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E2F-4 Switches from p130 to p107 and pRB in Response to Cell Cycle Reentry

KEN MOBERG, MICHELLE A. STARZ, AND JACQUELINE A. LEES*

Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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The E2F transcription factor couples the coordinate expression of cell cycle proteins to their appropriate transition points. Its activity is controlled by the cell cycle regulators pRB, p107, and p130. These bind to E2F at defined but distinct stages of the cell cycle. Using specific antisera, we have identified the DP and E2F components of each of these species. Although present at very different levels, DP-1 and DP-2 are evenly distributed among each of these complexes. In contrast, the individual E2Fs have distinctly different binding profiles. Consistent with previous studies, E2F-1, E2F-2, and E2F-3 bind specifically to the retinoblastoma protein. In each case, their expression and DNA binding activity are restricted to post-G_1/S fractions. Surprisingly, E2F-1 and E2F-3 make unequal contributions to the pRB-associated and free E2F activity, suggesting that these proteins perform different cell cycle functions. Most significantly, this study showed E2F-4 accounts for the vast majority of the endogenous E2F activity. In arrested cells, E2F-4 is sequestered by the p130 protein. However, as the cells pass the G_1-to-S transition, the levels of pRB and p107 increase and E2F-4 now associates with both of these regulators. Despite this, a considerable amount of E2F-4 exists as free E2F. In G_1 cells, this accounts for almost all of the free activity. Once the cells enter S phase, free E2F is composed of an equal mixture of E2F-4 and E2F-1.

E2F is a cellular transcription factor that plays a pivotal role in the regulation of cell division. Its responsive genes are either strongly implicated in or directly linked to the induction of cellular proliferation. E2F appears to act to tether the expression of these genes to the point in the cell cycle at which their products are known to act. E2F activity is tightly regulated by the physical association of key components of the cell cycle machinery. The best characterized of these is a known tumor suppressor, the retinoblastoma protein (pRB). The retinoblastoma gene (*RB-1*) was originally identified and subsequently cloned by virtue of its absence in retinoblastomas (19, 20, 38). However, further studies have identified *RB-1* gene mutations in 30% of all human tumors, and in each case, these result in either loss or functional inactivation of the retinoblastoma protein (59). Moreover, the transforming potential of the small DNA tumor viruses correlates closely with their ability to bind, and presumably sequester, pRB (14).

In normal cells, the growth-inhibitory properties of pRB are inactivated by phosphorylation (12, 29). This modification appears to be mediated by one or more of the cell cycle-dependent kinases, cyclin D/cdk4 or cyclin D/cdk6 (mid-G_1 specific), cyclin E/cdk2 (G_1/S specific), or cyclin A/cdk2 (S specific) (16, 29, 31, 40, 43, 46). This provides a simple mechanism to ensure that the growth inhibitory properties of pRB become inactivated once cells are triggered to reenter the cell cycle.

In 1991, a number of laboratories demonstrated that the retinoblastoma protein binds to E2F in vivo (2, 5, 8). Although many other pRB-binding proteins have been reported, E2F has all of the predicted characteristics of a major pRB-target. It binds specifically to the unphosphorylated form of pRB (8, 47). This association does not affect its DNA-binding activity but is sufficient to inhibit its transcriptional properties in a manner that can be specifically relieved by pRB phosphorylation (12, 24, 27, 41). In addition, E2F is capable of acting as an oncoprotein. E2F overexpression will drive quiescent cells to reenter the cell cycle (34) and in some situations is sufficient to bring about transformation (57, 62). More often, E2F expression induces cells to undergo apoptosis, suggesting that E2F is sufficient to induce cell cycle reentry even in the presence of conflicting growth signals (45, 51, 55, 61). In each case, genetic analyses suggest that these effects are directly dependent upon the inappropriate activation of one or more target genes.

E2F is also regulated by two other proteins called p107 and p130 (7, 10, 11, 56). These were originally identified and cloned by virtue of their association with the adenovirus E1A protein, and both share considerable sequence homology with the retinoblastoma protein (17, 23, 42, 44). Unlike pRB, these proteins also associate stably with the cell cycle-dependent kinases, cyclin A/cdk2 and cyclin E/cdk2, and this association does not prevent them from binding to E2F (7, 10, 11, 15, 18, 39, 47). Several studies have shown that pRB, p107, and p130 each bind to E2F at defined but different stages of the cell cycle (9, 53, 56). In arrested cells, the predominant E2F species appears to be a kinase-deficient form of the p130/E2F complex (9, 10). In contrast, pRB and p107 do not associate with E2F until the cells reach the G_1-to-S transition (39, 53). In each case, formation of the p130-E2F-kinase or p107-E2F-kinase complexes coincides exactly with the timing of appearance of either cyclin E/cdk2 at the G_1-to-S transition or cyclin A/cdk2 during S (39, 56). Although p107 and p130 are not tumor suppressor proteins, they appear to repress E2F in a similar manner to pRB (28, 63) and are also targeted by the small DNA tumor viruses (14). These data therefore suggest that pRB, p130, and p107 act in concert to confine the activation of E2F, and therefore E2F-responsive genes, to precise stages of the cell cycle.

In the last 3 years, we and others have cloned at least seven genes that encode components of E2F (21, 22, 25, 32, 35, 41, 52, 60). These genes fall into two distinct classes, termed E2F

* Corresponding author. Mailing address: Center for Cancer Research, Massachusetts Institute of Technology, Bldg. E17-517B, 77 Massachusetts Ave., Cambridge, MA 02139. Phone: (617) 252-1972. Fax: (617) 253-9863.

and DP, that share little or no sequence homology. E2F and DP proteins heterodimerize in vivo, and this association appears to be essential for both high-affinity DNA binding and transcriptional activation (4, 26, 37, 60). Several groups have shown that the individual E2F/DP heterodimers differ in their regulatory protein binding properties. Although both subunits are required for high-affinity binding, this specificity appears to be determined by the E2F moiety. We have shown that complexes containing E2F-1, E2F-2, or E2F-3 bind specifically to pRB and not p107 in vivo (13, 41). In contrast, E2F-4 and E2F-5 complexes have been reported to bind specifically to p107 and/or p130 (6, 21, 28, 58). Since the regulatory proteins bind to E2F at defined but different stages of the cell cycle, this specificity is likely to play an essential role in determining both the timing and length of activation of the individual E2F/DP heterodimers. It has also been suggested that differences in either the intrinsic properties of the E2F/DP heterodimers and/or the modulating effects of the associated regulatory protein might cause the pRB- and p107-/p130-specific E2Fs to activate different classes of responsive genes. Despite this speculation, we actually know very little about the cell cycle regulation of the individual E2F/DP complexes. To help address this issue, we have used specific antisera to analyze the temporal expression patterns and binding properties of the individual family members. These studies have revealed striking differences in the cell cycle regulation of E2F-1, -2, -3, and -4.

MATERIALS AND METHODS

Plasmid construction. The cDNA clones encoding E2F-1, E2F-2, E2F-3, E2F-5, DP-1, and DP-2 have been described previously (25, 28, 41, 52, 60). The E2F-4 cDNA clone was isolated by screening a Nalm-6 human pre-B cell library with a degenerate probe derived from sequences encoding the C-terminal 15 amino acids of the minimal DNA-binding domains of E2F-1, E2F-2, and E2F-3 (our unpublished data). The sequence of this clone is identical to those described previously (6, 21). Plasmids encoding the 6× His-tagged E2F proteins were each constructed with the vector pQE8 (Qiagen). pQE8-E2F-2 (1–437) was generated by subcloning a *Bgl*II fragment encompassing the complete E2F-2 open reading frame from pCMV-E2F-2 into the *Bam*HI site of pQE8. Sequences encoding amino acids 1 to 244 of E2F-3 and 147 to 413 of E2F-4 were amplified by PCR with Vent polymerase (New England BioLabs) and the following primers: 31.6 (GATCGGATCCATGGTGAGAAAGGGAATCCAGCCC) plus 31.3 (GATC GGATCCTCAGCCCATCCATTGGACGTTG) (E2F-3) and TPF4 (GATCGG ATCCAGATGCTTTGCTGGAGATAC) plus 4.15 (GATCGGATCCTCAGA GGTTGAGAACAGGCAGATC) (E2F-4). The resulting products were digested with *Bam*HI and subcloned into pQE8 to generate pQE8-E2F-3 (1–244) and pQE8-E2F-4 (147–413) respectively. The eukaryotic expression vectors were constructed in pCMV-Neo-Bam (3). pCMV-E2F-1 (1–437), pCMV-E2F-2 (1–437), and pCMV-HA-hDP-1 (1–410) have been described previously (25, 41, 60). The E2F-5 eukaryotic expression vector pcDNA3-E2F5 has been described previously (52) and was a kind gift of C. Sardet. Sequences encompassing the complete E2F-3 open reading frame (425 amino acids) were prepared by PCR with Vent polymerase and the primers 31.6 plus 31.15 (CTAGGATCCGGATC GAAGGAGAGTTCACACGAAGC). The amplified fragment was digested with *Bam*HI and subcloned into pCMV-Neo-Bam to generate pCMV-E2F-3 (1–425). The complete E2F-4 open reading frame was excised from pBKS-E2F-4 as an *Eco*RV-*Bam*HI fragment and transferred to pCMV-Neo-Bam by using *Bam*HI linkers (New England BioLabs).

Polyclonal and monoclonal antibody production. 6× His-tagged E2F-2 (amino acids 1 to 437), E2F-3 (amino acids 1 to 244), and E2F-4 (amino acids 147 to 413) polypeptides were expressed in bacteria, purified over Ni^{2+}-nitrilotriacetic acid-agarose resin (Qiagen), and used to immunize female BALB/c mice. The resultant polyclonal antiserum was monitored by testing its ability to specifically supershift transfected E2F-2, E2F-3, or E2F-4/DP-1 complexes in gel shift assays or to recover transfected E2F-2, E2F-3, or E2F-4/DP-1 complexes in immunoprecipitation assays.

For both E2F-2 and E2F-3, as little as 1 μl of a 1:100 dilution of the polyclonal antiserum was sufficient to detect the correct E2F/DP complex. These mice were sacrificed, and the spleens were removed. Hybridomas were generated by polyethylene glycol-mediated fusion of the recovered splenocytes to the SP2/O cell line. At 8 days postfusion, the tissue culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for their ability to detect the relevant 6× His-tagged purified proteins. These positive supernatants were then screened for their ability to specifically supershift transfected E2F-2/DP-1 or E2F-3/DP-1 complexes in gel shift assays and to recover transfected E2F-2/DP-1 or E2F-3/DP-1 complexes in immunoprecipitation assays or Western blot (immunoblot) assays. The positive hybridoma cell lines were separated from other hybridomas by limiting dilution followed by single-cell cloning.

The monoclonal antibodies KH20 (anti-E2F-1), WTH1 (anti-DP-1), XZ55, XZ77, and XZ91 (anti-pRB), and SD2, SD6, SD9, and SD15 (anti-p107) (13) were a gift of Nick Dyson and Ed Harlow.

Tissue culture. The human cell lines ML-1 (premyeloid leukemia) and C33-A (cervical carcinoma) were grown under standard conditions in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections into C33-A cells were carried out by standard $CaPO_4$ precipitation methods (1). Dishes (diameter, 10 cm) of C33-A cells at 50% confluence were transfected with 10 μg of each of the E2F and DP expression plasmids plus 10 μg of carrier DNA. Cells were washed 16 h posttransfection and refed with fresh medium. They were harvested 24 h later, and whole-cell extracts were prepared as described below.

For T-cell preparations, buffy coats from human blood were obtained from the Massachusetts General Hospital Blood Bank. The mononuclear cell layer was isolated by centrifugation on a Ficoll-Paque cushion (Pharmacia) and then washed twice with phosphate-buffered saline. Mononuclear cells were then resuspended at 2.0×10^6 cells per ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 1 μg of phytohemagglutinin (PHA; Murex UK) per ml to stimulate T-cell proliferation. Cells were harvested at the indicated times, and the majority were used to prepare whole-cell extracts as described below. At each time point, thymidine incorporation was also assayed by incubating 1 ml of cultured T cells for 30 min in the presence of 10 μCi of [^{3}H]thymidine. After being washed, these cells were lysed in 0.3 N NaOH, spotted onto GF/C glass filters (Whatman), precipitated with trichloroacetic acid, and counted.

Gel shift assays. Whole-cell extracts were prepared from ML-1, C33-A, and T cells by standard procedures. Briefly, cells were lysed at 5×10^6/0.1 ml in 0.5 M KCl–35% glycerol–100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–5 mM $MgCl_2$–0.5 mM EDTA (pH 8.0)–5 mM NaF–1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol–5 μg of aprotinin per ml–5 μg of leupeptin per ml and then centrifuged at 4°C for 45 min at 40,000 rpm in a TLA45 rotor (Beckman) to remove cell debris. Supernatants were removed, and protein concentrations were determined (protein assay reagent [Bio-Rad]). Gel shift reactions were performed as follows. The initial DNA-binding mixtures contained 1 μg of sonicated salmon sperm DNA and 5 to 8 μg of whole-cell extract (as indicated in the figure legends) in 20 μl of 50 mM KCl–20 mM HEPES (pH 7.4)–1 mM $MgCl_2$–8.5% glycerol–1 mM EDTA (pH 8.0). For experiments involving T-cell extracts, 75 ng of double-stranded mutant E2F oligonucleotide (ATTTAAGTTTCGatCCCTTTCTCAA) was also included in the binding reaction to inhibit the formation of nonspecific DNA complexes. Samples were incubated for 10 min on ice prior to the addition of 1 ng of ^{32}P-end-labeled, double-stranded, wild-type E2F oligonucleotide (ATTTAAGT TTCGCGCCCTTTCCAA). Binding-reaction mixtures were incubated for a further 10 min on ice followed by 15 min at room temperature. The samples were then analyzed by electrophoresis at 180 V at 4°C in 4% polyacrylamide gels buffered with 0.25× TBE (22 mM Tris, 22 mM borate, 0.5 mM EDTA).

Deoxycholate (DOC)-treated gel shift reactions were performed in an identical manner except for the presence of 0.6% sodium deoxycholate (Sigma) in the initial binding mixture and the addition of Nonidet P-40 to 1% at the beginning of the room temperature incubation step. In both standard and DOC-treated gel shift reactions, competitions were carried out by the addition of 100 ng of unlabeled double-stranded wild-type or mutant E2F oligonucleotide prior to the addition of cell extract. Where indicated, hybridoma supernatant or diluted polyclonal antiserum was also added to the DNA-binding reaction mixtures prior to the addition of cell extract.

Immunoprecipitation-DOC release assays. Immunoprecipitation-DOC-released proteins were generated from the standard ML-1 or T-cell whole-cell extracts. Extracts (500 to 5,000 μg) were incubated on a rocking platform for 60 min at 4°C with 200 μl of the indicated hybridoma supernatants in 1× IP-DOC buffer (20 mM HEPES [pH 7.4], 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0]) containing 3 mg of bovine serum albumin per ml. Immune complexes were recovered on protein A-Sepharose beads (Pharmacia) and then washed three times in 1× IP-DOC buffer. The associated proteins were released by the addition of 10 μl of 0.72% sodium DOC in 1× IP-DOC buffer. Nonidet P-40 was added to a final concentration of 1.5%, and the supernatants were assayed in the gel shift protocol described above.

Western blots. Whole-cell extract was prepared from T cells as described above. The indicated amounts of cellular protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% polyacrylamide) and transferred to Immobilon-P membranes (Millipore) by electrophoresis for 16 h at 20 V in 390 mM glycine–50 mM Tris–20% methanol. The membranes were blocked for 2 h at room temperature in 1× TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.2% Tween 20) containing 5% dry milk and then immunoblotted with the indicated antibodies. Horseradish peroxidase-linked sheep anti-mouse immunoglobulin and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (Amersham) were used as secondary antibodies, and the blots were developed by using the enhanced chemiluminescence system (Amersham).

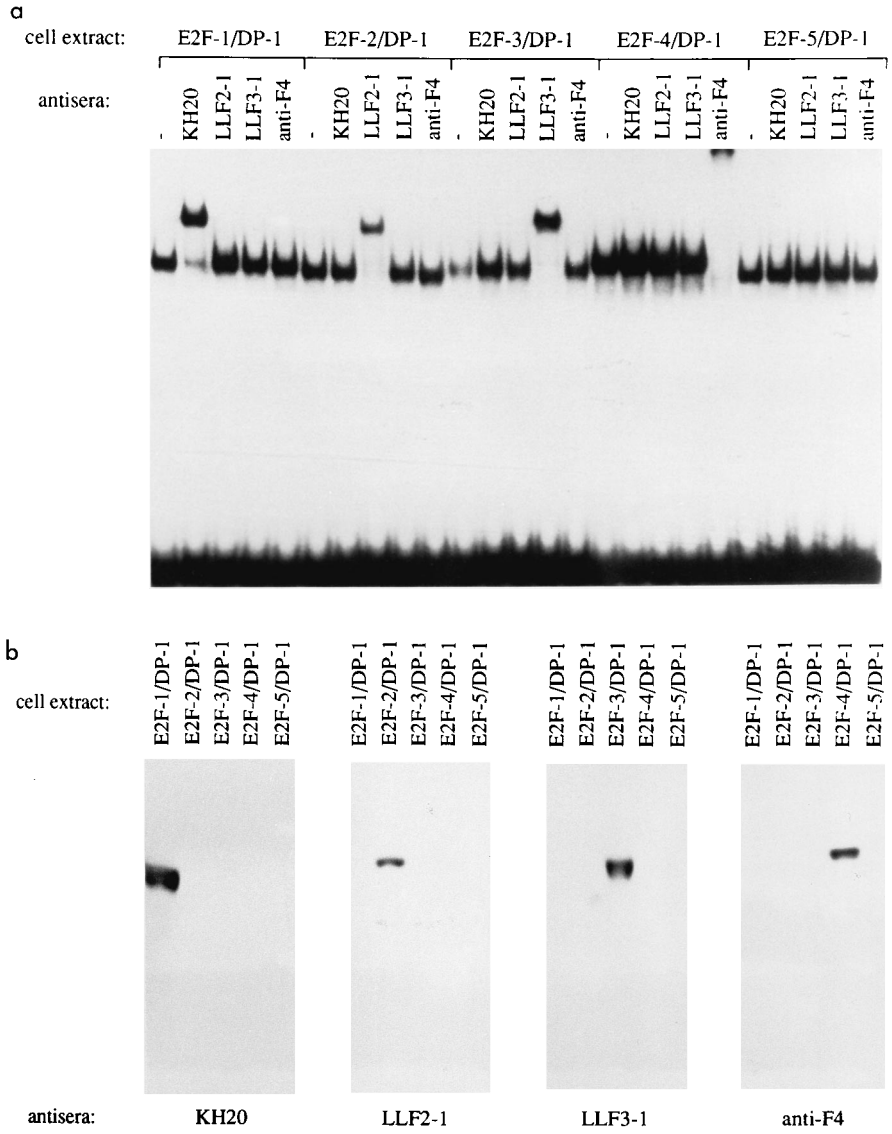

FIG. 1. Specificity of the E2F antisera. (a) Anti-E2F antibodies were tested in gel shift assays with whole-cell extracts (5 μg per lane) of C33-A cells that were transiently transfected with the indicated CMV-E2F and CMV-DP expression vectors. Where noted, gel shift reactions contained 1 μl of monoclonal antibody supernatant specific for E2F-1 (KH20), E2F-2 (LLF2-1), or E2F-3 (LLF3-1). The anti-E2F-4 lanes contain 1 μl of a 1:10 dilution of mouse polyclonal serum. (b) Anti-E2F antibodies were tested in a Western blot assay with whole-cell extract (20 μg per lane) derived from C33-A cells transiently transfected with the indicated CMV-E2F and CMV-DP expression vectors. Blots were probed with antisera specific to E2F-1 (KH20), E2F-2 (LLF2-1), E2F-3 (LLF3-1), or E2F-4 (Santa Cruz sc-512x) as indicated.

RESULTS

Generating antibodies that specifically recognize E2F-2, E2F-3, and E2F-4. In the last 3 years, it has become clear that the endogenous E2F activity arises from the concerted action of multiple E2F/DP heterodimers. Although there is considerable information about the basic properties of the E2F and DP proteins, we have yet to understand whether the individual E2F/DP complexes are required to mediate distinct functions in vivo or whether they are functionally redundant. To address this question, we need to be able to identify and monitor each of these complexes in vivo. To this end, BALB/c mice were immunized with purified, bacterially expressed proteins in which a tag of six histidines is fused to either full-length E2F-2, amino acids 1 to 244 of E2F-3, or amino acids 147 to 413 of E2F-4. The response of these mice was monitored by assaying successive bleeds for the ability to detect E2F/DP complexes in either gel shift or immunoprecipitation assays. For these experiments, C33-A cells were transiently transfected with pCMV-E2F-1, pCMV-E2F-2, pCMV-E2F-3, pCMV-E2F-4, or pcDNA-E2F-5 expression vectors in combination with pCMV-HA-DP-1. These cells were either labeled with [^{35}S]methionine for immunoprecipitation experiments or used to generate whole-cell lysates for gel retardation assays. The polyclonal antisera were tested for their ability to either supershift E2F/DP/DNA-bound complexes or coprecipitate both the labeled E2F and its associated DP protein. In each case, these antisera specifically recognized the relevant E2F-2-, E2F-3 (data not shown)-, or E2F-4 (Fig. 1a)-containing complexes in both gel retardation and immunoprecipitation assays.

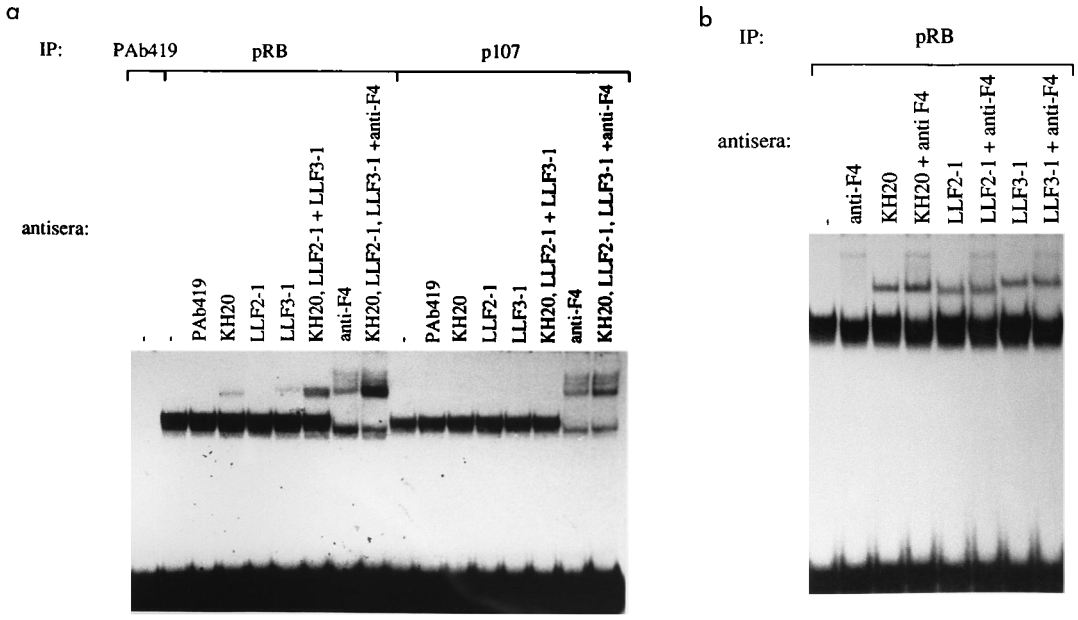

FIG. 2. E2F-1, -2, -3, and -4 associate with pRB in vivo. (a) Asynchronous ML-1 whole-cell extract (800 μg per lane) was immunoprecipitated by either PAb419 (anti-T antigen), XZ55 (anti-pRB), or SD6 (anti-p107), and the immune complexes were treated with 0.72% sodium DOC to release associated E2F activity. The supernatants were then analyzed in E2F gel shift assays in the presence of 1 μl of the anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), or anti-E2F-3 (LLF3-1) tissue culture supernatant or 1 μl of the diluted anti-E2F-4 polyclonal serum. Binding-reaction mixtures containing multiple antibodies included 1 μl of each of the indicated antibodies. (b) Asynchronous ML-1 whole-cell extract (5,000 μg per lane) was immunoprecipitated with XZ55 (anti-pRB), and the immune complexes were released as described for panel a. Supernatants were analyzed in E2F gel shift assays in the presence of 1 μl of anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), or anti-E2F-3 (LLF3-1) or 1 μl of the diluted anti-E2F-4 polyclonal sera. Reaction mixtures containing multiple antibodies included 1 μl of each of the indicated antibodies.

For E2F-2 and E2F-3, the polyclonal antisera were of sufficiently high titer (a strong positive reaction with 1 μl of a 1/100 dilution) to conduct hybridoma fusions. The resultant lines were initially screened by ELISA for their ability to secrete antibodies that recognized the relevant E2F immunogen. These primary screens identified multiple wells that produced either anti-E2F-2 or anti-E2F-3 antibodies. Subsequently, these ELISA-positive tissue culture supernatants were tested for their ability to specifically recognize the relevant E2F/DP complexes in either gel shift or immunoprecipitation assays. Exactly as described above, these experiments were conducted with extracts from C33-A cells transiently transfected with E2F and DP expression vectors. Most of the ELISA-positive E2F-2 and E2F-3 antisera specifically recognized either E2F-2 or E2F-3 in immunoprecipitation and/or gel retardation assays (data not shown). Figure 1 confirms that the monoclonal antibodies used in this study (KH20 [anti-E2F-1], LLF2-1 [anti-E2F-2], and LLF3-1 [anti-E2F-3]) specifically recognize their respective E2Fs in both gel retardation assays (Fig. 1a) and Western blots (Fig. 1b). In the former case, neither the binding capacity nor the specificity of these antibodies was affected when DP-1 was replaced by DP-2 or when the regulatory proteins, pRB or p107, were individually included in the DNA-bound complexes (data not shown).

E2F-4 associates with pRB in vivo. To date, five distinct members of the E2F gene family have been identified (6, 21, 25, 32, 35, 41, 52). Although these proteins share similar DNA-binding and transcriptional properties, numerous studies have reported distinct differences in their specificity of regulatory protein binding. E2F-1, E2F-2, and E2F-3 each bind specifically to the retinoblastoma protein in vivo (13, 41). In contrast, E2F-4 interacts in vivo with both p107 and p130 whereas E2F-5 associates specifically with the G_0-regulatory protein, p130 (6, 21, 28, 58). Since there is extensive evidence that pRB, but not p107 and p130, is a tumor suppressor, it is essential that we understand the functional significance of the different binding specificities of the individual E2F/DP complexes.

Initially, we tested our antibodies for their ability to detect the individual E2F/DP complexes in vivo. For these experiments, pRB or p107 was immunoprecipitated from asynchronous ML-1 cells and associated E2F activity was released by treating the precipitates with DOC and then detected in E2F gel shift assays in the absence or presence of our anti-E2F antibodies (Fig. 2a). While the control (anti-large T-antigen) monoclonal antibody failed to bring down any proteins capable of binding the E2F probe, a considerable amount of E2F activity was recovered from both the pRB- and p107-immunoprecipitates. In both cases, we were able to resolve this E2F DNA-binding activity into a series of bands, all of which can be specifically inhibited by a 50-fold molar excess of wild-type but not mutant unlabeled E2F probe (data not shown). Consistent with our previous findings, the monoclonal antibodies against E2F-1 (KH20), E2F-2 (LLF2-1), and E2F-3 (LLF3-1) supershifted E2F-DNA binding activity from DOC-treated pRB but not p107 immunoprecipitates. E2F-1 and E2F-3 were easily detectable in these assays and seemed to be present at roughly similar levels. In contrast, the anti-E2F-2 supershift was extremely weak, although it was clearly detectable when the input level of pRB-associated E2F activity was increased (Fig. 2b). Since several other E2F-2 monoclonal antibodies detected similarly low levels (data not shown), these data suggest that E2F-2 comprises a small proportion of the pRB-associated E2F activity.

To date, E2F-1, E2F-2, and E2F-3 are the only known pRB-associated E2Fs. To determine whether these three proteins are sufficient to account for all of the pRB-associated E2F activity, we tested how a mixture of these three antibodies affected the pRB-associated E2F activity. Surprisingly, the an-

tibody cocktail supershifted less than half of the pRB-associated E2F activity (Fig. 2a). Moreover, this supershift had no detectable effect on the most abundant, upper species of E2F activity. Since control experiments (with transfected E2F/DP complexes) were able to confirm that all three antibodies were present in at least 10-fold excess (data not shown), these data indicate that pRB must associate with one or more additional E2F species in vivo.

In an effort to identify this additional activity, we tested the effect of the E2F-4 polyclonal antiserum in this assay. Consistent with previous studies, the anti-E2F-4 serum supershifted a significant proportion of the p107-associated E2F activity (lane 16), confirming that we can detect the association between E2F-4 and p107 in vivo. In addition, this antiserum specifically supershifted a large proportion of the E2F activity released from the pRB immunoprecipitates (Fig. 2a). In this case, the presence of this antibody selectively depleted the prominent upper complex that had been unaffected by the E2F-1-, E2F-2-, and E2F-3-specific monoclonal antibodies (Fig. 2a). To determine whether E2F-1, E2F-2, E2F-3, and E2F-4 were sufficient to account for all of the pRB-associated E2F, we included a mixture of all four antibodies in the DNA-binding reaction mixture (Fig. 2a). Together, these reagents recognized all of the upper complex as well as a portion of the lower complexes. However, a significant fraction of the lower species was not supershifted by the antibodies, suggesting that they represented novel E2F species. Since forms of equal mobility also persisted when the p107-associated E2F preparation was treated with the E2F-1, E2F-2, E2F-3, and E2F-4 antibody cocktail, these novel E2Fs appeared to associate with both pRB and p107 in a manner similar to E2F-4. Obviously, E2F-5 is a good candidate to be one or both of these species, and we are currently raising E2F-5-specific antisera to determine whether this accounts for the remaining E2F activity or whether the cell contains other, as yet unidentified E2F species.

Although E2F-4 is generally considered to be a p107/p130-associated protein, these data suggest that it also forms a significant proportion of the pRB-associated E2F activity. Consistent with this finding, Vairo et al. have previously reported that their anti-E2F-4 polyclonal antiserum also disrupts a portion of the pRB-associated E2F activity (58). However, both of these studies have been conducted with polyclonal antisera. We therefore wished to confirm that our E2F-4 antiserum was unable to supershift the endogenous E2F-1, E2F-2, or E2F-3 species. Exactly as described above, we tested the different combinations of E2F antisera for their ability to supershift E2F activity that had been released from pRB immunoprecipitates. However, in this experiment, we increased the levels of pRB-released E2F activity by over fivefold (Fig. 2b). Although this reduced our ability to detect discrete E2F complexes, we were now able to supershift significant levels of E2F-1, E2F-2, or E2F-3 from the pRB precipitates. Coaddition of the E2F-4 antiserum had no effect upon these E2F-1, E2F-2, or E2F-3 supershifts (Fig. 2b), confirming that our E2F-4 polyclonal antibody did not cross-react with any of these pRB-specific E2Fs. On the basis of this and other studies, we conclude that E2F-4 makes up a significant proportion of the pRB-associated E2F activity.

The E2Fs are differentially expressed in a cell cycle-dependent manner. Previous studies have shown that pRB, p107, and p130 bind to E2F at defined but distinct stages of the cell cycle. Having confirmed that our antisera effectively recognize endogenous E2F activity, we were able to compare the cell cycle regulation of the individual E2F/DP complexes with that of the regulatory proteins. Human T cells were selected for these experiments for three reasons. First, these cells reenter the cell cycle in a highly synchronous manner and the staging is maintained throughout the first round of division. Second, the timing of pRB, p107, and p130 complex formation in this system has already been well documented (9). Finally, because these are primary cells, their cell cycle regulation should most closely parallel that found in vivo.

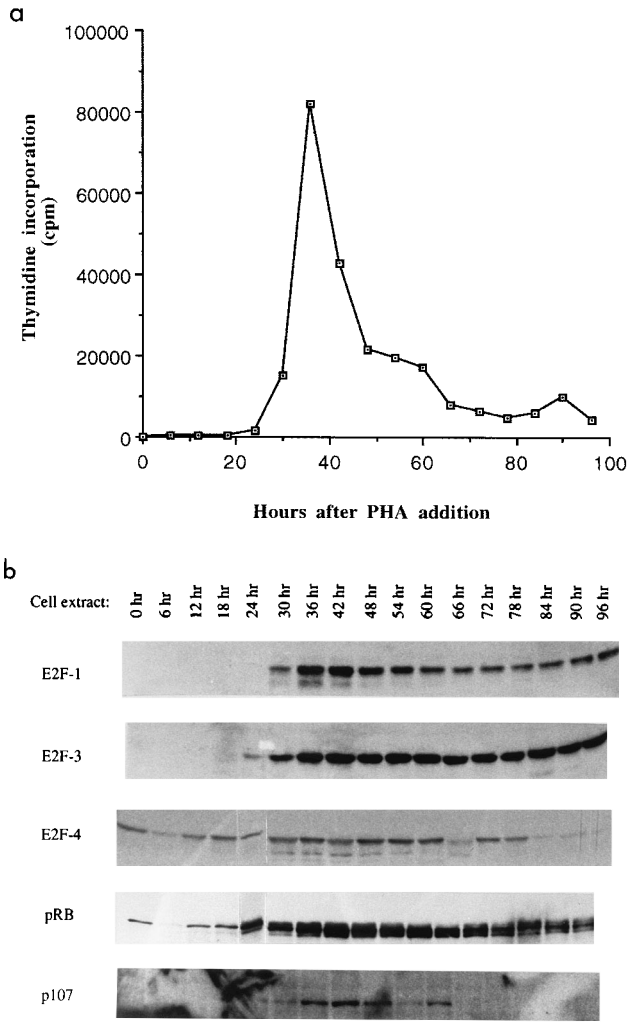

FIG. 3. Cell cycle expression of the E2F proteins. (a) [^{3}H]thymidine incorporation into T cells at the indicated times following addition of PHA. (b) The level of E2F-1, E2F-3, E2F-4, pRB, and p107 in each of the T-cell fractions was assessed by Western blotting. Each lane contained 15 μg of whole-cell extract. The filters were probed with a 1/5 dilution of the E2F-1 (KH20), E2F-3 (LLF3-1), pRB (cocktail of XZ77 and XZ91), or p107 (cocktail of SD2, SD6, SD9, and SD15) tissue culture supernatant or a 1:1,000 dilution of the anti-E2F-4 mouse polyclonal antiserum, Santa Cruz sc-512x.

Human T cells were isolated from peripheral blood and stimulated to proliferate by the addition of PHA. At each time point, the level of DNA replication was monitored by assessing the uptake of tritiated thymidine (Fig. 3a). In this experiment, [^{3}H]thymidine incorporation peaked 36 h poststimulation and declined to near basal levels by the 66-h time point. The sharp increase between the 30- and 36-h time points indicates that the majority of the cells traversed the G_1-to-S transition in this time interval; the narrowness of the peak and the rapid decline to near basal levels suggested that the cells responded to the proliferative signal in a synchronous manner.

Expression of the E2F proteins was assessed by Western blot analysis of whole-cell extracts from each of the time points (Fig. 3b). In a similar manner to the ML-1 cells, E2F-2 was expressed at extremely low levels in the T cells (see the discussion of the gel shift assays [below]) and was undetectable in the Western blot experiments. In contrast, E2F-1, E2F-3, and E2F-4 were all present at reasonable levels in the T-cell fractions. E2F-1 was first detected at 30 h, coincident with the beginning of S phase. At this time point, it existed as a clear doublet. The level of the more prominent, upper species reached a maximum at 42 h and then declined to a moderate level that was maintained throughout the remainder of the cell cycle. In contrast, the smaller species was detected only in fractions undergoing DNA replication. Both the timing and the rapid induction of E2F-1 protein synthesis were consistent with the known E2F-dependent, G_1/S-specific transcriptional activation of this gene (30, 33, 48).

In contrast to E2F-1, our studies show that the E2F-3 mRNA is present in both the arrested and PHA-stimulated T-cell fractions at similar low levels (data not shown). We were therefore surprised to find that expression of the E2F-3 protein required cell cycle reentry. In fact, E2F-3 was not detected until 24 h after PHA stimulation, just 6 h prior to the initiation of DNA synthesis and the induction of E2F-1 expression. Following its appearance, the level of E2F-3 rose quickly to reach a maximum at 36 h that was maintained throughout the remainder of the cell cycle. Although the profile of E2F-3 expression closely mirrored that of E2F-1, this protein was detected as a single species throughout the time course of the experiment.

Consistent with previous studies (58), the E2F-4 protein was detected at significant levels at all the time points. In G_0 cells, E2F-4 was present as a single species whose levels increased steadily as the cells progressed through G_1 and S phases and then returned to the G_0 levels toward the end of the cell cycle. At 30 h, we first detected two additional E2F-4 species, whose presence appeared to be specific to fractions that were undergoing DNA synthesis. In fact, the appearance of these species was entirely coincident with the appearance of the minor form of E2F-1.

For comparison, we also screened the T-cell fractions for the presence of the regulatory proteins, pRB and p107. As previously described (59), pRB was detected as a single species in G_0 cells that corresponds to the underphosphorylated form (Fig. 3b). Once the cells reentered the cell cycle, the overall level of pRB increased and slower-migrating species were also detected. These first appeared 24 h after PHA addition and persisted through the remainder of the cell cycle, entirely consistent with the known cell cycle-dependent phosphorylation of pRB that is initiated at the G_1-to-S transition.

The p107 protein was first detected 24 h after PHA addition. From this time on, its levels continued to rise, reached a peak at 42 h, and then dropped to undetectable levels at 66 h. Since the level of this species was only just within the detection limits of the experiments, we cannot conclude that p107 was absent from the early and late time points. However, these experiments clearly show that expression of the p107 protein is induced upon cell cycle reentry. The concomitant increase in the levels of the E2Fs and their regulatory proteins was consistent with recent reports that the *RB-1* and *p107* genes both contain E2F-responsive elements within their promoters (54, 64).

Cell cycle-dependent E2F DNA-binding activities. The Western blotting data indicated that E2F-1, E2F-3, and E2F-4 were each expressed at defined but different points of the cell cycle. However, since monomeric E2Fs are unable to bind to DNA in the absence of an associated DP protein, it was important to determine when each of the E2F proteins became competent to bind to DNA. To abolish any possible influence of the regulatory proteins, the T-cell extracts were treated with DOC to dissociate the higher-order complexes, and the total "free" E2F activity was assayed by gel shift (Fig. 4). E2F activity was detected at all of the time points, but the levels increased significantly as the cells reentered the cell cycle to reach a maximum in the S-phase fractions. Wild-type but not mutant E2F oligonucleotides inhibited all of the upper species (labeled as free E2F in Fig. 4) but failed to alter the strong lower band, indicating that this corresponds to a nonspecific binding activity (data not shown).

As expected, addition of a control monoclonal antibody (PAb419) had no effect on the DNA-binding properties of any of these bands. In contrast, the E2F-1-, E2F-2-, E2F-3-, or E2F-4-specific antibodies were all capable of supershifting a proportion of the free E2F activity from one or more of the T-cell fractions. The three pRB-specific E2Fs, E2F-1, E2F-2, and E2F-3, were each detected in a cell cycle-dependent manner. E2F-1 activity was first supershifted in the 30-h extract, and its levels peaked in the 36- and 42-h samples and then declined to a low level at the remainder of the time points. E2F-2 DNA-binding activity was also detected in this assay but at extremely low levels, beginning at the 36-h time point and declining to almost undetectable levels at the later time points. Finally, an LLF3-1-specific supershift was first identified 24 h after PHA addition. Although this activity did increase during S phase, E2F-3 remained clearly detectable in all subsequent fractions.

In each case, the timing and level of these DNA-bound complexes were nearly identical to the timing and level of the individual E2Fs detected in our Western blotting experiments. (The apparent discrepancy in our ability to detect E2F-2 in the DNA-binding assay but not the Western blot simply reflects the greater sensitivity of the gel shift assay and the extremely low levels of this protein and is entirely consistent with our analysis of other cell lines, including ML-1 and C33-A [data not shown].) In light of these findings, these experiments strongly suggest that there is little delay between the synthesis of the individual E2Fs and their ability to associate with a DP protein to form competent DNA-binding complexes.

Consistent with its expression pattern, E2F-4 dependent DNA-binding activity was detected at all the time points (Fig. 4). In the early stages of the cell cycle, E2F-4 made up almost all of the total E2F activity. However, as the cells progressed through the cell cycle, significant levels of nonsupershifted bands appeared at those time points when E2F-1, E2F-2, and/or E2F-3 activity had previously been detected. As in our analysis of ML-1 cells (Fig. 2), E2F-4 seemed to correspond to the major, higher-mobility species while E2F-1, E2F-2, and E2F-3 corresponded to the collection of bands migrating immediately below.

Our preliminary experiments with ML-1 cells indicated that E2F-1, -2, -3, and -4 were not sufficient to account for all of the endogenous E2F activity (Fig. 2). We were therefore interested to determine whether the unidentified E2Fs are present at all stages of the cell cycle, like E2F-4, or whether their presence is also cell cycle regulated in a similar manner to the pRB-specific E2Fs. To this end, the gel shift reactions were performed in the presence of a cocktail of all three monoclonal reagents and the polyclonal serum (Fig. 4). Consistent with the results in ML-1 cells, supershifting E2F-1, -2, -3, and -4 revealed the presence of two distinct bands that were unaffected by the presence of these antisera. One of these novel T-cell E2F activities is present at all the time points, suggesting that

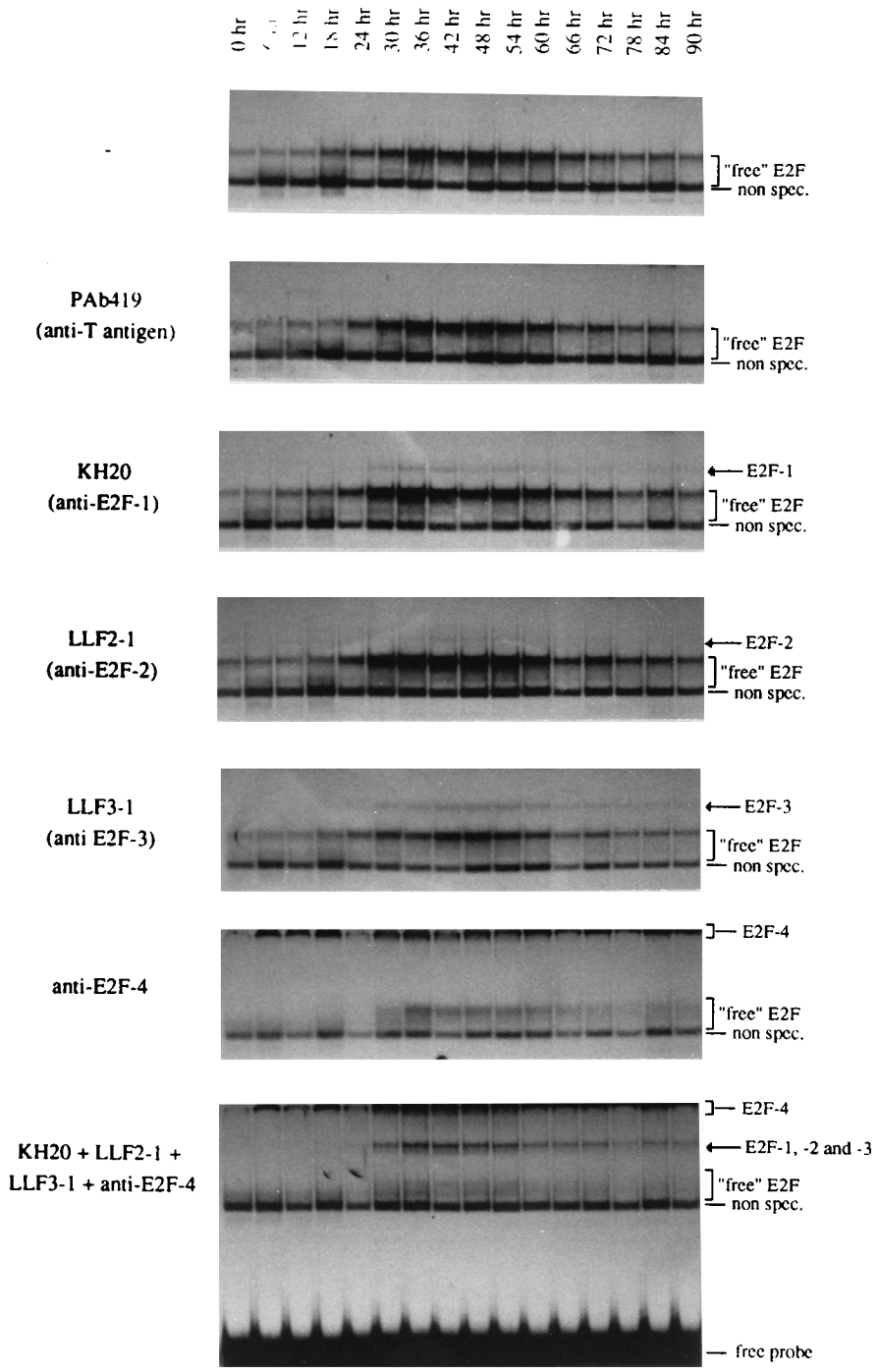

FIG. 4. Cell cycle-dependent E2F DNA-binding activity. The T-cell whole-cell extracts (6 μg per lane) were preincubated with 0.6% sodium DOC to dissociate higher-order complexes. These samples were then analyzed in E2F gel shift assays in the presence of 2 μl of tissue culture supernatant (PAb419 [anti-T antigen], KH20 [anti-E2F-1], LLF2-1 [anti-E2F-2], or LLF3-1 [anti-E2F-3]) and/or 1 μl of the diluted polyclonal anti-E2F-4 antiserum as indicated. Brackets denote positions of the "free" E2F, as determined by competition with unlabeled wild-type E2F-binding site. The noninhibitable activity is also indicated. In each case, the position of the supershifted complex(es) is marked with an arrow. All gels were exposed for 4 days, except the anti-E2F-2 gel, which was exposed for 8 days.

its expression is regulated in a similar manner to E2F-4; it may therefore correspond to the recently described p130-specific E2F, E2F-5. The second, more slowly migrating activity is completely absent in G_0/G_1 extracts, appears strongest in the S-phase extracts (30 through 54 h), and declines to low levels at the remainder of the time points in a similar manner to the three pRB-specific E2Fs.

E2F-1, E2F-3, and E2F-4 associate with their regulatory protein(s) in a cell cycle-dependent manner. Having determined the timing of synthesis and DNA-binding activity of the individual E2Fs, it was important to establish whether these DNA-binding complexes were free to activate transcription or whether their activity was blocked by the association of the regulatory protein(s). We therefore used gel shift assays to

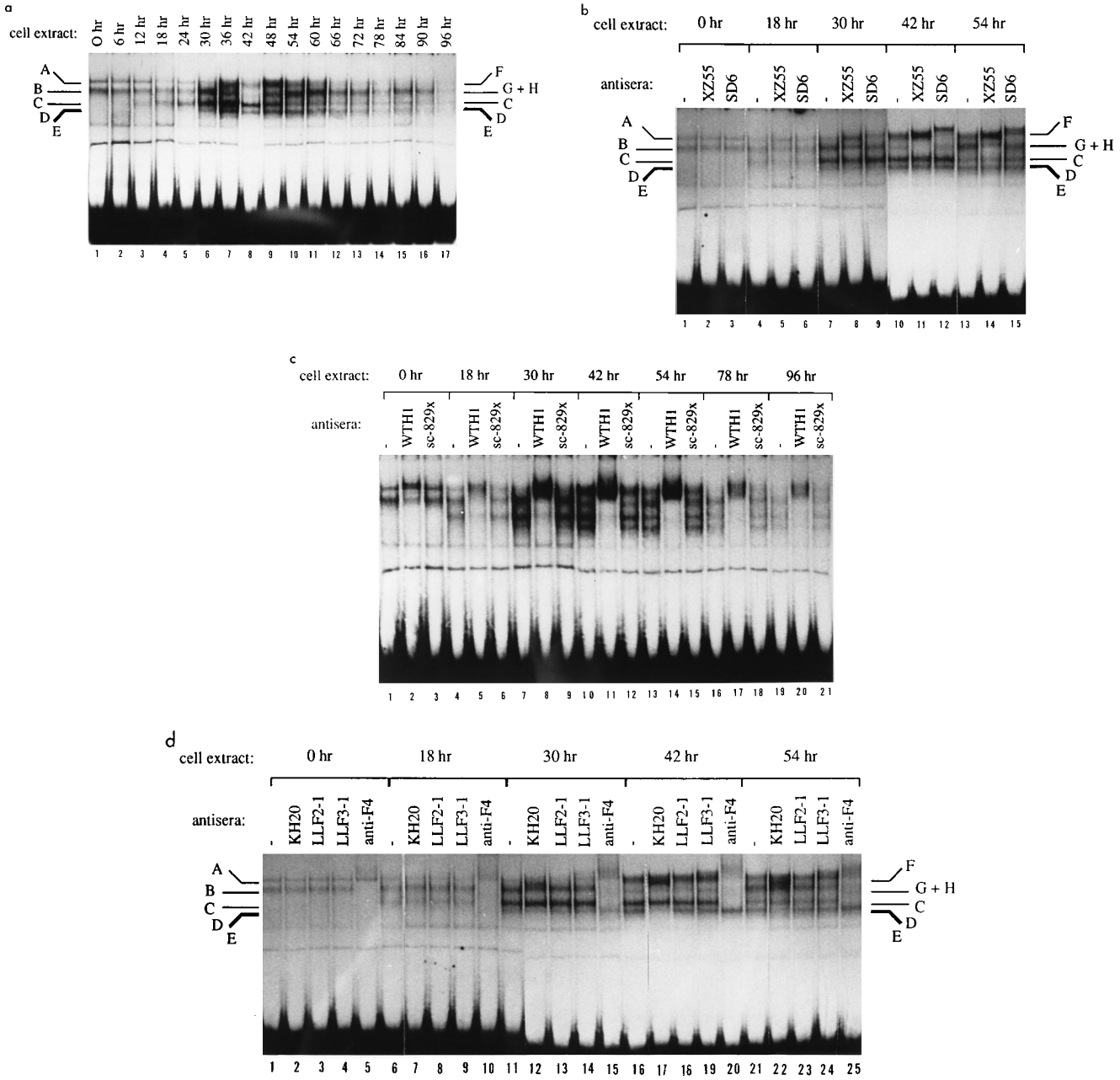

FIG. 5. Cell cycle regulation of the individual E2F complexes. The T-cell whole-cell extracts (8 μg per lane) were analyzed in the E2F gel shift assay in either the absence (a) or the presence of the following antisera: (b) 2 μl of the anti-pRB (XZ55) or anti-p107 (SD6) tissue culture supernatant; (c) 2 μl of the anti-DP-1 (WTH1) tissue culture supernatant or 1 μl of the anti-DP-2 (Santa Cruz sc-829x) polyclonal antiserum; or (d) 2 μl of the anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), or anti-E2F-3 (LLF3-1) tissue culture supernatant or 1 μl of the diluted polyclonal anti-E2F-4. In each case, complexes are labeled as described in the text.

examine the pattern of complexes arising from each of the T-cell time points (Fig. 5a). For clarity, we have labeled each of these complexes in the order of their appearance. In G_0 cells, E2F activity was detected as two discrete complexes, labeled A and B, respectively (Fig. 5a, lane 1). During the first 12 h, the levels of the A and B complexes declined steadily. At the same time, we began to detect a third novel species, labeled C, whose levels increased steadily (lane 3). At the G_1-to-S transition (between 30 and 36 h after PHA addition), this pattern altered considerably (compare lanes 6 and 7). First, we detected the formation of two novel complexes that migrated with increased mobility. The larger complex (labeled D) was present at reasonably high levels, while the smallest complex (labeled E) was barely detectable. At the same time, we detected significant changes in both the A and B complexes. Despite the previous steady decline, the levels of the upper, A complex appeared to increase dramatically between 30 and 36 h. In fact, the antibody experiments described below indicate that the increase in the level of this band was actually caused by the appearance of a novel, similarly sized species (labeled F) that replaced the G_0-A complex. In a similar manner, the levels of the B complex declined and disappeared between 30 and 36 h, to be replaced

by another novel species (labeled G) that has increased mobility. These five complexes (C through G) persisted in all of the fractions (36, 42, 48, and 54 h) containing S-phase cells (lanes 7 to 10). However, as the cells exited S phase (between 54 and 60 h), the pattern of these complexes altered once more. The S-phase-specific G complex disappeared rapidly and was replaced by an additional species, labeled H, that migrates with the same mobility as the original G_0-B complex. At the same time, the levels of other complexes declined. Although this reduction affected all four of these species, the E and F complexes appeared to be lost preferentially. The strong peak of E2F activity in S phase coincided exactly with the increased levels of E2F detected in both the Western blotting (Fig. 3b) and DOC release gel shift (Fig. 4) assays.

To determine the identity of each of these complexes, the T-cell extracts were analyzed in the presence of antibodies against the regulatory proteins in gel shift assays. The most interesting time points are shown in Fig. 5b. Consistent with previous studies, a p130 polyclonal antiserum specifically supershifted both the A and the B complexes (data not shown), confirming that p130 is contained within both of the G_0 complexes and that free E2F is absent at this time (9, 10). As the cells reentered the cell cycle, the level of these p130 complexes declined steadily, and they disappeared between 30 and 36 h after PHA addition. Low levels of p107- and pRB-specific supershifts were first detected at 30 h. These supershifts caused a partial reduction in the upper two bands, indicating that they both contain a mixture of two species, A (p130) plus F (p107) and B (p130) plus G (pRB). By the time DNA replication had reached maximum levels (42 h), the p107- and pRB-specific antibodies were able to supershift all of the upper two bands (lanes 10 to 12), indicating that the p130 complexes (A and B) had been completely replaced by p107 (complex F) and pRB (complex G). By 54 h, most of the cells had completed DNA replication (Fig. 3a). Although the pRB-specific antibodies continued to supershift a significant amount of E2F activity from this later time point, they shifted only the lower half of the second band (Fig. 5b, compare lanes 13 and 14), indicating that once again it was derived from two distinct species, G (pRB) and H. Consistent with this observation, antibodies against p107 suddenly generated two distinct supershifted species, one abolishing the F complex and the other affecting the upper half of this G (pRB) plus H (p107) band. Throughout the experiment, antibodies against either pRB, p107, or p130 failed to alter the C and D complexes, indicating that they make up the free, transcriptionally active E2F.

Having determined the regulatory protein component of each of the T-cell complexes, we wished to establish the identity of their associated E2Fs. The fractions were therefore treated with antisera specific to either the DP (Fig. 5c) or E2F (Fig. 5d) proteins. We have previously investigated the relative levels of DP-1 and DP-2 in asynchronous ML-1 cell extracts (60). This study showed that DP-1 is the major species within these cells and, together with DP-2, is sufficient to account for all of the endogenous E2F DNA-binding activity. Our analysis of the T-cell fractions was highly consistent with these data (Fig. 5c). In fact, DP-1 seemed to make up at least 80% of the B, C, D, E, G, and H complexes. Although the DP-1 supershift comigrates with the higher-mobility complexes (A and F), two distinct supershifted species were detected at the G_0 time point (compare lanes 1 and 2), suggesting that the A complex also contained a large amount of this protein. These data therefore suggest that the DP-1 was distributed equally among all of the complexes and was not limited to particular species. Because it is a polyclonal antiserum, the DP-2 antibody does not give rise to a discrete supershifted band(s). However, we did detect a reduction in the level of some early complexes (for example, B and C in lanes 4 and 6), as well as a minor supershift at the later time points (compare lanes 16 and 18 and lanes 19 and 21), indicating that DP-2 makes some contribution to many of these species. Together, these data suggest that DP-1 and DP-2 do not participate preferentially in any specific complex(es).

In contrast to the DP data, we detected clear differences in the identity of the E2F component of these complexes. At the early time points (prior to 30 h), we failed to detect any evidence of the pRB-specific E2Fs. In contrast, E2F-4 was present at high levels. In G_0, the E2F-4 polyclonal antiserum specifically supershifted all of the B complex (Fig. 5d, lane 5), indicating that this corresponds to p130/E2F-4. In addition, this antiserum recognized the complex that first appeared in mid-G_1. This C complex persisted at all subsequent time points and in each case was fully supershifted by the E2F-4 antisera. Since this complex did not contain an associated regulatory protein, these experiments suggest that E2F-4 is transcriptionally active from mid-G_1 to late S phase. In addition to E2F-4, our analysis of DOC-treated cell extracts detected a second G_0/G_1-specific E2F of unknown identity. Consistent with this observation, the G_0/G_1-specific p130-A complex was unaffected by the E2F-1, E2F-2, E2F-3, or E2F-4 antisera. In light of recent observations that E2F-5 binds specifically to p130 (28), it seems likely that the A complex corresponds to p130/E2F-5.

At the G_1-to-S transition (30 h after PHA addition), we detected major changes in the binding properties of both the regulatory proteins (as described above) and the E2Fs. Consistent with both the Western blotting and DOC release data, E2F-1- and E2F-3-specific supershifts were first detected at this time (Fig. 5d, lanes 12 and 14). Although the initial supershifts were weak, the levels of E2F-1 and E2F-3 continued to rise as the proportion of S-phase cells increased. By 42 h, it was clear that the E2F-1 antiserum had specifically supershifted almost all of the D complex (compare lanes 16 and 17). This finding is consistent with previous data (9) and suggests that E2F-1 is a significant component of the free E2F and is likely to play an important role in activating G_1/S- and S-specific transcription. Although E2F-1 and E2F-3 seem to be present at similar levels, it was almost impossible to identify the origin of the E2F-3 supershift. Even in the peak S-phase fractions, the presence of the E2F-3 antiserum did not significantly alter the intensity of any of the original complexes (compare lanes 16 and 19), suggesting that E2F-3 is a minor component of one or more of these species. Consistent with its low level of expression (Fig. 3b and 4), we were also unable to detect any E2F-2 supershift in the presence of the undissociated complexes.

Although our previous experiments had shown that E2F-4 also bound pRB in vivo, we were extremely surprised by the extent to which E2F-4 continued to dominate the latter E2F activity. In both the G_1/S and S fractions (30 and 42 h, respectively), E2F-4 was detected as the predominant component of both the p107 (complex F) and pRB (complex G) species (Fig. 5d, lanes 15 and 20). At the same time, the level of the free E2F-4 (complex C) also continued to rise as the proportion of S-phase cells increased. Together, these data indicate that the E2F-4 protein exists in excess of the pRB-specific E2Fs at all stages of the cell cycle. These high levels may help to explain why E2F-4 binds pRB in the presence of E2F-1, E2F-2, and E2F-3.

pRB does not appear to bind to E2F during G_1. Our cell cycle experiments clearly demonstrate that the G_1 T-cell fractions contain both pRB (as judged by Western blotting) and free E2F-4 (as judged by gel retardation assays). In light of these findings, we might expect pRB and E2F-4 to associate

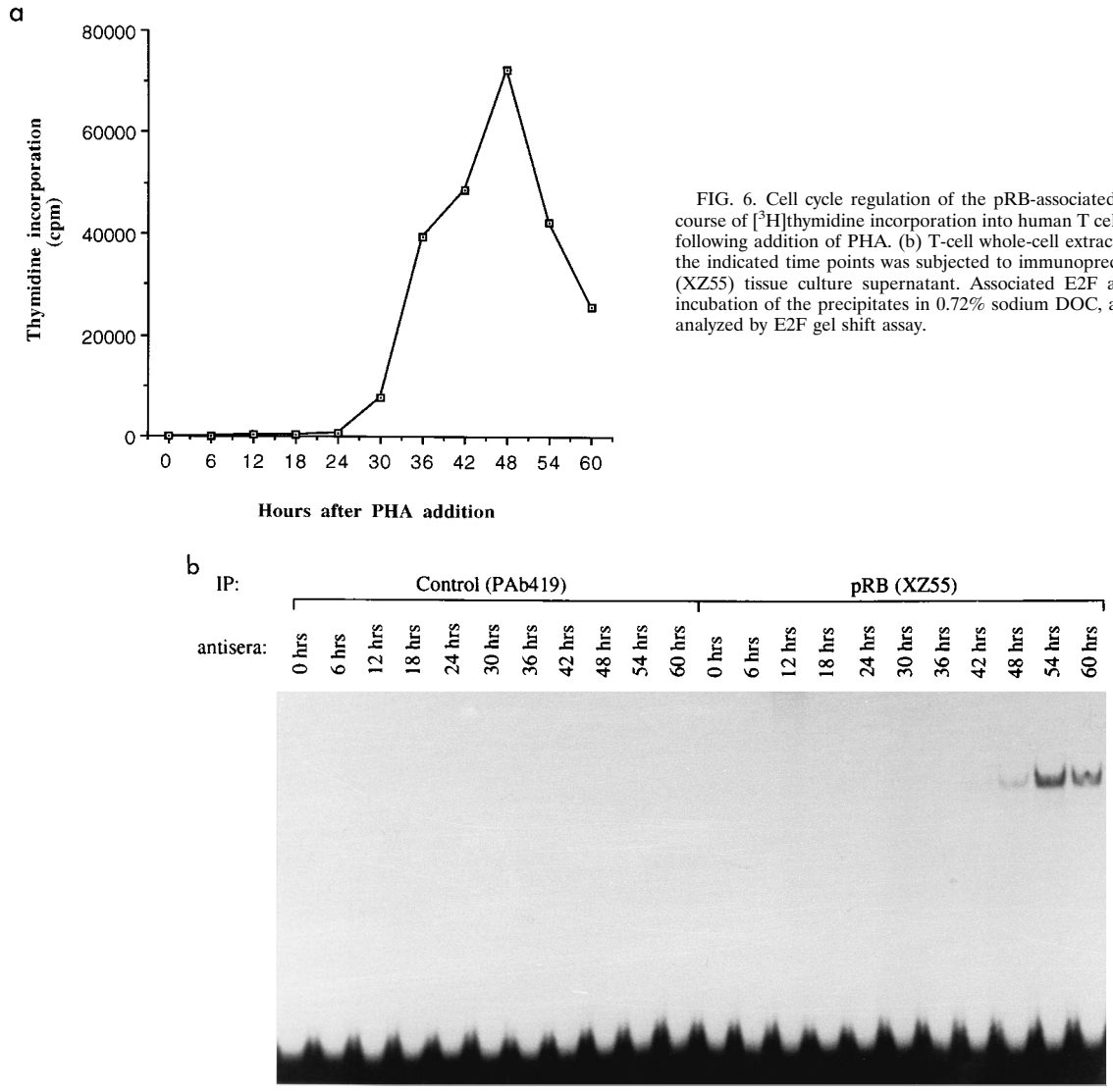

FIG. 6. Cell cycle regulation of the pRB-associated E2F activity. (a) Time course of [^{3}H]thymidine incorporation into human T cells at the indicated times following addition of PHA. (b) T-cell whole-cell extract (500 μg) from each of the indicated time points was subjected to immunoprecipitation with anti-pRB (XZ55) tissue culture supernatant. Associated E2F activity was released by incubation of the precipitates in 0.72% sodium DOC, and the supernatant was analyzed by E2F gel shift assay.

during the early cell cycle stages. However, our gel retardation assays failed to detect any pRB-containing complexes until after the cells had traversed the G_1-to-S transition. We therefore used the immunoprecipitation-DOC release assay to screen the early T-cell fractions for any evidence of pRB-E2F complexes. To ensure that we would detect rare complexes, a second batch of PHA-activated T cells was prepared specifically for this experiment. In this instance, the profile of E2F complexes was almost identical to that in the previous batch of T cells (data not shown), with maximum DNA synthesis being detected approximately 48 h after PHA addition (Fig. 6a). A significant proportion of cell extract from each time point was then immunoprecipitated with either a control (PAb419) or an anti-pRB (XZ55) monoclonal antibody. Any associated E2F activity was released by treating the precipitates with DOC and detected in E2F gel shift assays (Fig. 6b). While the control (anti-large T-antigen) monoclonal antibody failed to bring down any proteins capable of binding the E2F probe, E2F activity was detected within some of the pRB immunoprecipitates. However, consistent with our gel shift assays, this activity was specifically detected in post-G_1/S fractions (lanes 19 to 22) and was most abundant in late S-phase cells. The appearance of these S-phase-specific pRB/E2F complexes is highly consistent with the apparent increase in the rate of pRB synthesis that occurs at this point in the cell cycle. Although we cannot rule out that pRB/E2F complexes are present at low levels, these data suggest that the pRB species that are present in G_0/G_1 cells are unable to associate with any E2F DNA-binding activity.

E2F-1 and E2F-3 display different pRB-binding properties. Our cell cycle experiments suggest that the pRB-specific E2Fs also act after the cells have traversed the G_1-to-S transition (Fig. 3b, 4, and 5d). However, there is some indication that these proteins participate in different complexes. The E2F-1-specific monoclonal antibody produces a clear supershift that arises at the expense of the S-phase-specific, free E2F complex (Fig. 5d, lane 17). Since this antibody had little detectable effect upon the pRB/E2F complexes, these data suggest that E2F-1 is present in the predominantly free form. In the same experiment, the E2F-3-specific monoclonal antibody also produced a clear supershifted species. However, in this case, we were unable to detect a significant reduction in any of the E2F

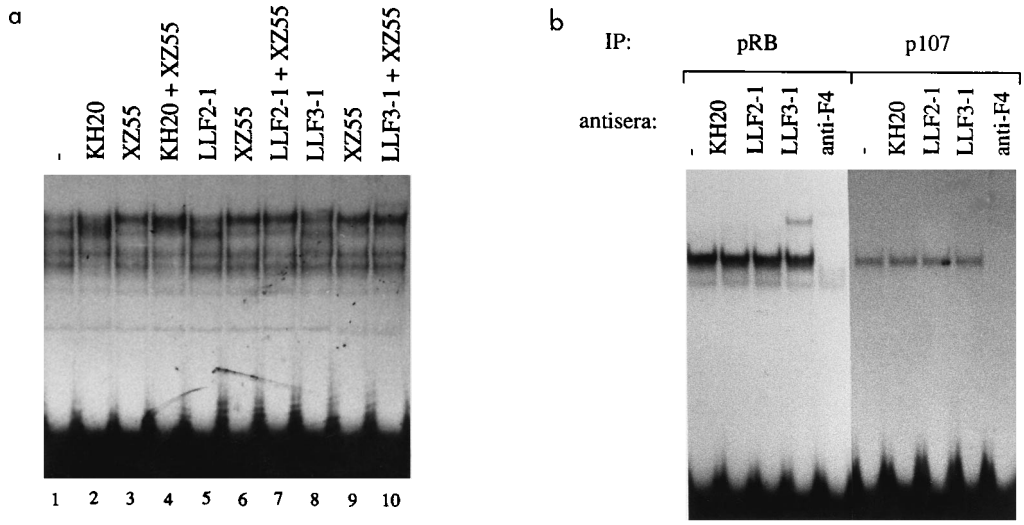

FIG. 7. Identification of the pRB-associated E2F activity. (a) Whole-cell extracts (8 μg per lane) from the 54-h time point of the first batch of T cells were analyzed in E2F gel shift assays in the presence of 2 μl of the indicated anti-E2F monoclonal antibody and/or the pRB-specific XZ55. (b) Whole-cell extracts containing the peak pRB-associated E2F activity of the second batch of T cells (54 and 60 h) were pooled, and 500 μg of total protein was subjected to immunoprecipitation with either the anti-pRB (XZ55) or the anti-p107 (SD6) tissue culture supernatant. Associated E2F activity was released by incubation in the presence of 0.72% sodium DOC, and the supernatant was analyzed in E2F gel shift assays in the absence or presence of either 1 μl of the anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), or anti-E2F-3 (LLF3-1) tissue culture supernatant or 1 μl of the diluted polyclonal anti-E2F-4.

complexes. We have therefore used two distinct assays to try and identify the nature of the E2F-3 complex. Initially, we tested whether the pRB-specific monoclonal antibody, XZ55, was able to alter the mobility of the individual E2F supershifts. In this experiment, the T-cell fractions were tested in a gel shift assay with a mix of E2F- and pRB-specific antibodies. Since similar results were obtained at each time point, only data from the peak S-phase fraction are shown (Fig. 7a). As described above, the E2F-1- and E2F-3-specific antibodies both gave rise to a discrete supershifted band (lanes 2 and 8). However, these bands were differentially affected by the addition of the anti-pRB monoclonal antibody. Inclusion of both KH20 and XZ55 (lane 4) gave rise to two discrete supershifted species that comigrated with the single E2F-1 (lane 2) and pRB (lane 3) shifts. This therefore supports our previous conclusion that pRB is not a component of the E2F-1 complex. In contrast, a mixture of LLF3-1 and XZ55 gave rise to a novel complex (lane 10) that migrated with reduced mobility relative to the individual E2F-3 (lane 8) or pRB (lane 9) supershifted species. The formation of this band suggests that, unlike E2F-1, most of the E2F-3 protein is associated with pRB. Moreover, this partitioning was apparent at every stage of the cell cycle at which the E2F-1 and E2F-3 DNA-bound complexes were detected (data not shown).

To confirm this difference, we have used the immunoprecipitation-DOC release assay to examine the E2F content of the pRB-associated T-cell E2F activity. For this experiment, we pooled the two T-cell fractions (54 and 60 h) that were previously shown to contain most of the pRB-associated E2F activity (Fig. 6b) and immunoprecipitated either pRB or p107 complexes. The associated E2F activity was then released by DOC treatment and detected in gel shift assays in the presence of the E2F antiserum (Fig. 7b). Consistent with our previous experiments, antibodies against E2F-1 or E2F-2 failed to supershift any of the pRB- or p107-associated E2F activity. In contrast, LLF3-1 specifically detected a significant amount of E2F-3 in the pRB but not the p107 immunoprecipitate. Not surprisingly, the E2F-4 polyclonal antiserum recognized most of both the pRB- and p107-associated E2F activity, giving rise to a large smear of supershifted bands. Although the apparent lack of association between pRB and either E2F-1 or E2F-2 may be due to the detection limits of this assay, these experiments reveal a major difference in the binding profile of E2F-1 and E2F-3. Although these two proteins are present at similar levels, E2F-1 remains predominantly free in all fractions in which it was detected whereas most of E2F-3 seems to remain bound to the retinoblastoma protein.

DISCUSSION

E2F is known to play a pivotal role in coupling the coordinate expression of cell cycle proteins to their appropriate transition points. Although there has been considerable progress in establishing both the properties and regulation of the individual E2F/DP heterodimers, we do not understand how these complexes bring about the differential activation of different target genes. Several groups have reported that the individual E2F/DP heterodimers bind specifically to either pRB (E2F-1, E2F-2, and E2F-3) or p107-p130 (E2F-4 and E2F-5) in vivo (6, 14, 21, 28, 41, 52, 58). It is generally accepted that this specificity will play a major role in determining both the timing and length of activation of these E2F complexes. It has also been suggested that differences in either the intrinsic properties of the E2F/DP heterodimers and/or the modulating effect of the associated regulatory protein(s) would be sufficient to somehow ensure that these different E2F subclasses target different E2F-responsive genes. Differences in E2F target gene specificity could also explain why pRB and not p107 or p130 is a tumor suppressor.

In this study, we document the cell cycle regulation of the individual E2F/DP heterodimers and their associated regulatory proteins. Both the components of the complexes and the timing of their appearance are summarized in Fig. 8. These findings considerably alter our interpretation of the relative roles of the individual E2Fs. Most surprisingly, our data indicate that a single E2F/DP complex, E2F-4/DP-1, is responsible for most of the endogenous E2F DNA-binding activity. Moreover, this complex interacts in turn with each of the known E2F

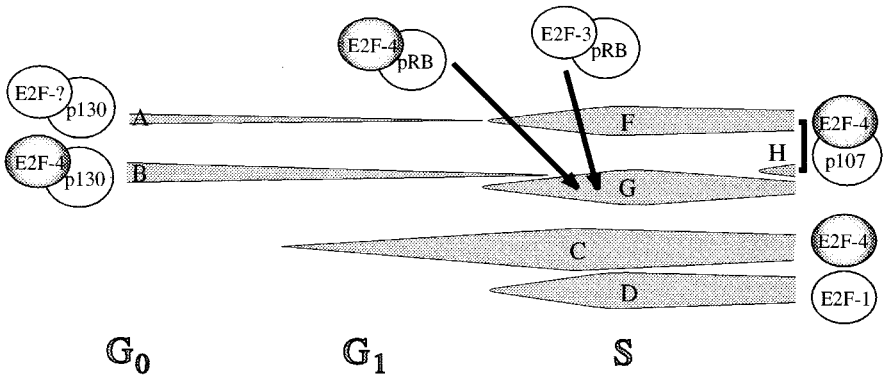

FIG. 8. Diagrammatic representation of the cell cycle dependence and identity of the various E2F complexes. Complexes are labeled A to H according to the nomenclature used in the text; their constituent E2F and pocket protein moieties are indicated.

regulatory proteins, including the retinoblastoma protein. In G_0 cells, E2F-4 and an unidentified E2F that has properties similar to those described for E2F-5 were sufficient to account for all of the endogenous E2F activity. In both cases, these complexes were associated with p130 and presumably were transcriptionally inactive. Once the cells reentered the cell cycle, the unidentified E2F complex disappeared rapidly while the levels of E2F-4 increased continually. By mid-G_1, we were able to detect significant quantities of free, presumably transcriptionally active E2F-4/DP-1. Although the levels of the p130–E2F-4 complex were in decline, this was insufficient to account for the rise in levels of free E2F-4/DP-1. The mid-G_1 "free" E2F must therefore be composed of a combination of p130-released and newly synthesized protein. Most known E2F-responsive genes are first transcribed at the G_1-to-S transition (49), considerably later than the appearance of the free E2F-4. In fact, Northern (RNA) analysis confirms that the transcription of at least one known E2F-responsive gene, *E2F-1*, is not detected until 12 h after the appearance of the free E2F-4 (our unpublished data). This suggests that this initial free E2F-4 does not activate the transcription of the known G_1/S-responsive genes. Obviously, it will be important to determine whether this E2F-4 complex activates a different set of responsive genes or whether it requires an additional modification (for example, phosphorylation) to give rise to a transcriptionally active complex.

Once cells reach the G_1/S boundary, we detected a significant increase in the total level of E2F activity that continues throughout S phase. Consistent with the induction of p107 and pRB expression, this increase arises largely from the appearance of p107- and pRB-containing complexes. Surprisingly, both of these complexes are almost entirely composed of E2F-4/DP-1, and this occurs without any apparent reduction in the level of free E2F-4 species. This suggests that the increased synthesis of this protein is sufficient to match the increasing demand for E2F-4. Our data have suggested that the mid-G_1, free E2F-4 (consisting of p130-released and newly synthesized protein) does not activate the "G_1/S" class of E2F responsive genes. This raises questions about the target specificity of the S-phase free E2F-4 complex. At this time, we assume that the free E2F-4 may include forms that have been released from association with p107 and/or pRB. If the regulatory proteins influence the target specificity of their associated E2Fs, this S-phase E2F-4/DP-1 activity could activate the transcription of responsive genes that the mid-G_1 complex was unable to target.

The S-phase activity is further complicated by the appearance of E2F-1, E2F-2, and E2F-3, as well as an additional E2F that is clearly detectable in DOC release experiments. Unlike the G_0 complex, there are no good candidates for this novel species, suggesting that it may correspond to an as yet unidentified, sixth E2F. The E2F-1, E2F-2, and E2F-3 proteins are absent in arrested cells and are not expressed until the G_1-to-S transition. Once synthesized, these proteins are quickly detected in DNA-dependent assays, suggesting that DP association is not a rate-limiting step in the formation of active E2F/DP complexes. In all cell types examined (ML-1, C33-A, and human thymocytes), E2F-2 seems to be a very minor component of the endogenous E2F activity, and we were unable to determine the relative levels of the bound and free forms of this protein. In contrast, E2F-1 and E2F-3 were both clearly detectable. Strikingly, although these proteins were present at roughly similar levels (as judged by both Western blots and DOC release assays), they appear to be present in very different forms. Through S, G_2, and M, most of E2F-3 remains associated with the retinoblastoma protein in a presumably inactive form. In contrast, E2F-1 is detected predominantly within a free E2F complex, in agreement with previous studies (9). In fact, although the total S-phase levels of E2F-4 vastly exceed those of E2F-1, these proteins seem to contribute almost equally to the amount of free E2F that is present at this stage of the cell cycle.

These observations raise important questions about the underlying mechanism(s) that determines whether a particular E2F is free. At this time, it is not clear how the p130-E2F and p107-E2F complexes are regulated. In contrast, it is generally accepted that dissociation of the pRB-E2F is induced by the cell cycle-dependent phosphorylation of the retinoblastoma protein. Since our data show that pRB is being continually synthesized throughout S phase, the presence of these late pRB-E2F complexes does not challenge the model. However, it does not help to address why we detect major differences in the bound-to-free ratio of E2F-1, E2F-3, and E2F-4 or why pRB does not bind to E2F during G_1. It is possible that the level of these E2Fs exceeds that of the unbound retinoblastoma protein and that these differences reflect differences in its affinity or avidity for the three E2Fs. Although we cannot rule this out, both the levels of pRB and the in vitro-binding properties of the individual E2F/DP species are inconsistent with this model. Alternatively, one has to argue that these complexes are modified in different ways. It has recently been reported that phosphorylation of E2F can increase its affinity for pRB (50). If this is true in vivo, differential phosphorylation of the individual E2F/DPs would significantly influence their

relative pRB-binding properties. Finally, one could imagine that the E2F-pRB complexes are formed with equal efficiency but dissociated at different rates because the cyclin-dependent kinases somehow recognize the individual E2F-pRB complexes with different degrees of efficiency. Understanding what determines the bound-to-free ratio of the individual E2F/DPs will become increasingly important if these complexes are found to activate different target genes.

In light of the models discussed above, it is of note that additional forms of E2F-1 and E2F-4 appeared in the Western blots in the S-phase fractions. At the same time, E2F-3 was consistently detected as a single species. It is interesting to speculate that these differences reflect changes in the phosphorylation of these proteins that specifically affect the free E2F species. This would be consistent with the recent finding that the free E2F-1/DP complex is specifically phosphorylated by the S-phase kinase, cyclin A/cdk2, and that this is sufficient to inhibit its DNA binding and transcriptional activity (12, 36). Obviously, further studies are required to determine both the nature and the functional consequences of these in vivo modifications.

A long-term goal of the field has been to understand why pRB but not p107 or p130 is a tumor suppressor. This is particularly confusing because these proteins display many of the same characteristics: all three contribute to the regulation of E2F, and all are targeted by the transforming proteins of the small DNA tumor viruses. The finding that the individual E2Fs bound with high specificity to either pRB or p107-p130 offered a possible explanation for the unique properties of pRB. This model proposes that the pRB- and p107-p130-specific E2Fs activate different E2F responsive genes and that the pRB-E2F targets are the ones that confer a growth advantage. The observation that E2F-4 is also regulated by the retinoblastoma protein suggests that pRB may be the tumor suppressor because only its loss is sufficient to mobilize the vast majority of the endogenous E2F/DP species.

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