactivate the $E2F_4$-BCAT reporter.

As before, the various E2F/DP combinations all increased transcription of this reporter by at least 80-fold (Fig. 5). This activation was not significantly altered by the presence of the mutant non-E2F-binding form of pRB. In contrast, the wild-type pRB was able to reduce the transcriptional activity of all of these complexes by at least 10-fold. This finding suggests that pRB is able to block transcriptional activation of all of these E2F/DP heterodimers.

hDP-1 and hDP-2 are associated with all of the endogenous E2F DNA binding activity in ML-1 cells. The studies described above demonstrate that hDP-1 and hDP-2 are capable of heterodimerizing with E2F-1, E2F-2, or E2F-3 to generate a functional E2F transcriptional activator. Since all of these assays are dependent upon the overexpression of the relevant proteins following transfection, they fail to demonstrate whether these individual complexes exist in vivo and what fraction they contribute to the endogenous E2F activity. To analyze complexes formed by the endogenous hDP-1 and hDP-2, we prepared mouse polyclonal and monoclonal antibodies that were specific for these proteins. Mice were immunized with either a GST fusion protein that contains the first 204 residues of hDP-1 (29) or polyhistidine-tagged hDP-1 or hDP-2 protein. Polyclonal antibodies against both hDP-1 and hDP-2 and monoclonal antibodies against hDP-1 were prepared.

Initially, we tested these antibodies for their specificity. hDP-1 or hDP-2 protein was synthesized from an in vitro transcription-translation reaction. In immunoprecipitations, the antibodies were specific for the appropriate immunogen and showed no evidence of cross-reaction to the other DP (data not shown). Next we tested both these antibodies and those raised against the E2F proteins (29, 41) for the ability to

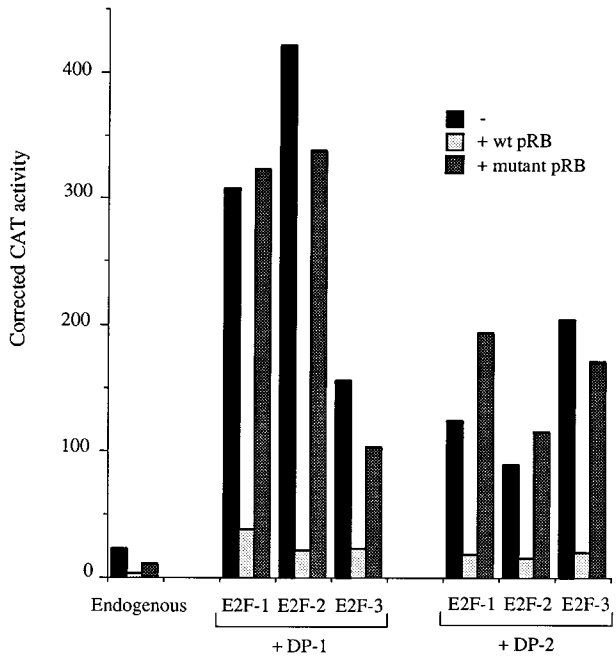

FIG. 5. pRB suppresses E2F/DP transactivation. CAT assays were performed with C33-A cells as described for Fig. 4. Each dish of cells was transfected with 30 ng of pCMV-E2F and pCMV-DP plus 300 ng of pCMV-pRB or pCMV-Δ22. CAT activity was calculated as arbitrary units of CAT counts divided by luciferase activity. Each value is the average of the duplicate transfections.

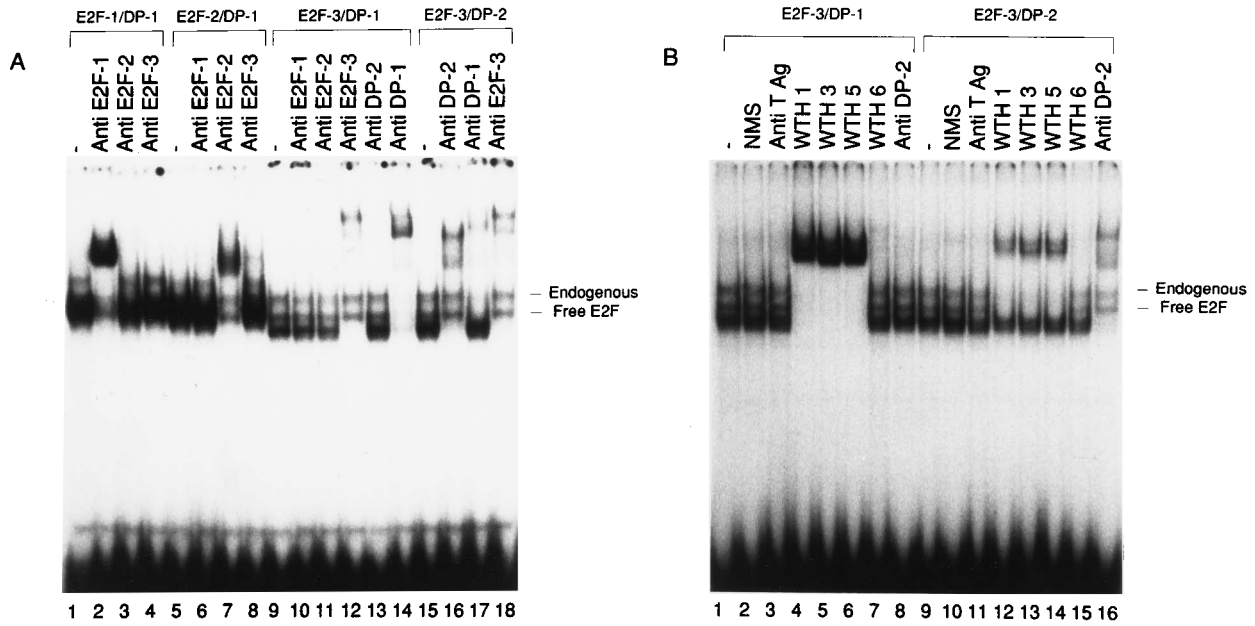

FIG. 6. Specificity of DP and E2F antibodies. (A) Three E2F and two DP antibodies were tested in gel shift assays with whole-cell extract made from cells cotransfected with E2F/DP genes as indicated. Anti-E2F-1 is a monoclonal antibody (KH20); 1 μl of this tissue culture supernatant of the hybridoma cells was used in the DNA binding reactions. Other antibodies were mouse polysera raised against recombinant proteins as described in Materials and Methods; 1 μl of each mouse serum was used per reaction. (B) hDP-1 monoclonal antibodies were tested in gel shift assays with cell extract made from cells cotransfected with E2F-3/hDP-1 (lanes 1 to 8) or E2F-3/hDP-2 (lanes 9 to 16). Control antibodies are normal mouse serum (NMS), Pab419 (anti-T antigen), and anti-hDP-2 mouse serum.

recognize native E2F and DP proteins in gel retardation assays (Fig. 6). In each case, these antibodies were mixed with the lysates of cells that had been transfected with combinations of E2F and DP, and the resultant complexes were analyzed by gel shift assays. As shown previously (Fig. 3), the E2F-1/DP and E2F-2/DP complexes migrated with the endogenous free E2F, whereas E2F-3, which is expressed as an amino-terminally truncated protein, migrated faster than the endogenous free E2F when bound by either DP subunit. The antibodies raised against E2F-1, E2F-2, and E2F-3 were able to shift their respective protein-DNA complexes specifically (Fig. 6A, lanes 2, 7, 12, and 18). hDP-1 antiserum and three of the four hDP-1 monoclonal antibodies used in this assay shifted the transfected hDP-1/E2F-3 complex, as well as the bulk of the endogenous free E2F, but did not alter the hDP-2/E2F-3 complex (Fig. 6A, lanes 14 and 17; Fig. 6B, lanes 4 to 7 and 12 to 15). This finding confirmed that the hDP-1 antibodies were specific and also indicated that the endogenous free E2F contains a significant amount of hDP-1. In the same experiment, hDP-2 antibody specifically shifted the hDP-2/E2F-3 complex but not the hDP-1/E2F-3 complex or the endogenous free E2F (Fig. 6A, lanes 13 and 16; Fig. 6B, lanes 8 and 16).

Having generated antibodies that are capable of specifically supershifting all of the relevant complexes, we were then able to use these antibodies to analyze the composition of the endogenous E2F complexes. ML-1 cells were chosen for these assays because they showed high levels of both hDP-1 and hDP-2 mRNAs, as judged by Northern blotting. ML-1 cell lysates consistently generated four distinct E2F/DNA bands in gel shift assay that correspond to the p107/cyclin A or E/cdk2/E2F complex, the pRB/E2F complex, and the two free E2F bands (Fig. 7, lane 1).

In earlier experiments, we noted that the anti-hDP-1 antibodies appeared to shift a large proportion of the free E2F as well as the majority of the pRB/E2F band and a portion of the

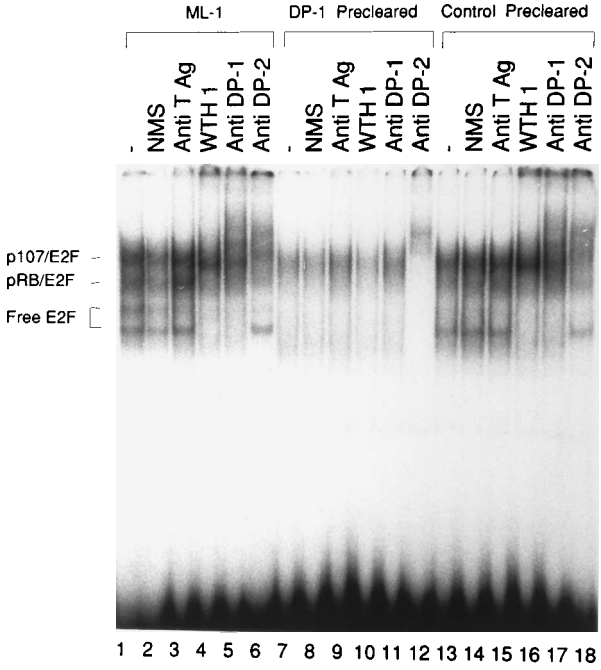

FIG. 7. hDP-1 and hDP-2 are associated with most E2F binding activity in vivo. ML-1 protein (6 μg) was used in E2F gel shift assays and subjected to supershift by normal mouse serum (NMS), anti-T-antigen (Anti T Ag) monoclonal antibody Pab419, anti-hDP-1 monoclonal antibody WTH1, anti-hDP-1 mouse serum, and anti-hDP-2 mouse serum (lanes 1 to 6). Equivalent ML-1 proteins depleted by hDP-1 antibodies (lane 7 to 12) or by Pab419 antibody (lane 13 to 18) were used in the same experiment. The p107/E2F and pRB/E2F complexes were identified by specific antibody supershift (data not shown).

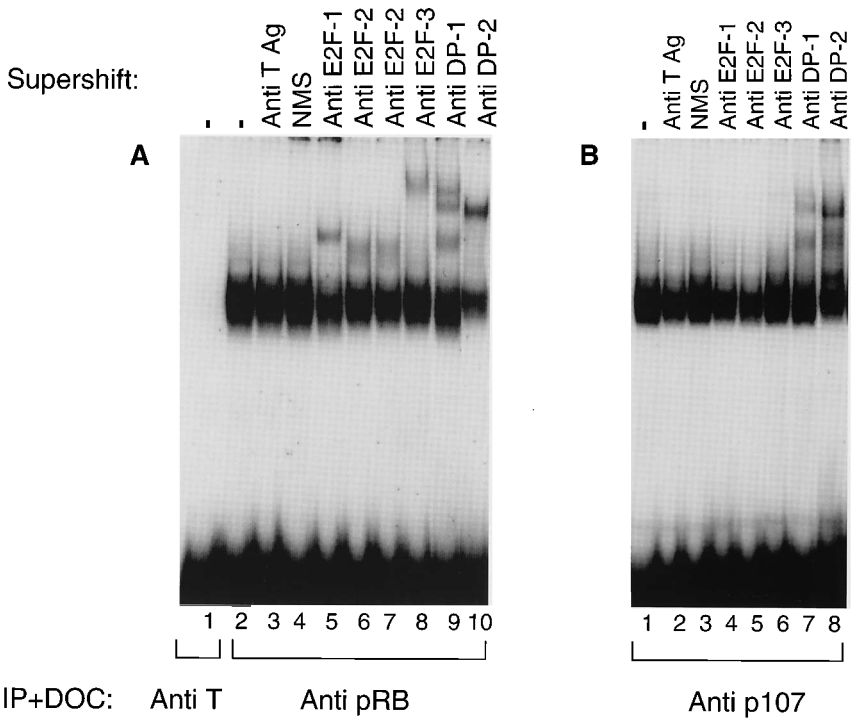

FIG. 8. Both hDP-1 and hDP-2 are associated with pRB and p107 in vivo. ML-1 cell extract was immunoprecipitated (IP) by anti-simian virus 40 T antigen (Anti T Ag) Pab416, anti-pRB XZ77, or anti-p107 antibodies (a combination of SD2, SD4, SD6, SD9, and SD15), and the immune complexes were incubated with 0.8% deoxycholate (DOC) to release associated E2F activity. The samples were then used in gel shift assays and were incubated with 1 μl of tissue culture supernatants (anti-T-antigen monoclonal antibody Pab419 and anti-E2F-1 monoclonal antibody KH20) or 1 μl of mouse sera (anti-E2F-2, anti-E2F-3, anti-hDP-1, and anti-hDP-2), except for lane 7 in panel A, in which case 2 μl of anti-E2F-2 mouse serum was used.

p107/E2F bands (Fig. 7, lanes 4 and 5). The addition of hDP-2 antibodies appeared to change the pattern of bands but did not clearly supershift a large portion of the available E2F (Fig. 7, lane 6). To make this distinction more clearly, we removed all of the hDP-1-immunoreactive proteins by preclearing the ML-1 lysates with hDP-1 monoclonal antibodies prior to the gel shift. This made the activities of the hDP-2 antibodies easier to see. As shown in Fig. 7 (lane 12), the anti-hDP-2 antibodies were able to supershift all remaining E2F activities. Together, these results suggest that hDP-1 and hDP-2 are associated with most or all the endogenous E2F DNA binding activity in these cells.

hDP-1 and hDP-2 are associated with both pRB and p107 in vivo. We and others have previously shown that E2F-1, E2F-2, and E2F-3 display a strong specificity for binding to pRB but not p107 in both in vivo and in vitro assays. This specificity is maintained when E2F-1, E2F-2, and E2F-3 are complexed with either hDP-1 or hDP-2 (see above). However, analysis of the endogenous E2F complexes suggests that these DP proteins might bind to both pRB and p107 in vivo. We therefore tested whether hDP-1 and hDP-2 could be detected in immunoprecipitates of pRB and p107. E2F/DP complexes that were associated with pRB and p107 were immunoprecipitated from ML-1 cell lysate by using monoclonal antibodies that are specific to either pRB or p107. The proteins were then released by detergent (deoxycholate) treatment and analyzed in band shift assays. As has been reported previously (9, 10, 39, 51), pRB and p107 immunoprecipitates contain a substantial quantity of E2F DNA binding activity (Fig. 8). Using the specific antibodies, we were then able to test the composition of the released E2F. Exactly as expected, E2F-1, E2F-2, and E2F-3 antibodies shifted the E2F/DNA complex associated with pRB (Fig. 8A, lanes 5 to 8) but not with p107 (Fig. 8B, lanes 4 to 6). In contrast, hDP-1 and hDP-2 antibodies shifted both pRB- and p107-bound E2F (Fig. 8A, lanes 8 and 9; Fig. 8B, lanes 7 and 8). This finding demonstrates that both hDP-1 and hDP-2 associate with pRB and p107 in vivo.

DISCUSSION

The cellular transcription factor E2F plays a key role in regulating the expression of genes that are required for cell cycle progression (46). This transcriptional activity appears to arise from the concerted action of a family of proteins that can be subdivided on the basis of their sequence homology into two distinct groups called E2F and DP. Recent studies suggest that formation of functional E2F is dependent upon the association of E2F and DP subunits (4, 29, 38). This heterodimerization appears to be essential for both high-affinity DNA binding and efficient transcriptional activation. However, all of these studies have relied on either in vitro binding or overexpression assays. We therefore know very little about the composition of the endogenous E2F or the relative functions of the individual E2F proteins in vivo.

Here we have shown that the endogenous DP activity is due to the action of at least two proteins, hDP-1 and hDP-2. These proteins are both capable of binding to E2F-1, E2F-2, and E2F-3 in vitro and in vivo, and this interaction leads to high-affinity DNA binding and increased transcriptional activation in vivo. Consistent with this observation, we can readily detect both hDP-1 and hDP-2 in association with pRB and p107 in vivo. Since we are unable to detect any association between p107 and E2F-1, E2F-2, or E2F-3, hDP-1 and hDP-2 also must heterodimerize with the p107-specific E2F(s). The recent clon-

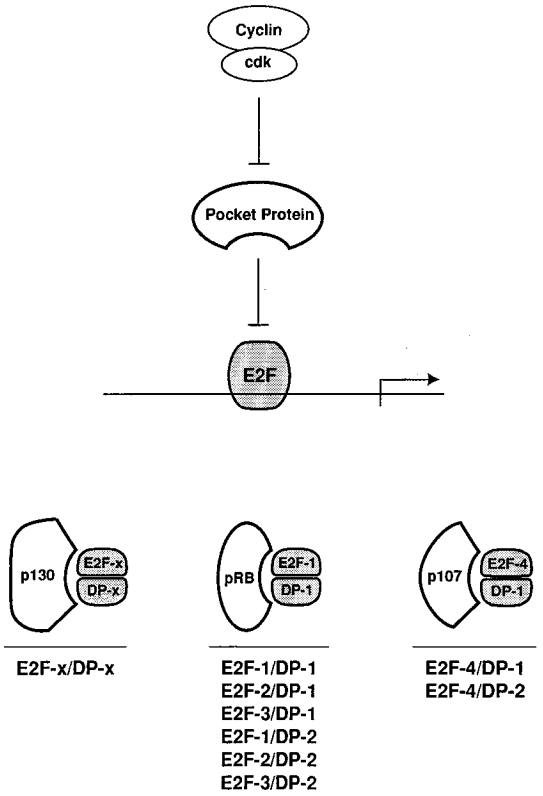

FIG. 9. Schematic illustration of components of E2F complexes described in this study or referenced in the text.

ing and characterization of E2F-4 has indicated that it is one of these p107-specific E2Fs (7, 19, 48a, 52a).

By using specific monoclonal and polyclonal antibodies, we have shown that all of the endogenous E2F DNA binding activity contains either hDP-1 or hDP-2. Although we cannot rule out the possibility that there are additional DP molecules which are immunologically similar to hDP-1 and hDP-2, this finding suggests that these proteins account for most or all of the observed DP activity in vivo in the cell lines that we have tested (ML-1, HL60, 293, CEM, HeLa, etc.). The data described above suggest that the DP subunits make little or no contribution to the specificity of pRB/p107 binding to the E2F complex but act as an essential partner for all of the E2F molecules.

We have found considerable variation in the expression patterns of the two hDP proteins. In a manner similar to that of many of the cell lines that we have analyzed, hDP-1 and hDP-2 are clearly coexpressed in muscle, kidney, placenta, and liver. In contrast, some tissues appear to express only hDP-1 (brain) or hDP-2 (heart). In light of the apparent differences in the contribution of these proteins to the free, transcriptionally active E2F, the levels and activities of the functional E2F may vary enormously in these different tissues.

It is attractive to think that each of the individual E2F/DP complexes is differentially regulated by its specific association with either pRB-, p107-, or p130-containing complexes in a manner that would give rise to the differential activation of different responsive genes. Structures of the complexes are shown in Fig. 9. We are currently trying to understand how the individual E2F/DP complexes contribute to the activation of known target genes.

Our E2F antibodies appear to be extremely efficient at supershifting complexes generated by transfecting cells with DP/E2F-1, DP/E2F-2, or DP/E2F-3. We were therefore surprised to find that, either separately or together, they were unable to supershift a large fraction of the free E2F complex (Fig. 6). There are several possibilities to explain this observation. First, the endogenous E2F proteins may be modified in some way so that they are no longer recognized by the antibodies. Second, the endogenous free E2F may be a mixture of many different E2Fs, each of which contributes only a small fraction of the total free E2F. If this is true, in order to be undetectable in the gel shift assay, E2F-1, E2F-2, and E2F-3 must contribute less than 10% of the total binding activity. Finally, it is possible that the endogenous free E2F consists of an E2F molecule or molecules distinct from E2F-1, E2F-2, and E2F-3. Since we believe that E2F-1, E2F-2, and E2F-3 account for all of the pRB-bound E2F activity, these observations pose interesting questions about the relative contributions that the pRB-, p107-, and p130-regulated E2Fs make to the pool of the free transcriptionally active E2F.

While this report was under review, the cloning and characterization of two new DP-1 related polypeptides from *Xenopus laevis* were reported (20). These clones were named xDP-1 and xDP-2. The xDP-1 and hDP-1 polypeptides are clearly related, having similar lengths and 86% amino acid identity. xDP-2 and hDP-2 are also related, again having similar lengths but with only 72% amino acid identity. The strong identity between xDP-1 and hDP-1 indicates that these genes are true homologs, and it seems unlikely that a more closely related *Xenopus* relative of human DP-1 will be found. While the xDP-2 and hDP-2 polypeptides are also clearly related and are more closely related to each other than to either xDP-1 or hDP1, we believe that their relationship as homologs should be regarded carefully. In this report we have used the designation hDP-2 rather than hDP3 for a number of reasons. First, the hDP-2 designation has been used in published reports for over a year, and we feel that this fact establishes some priority. Second, the hDP-2 cDNA and antibodies have been freely available from our laboratory for some time, and we know of several manuscripts in preparation in which this name is used. Finally, there are already several published references in which the DP-2 designation is used to describe the clone and protein characterized here. For these reasons, we believe that changing the name of hDP-2 would generate confusion in the literature. Nonetheless, researchers should be aware that the xDP-2 and hDP-2 cDNAs have not been formally shown to be homologs, and we recommend adhering closely to the use of the h and x prefixes for the human and *Xenopus* versions until more experimental evidence can be established to determine whether these genes are true homologs.

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