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Cell Cycle Analysis of E2F in Primary Human T Cells Reveals Novel E2F Complexes and Biochemically Distinct Forms of Free E2F

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The transcription factor E2F activates the expression of multiple genes involved in cell proliferation, such as c-*myc* and the dihydrofolate reductase gene. Regulation of E2F involves its interactions with other cellular proteins, including the retinoblastoma protein (Rb), the Rb-related protein p107, cyclin A, and cdk2. We undertook a detailed analysis of E2F DNA-binding activities and their cell cycle behavior in primary human T cells. Three E2F DNA-binding activities were identified in resting (G_0) T cells with mobilities in gel shift assays distinct from those of previously defined E2F complexes. One of these activities was found to be a novel, less abundant, Rb-E2F complex. The most prominent E2F activity in resting T cells (termed complex X) was abundant in both G_0 and G_1 but disappeared as cells entered S phase, suggesting a possible role in negatively regulating E2F function. Complex X could be dissociated by adenovirus E1A with a requirement for an intact E1A conserved region 2. However, X failed to react with a variety of antibodies against Rb or p107, implicating the involvement of an E1A-binding protein other than Rb or p107. In addition to these novel E2F complexes, three distinct forms of unbound (free) E2F were resolved in gel shift experiments. These species showed different cell cycle kinetics. UV cross-linking experiments suggested that a distinct E2F DNA-binding protein is uniquely associated with the S-phase p107 complex and is not associated with Rb. Together, these results suggest that E2F consists of multiple, biochemically distinct DNA-binding proteins which function at different points in the cell cycle.

The transcription factor E2F was originally identified as an element needed for the activation of a specific adenoviral early promoter (pE2A) (20, 21, 44). E1A stimulation of the viral E2 transcription unit requires the interaction of E2F with the sequence TTTCGCG, represented twice within the E2 promoter (23, 43). A second viral protein, the 19-kDa E4 protein, interacts with E2F and promotes the formation of a stable DNA-binding complex on the E2 promoter binding sites (17, 25, 29, 36).

While adenovirus recruits E2F during infection to activate viral promoters, one normal cellular function of E2F is to contribute to the transcriptional regulation of multiple growth-responsive cellular genes, many of which contribute to DNA synthesis. E2F binding sites have been identified in the promoters of the c-*myc*, c-*myb*, N-*myc*, dihydrofolate reductase (DHFR), thymidine kinase, DNA polymerase α, and *cdc2* genes (4, 9, 14, 16, 28, 30, 33, 39). The E2F binding site in the P2 promoter of c-*myc* is required for activation of *myc* following serum stimulation (28); in the case of the DHFR gene, the E2F site is important for increased expression at the G_1/S boundary (26). These observations suggest that E2F plays an important role in imparting appropriate cell cycle regulated transcription to genes that govern cell growth.

Regulation of E2F involves interactions with multiple cellular proteins (reviewed in reference 30). The retinoblastoma susceptibility protein (Rb), which has served as a paradigm for a tumor suppressor, forms a specific complex with E2F (2, 3, 6, 8). Loss of Rb function, by deletion or mutation, occurs in all retinoblastomas and is a frequent event in certain other human tumors as well (24). Rb-E2F binding may be an important component of the mechanism whereby Rb negatively regulates cell growth, since loss-of-function mutant species of Rb isolated from human tumors can no longer interact with E2F (15, 34). Moreover, the ability of Rb to suppress cell growth in some assays appears to correlate with its capacity to bind E2F (34). There is mounting evidence that Rb-E2F complexes may negatively regulate E2F function, perhaps by sequestering E2F or by functioning as a repressor of E2F-responsive promoters (2, 9, 12, 15, 41, 45).

The biological activity of adenovirus E1A is consistent with this possibility. E1A dissociates Rb-E2F complexes, releasing unbound or free E2F, which is then able to interact with adenovirus E4 protein and transactivate the E2 promoter (1). E1A sequences necessary for dissociation of Rb-E2F complexes reside within conserved region 2. These sequences are also required for direct binding to Rb and for E1A-mediated cell transformation (35). The capacity to disrupt Rb-E2F complexes is shared by two additional viral transforming proteins which interact with Rb, the simian virus 40 large T antigen and the human papillomavirus E7 protein (7, 32), indicating that E2F is a common cellular target of DNA tumor viruses.

The Rb-related protein p107 forms an independent complex with E2F (5, 10, 38). The predominant p107-E2F complex identified in cell extracts also contains cyclin A and an associated kinase, cdk2 (5, 10, 31). Adenovirus E1A also binds p107, with a requirement in both cases for E1A conserved region 2 sequences (42), and it can also dissociate p107-E2F complexes (7, 38). A less abundant p107-E2F complex, containing cyclin E and cdk2, has also been described (22). While it is not yet known how these interac-

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tions affect E2F function, the association of E2F with components of the cell cycle machinery likely reflects a mechanism linking E2F activity with its suspected role in cell cycle control.

Analysis of E2F complexes in the cell cycle has shown that the Rb-E2F and p107-E2F complexes are differentially regulated. The p107-cyclin A-E2F complex is absent in G_1 and is detected at the onset of S phase (38), whereas the p107-cyclin E-E2F complex largely appears in late G_1 (22). The Rb-E2F complex is present in G_1 and in S (38). Therefore, E2F does not appear to be simply handed off from Rb to p107 as a cell progresses from late G_1 into S.

To better understand the regulation of E2F, it is important to learn which species of E2F are present at certain key points in the cell cycle. In an effort to address this question, we have attempted to identify and characterize E2F species in primary, diploid human cells with respect to their composition and temporal abundance. In this study, we have undertaken a more detailed analysis of both predominant and less abundant E2F DNA-binding activities in primary human T lymphocytes from healthy donors. We chose to perform these studies with primary human cells since, unlike most established cell lines, these cells are diploid and should produce wild-type E2F and E2F-binding partners. Our analysis has identified several novel E2F complexes, one of which is restricted to G_0/G_1 and appears to involve an interaction with a heretofore unrecognized E2F-binding protein. In addition, both gel shift and UV cross-linking experiments suggested the existence of biochemically distinct E2F DNA-binding species, at least one of which interacts with p107 but not with Rb. These results point to additional levels of complexity in the regulation of and by E2F in the cell cycle.

MATERIALS AND METHODS

Preparation of human T lymphocytes and cell extracts. Primary human T lymphocytes were isolated from peripheral blood by centrifugation through Ficoll-Hypaque gradients (Pharmacia). Adherent cells were depleted from these preparations by incubation on plastic dishes for at least 2 h. Cells were harvested immediately thereafter for preparation of whole cell extracts. In some cases, cells were cultured in RPMI 1640 (GIBCO) with 10% fetal calf serum (GIBCO) at 37°C in a 5% CO_2-containing atmosphere. They were stimulated by the addition of phytohemagglutinin (1 μg/ml) for the indicated times prior to being extracted. Centrifugal elutriation of T cells was performed as described previously (38). U937 cells were grown in suspension in RPMI 1640 containing 10% fetal calf serum. Whole cell extracts were prepared as described previously (27, 38).

Electrophoretic mobility shift assays (EMSAs). DNA binding assays were performed as described previously (38), with some modifications. DNA binding reaction mixtures contained 20 μl of binding buffer (50 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.6], 10 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.1% Nonidet P-40), 2 μg of sonicated salmon sperm DNA, 5 to 10 μg of whole cell extract, and 1 ng of ^{32}P-end-labeled oligonucleotide probe (E2pro; see below). Approximately 50 ng of the relevant purified, *Escherichia coli*-synthesized glutathione *S*-transferase (GST)-E1A and/or GST-E4 fusion protein (19, 29, 38) was added to reactions where indicated. In some experiments, 1 μl (0.5 μg) of purified monoclonal antibody (XZ55 or C36; Pharmingen) or 1 μl of rabbit polyclonal antisera (anti-p107, N9, and preimmune; [38]) was added to DNA binding reactions. Affinity-purified polyclonal antiserum to E2F-1 (19) was provided by W. Krek. Where indicated, 2 μl of monoclonal antibody tissue culture supernatant SD4, SD6, SD15, XZ140, HE111, or 423 was added. The p107 monoclonal antibodies SD4, SD6, and SD15 were generously provided by N. Dyson and E. Harlow (10b); XZ140 and HE111 were the kind gifts of E. Harlow and E. Lees, respectively. Binding reaction mixtures were incubated at room temperature for 20 min and then analyzed by electrophoresis at 300 V and 4°C in 4% polyacrylamide–0.25× Tris-borate-EDTA gels.

Oligonucleotides. The DNA sequences of the E2F DNA binding site oligonucleotides E2pro, DHFR WT, and DHFR MUT were described previously (38), as were C9 and BK (8). E2pro, used as a probe in E2F DNA binding assays, contains the −70 to −32 region of the adenoviral E2 promoter, encompassing both E2F binding sites. The sequence of the E2BDR oligonucleotide used for UV cross-linking experiments (see below) is 5′-CTAGTTTCGCG-3′.

UV cross-linking experiments. UV cross-linking of E2F complexes was carried out as described by Thompson et al. (40). A bromodeoxyuridine (BUdR)-substituted ^{32}P-labeled probe was prepared by annealing the oligonucleotide primer E2BDR with the plus strand of E2pro. One microgram of the annealed oligonucleotides was incubated with DNA polymerase I (Klenow fragment) in the presence of 0.1 mM each dATP, dGTP, and bromo-dUTP (Sigma), 5 μM dCTP, and 200 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol). Unincorporated nucleotides were removed by Sephadex G-25 chromatography. EMSAs were performed with the BUdR-substituted probe (10^5 cpm per binding reaction) as described above except that the amount of cell extract was increased twofold. Immediately after electrophoresis, the wet gel was covered with plastic wrap and exposed to UV light (500 mJ) with a Stratalinker 1800 (Stratagene). The positions of various E2F complexes were determined by overnight autoradiography at 4°C. Gel slices containing various E2F bands were excised and crushed with 50 μl of 2× sodium dodecyl sulfate (SDS) sample buffer between two glass slides. The gel slurries were then boiled for 5 min and electrophoresed through an SDS–8% polyacrylamide gel along with prestained molecular weight markers (Sigma).

RESULTS

Identification of additional E2F DNA-binding activities in unstimulated T-cell extracts. Previous experiments using EMSA led to the identification of four specific E2F activities in mitogen-stimulated T cells (38) (Fig. 1, stimulated). They have been designated complexes C, B, A, and A′. It was previously demonstrated that B contains E2F bound to Rb (38). The most slowly migrating of these activities, C, is composed of E2F in complex with the Rb-related protein p107 and cyclin A (38). In addition, it is likely that C also contains the kinase cdk2, which was shown to be a component of a similar complex found in other human cells (5, 10). A and A′ have been characterized as species of free E2F (38). For the purpose of our studies, "free" is defined as forms of E2F that are free to interact with Rb (or p107) and the adenovirus E4 protein in gel shift assays (38; see below).

We undertook a detailed analysis of E2F activities in primary T cells with particular emphasis on resting T cells. In unstimulated T cells, which are arrested in G_0, we detected three E2F activities (labeled X, Y, and Z) with gel mobilities distinct from those of the previously defined species (Fig. 1). In some experiments, the activity labeled Z

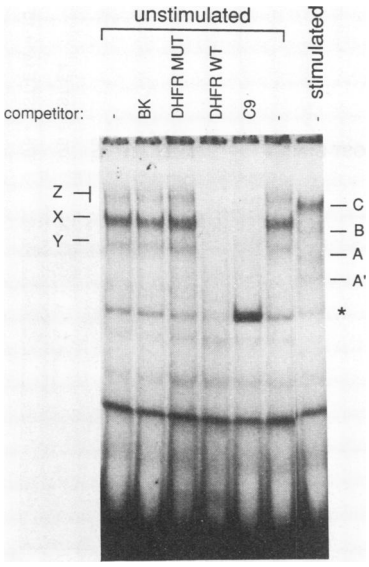

FIG. 1. E2F DNA-binding activities in resting T cells. Unstimulated (G_0) T-cell extracts were incubated with a 100-fold molar excess of the indicated competitor oligonucleotides (− indicates no competitor added) and a ^{32}P-labeled E2F probe (the E2pro oligonucleotide). DHFR WT and C9 encode functional E2F binding sites. DHFR MUT and BK encode a mutant E2F site (unable to bind E2F) and an unrelated DNA sequence, respectively. The DNA binding reactions were analyzed by EMSA. X, Y, and Z denote specific E2F DNA-binding species. E2F DNA-binding activities present in T cells stimulated for 40 h (complexes C, B, A, and A′) were analyzed in parallel for comparison (rightmost lane). The asterisks in all figures indicate a nonspecific DNA-binding activity. (The data shown in the lane labeled stimulated are also shown in Fig. 2C [lane marked stimulated]. The data in Figs. 1 and 2C are all derived from the same gel. In Fig. 1, the stimulated lane was cropped from the picture of the intact gel and apposed to the flanking lanes. In Fig. 2C, they remained in their original positions in the picture.)

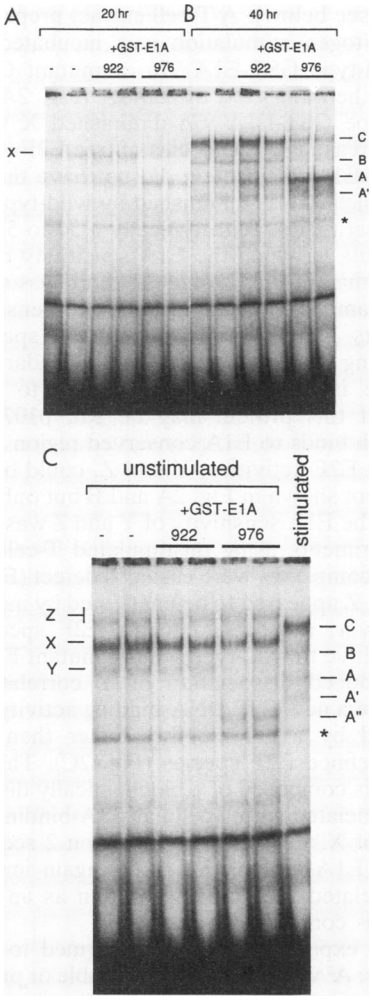

FIG. 2. Dissociation of E2F complexes by E1A. (A and B) Analysis of extracts prepared from G_1 and S-phase cells. T-cell extracts prepared after 20 h (mostly G_1 cells) or 40 h (containing S-phase cells) of mitogen stimulation were incubated with the indicated GST-E1A fusion proteins (see Materials and Methods). The reactions were then analyzed by EMSA. The mobilities of the various E2F-specific activities are indicated (X, C, B, A, and A′). (C) Analysis of a G_0 cell extract. An extract prepared from unstimulated human T cells was incubated with the indicated GST-E1A fusion proteins as described above and analyzed by EMSA. The rightmost lane shows the E2F activities detected in T cells 40 h after mitogen addition.

was resolved into two bands (see also Fig. 6B). The most prominent species, X, had been noted in a previous study but was not characterized (38). Competition experiments (Fig. 1) established that X, as well as the less prominent bands Y and Z, result from specific interactions with the E2F DNA binding site. X, Y, and Z all disappeared upon the addition of oligonucleotides corresponding to either the E2F binding site within the DHFR promoter (DHFR WT) or an E2F binding site isolated by an in vitro binding site selection strategy (C9). X, Y, and Z were not eliminated by an oligonucleotide of unrelated sequence (BK) or by a DHFR oligonucleotide containing a mutated E2F binding site (DHFR MUT). Thus, X, Y, and Z are all specific E2F DNA-binding activities, different from the A′-C set predominant in stimulated T-cell extracts.

X and Y are dissociated by adenovirus E1A and appear to contain different forms of E2F. E1A has the ability to dissociate Rb-E2F and p107-E2F complexes (B and C) with a resulting increase in free E2F (1, 7, 38). This activity requires E1A sequences necessary for binding to Rb and p107 (E1A conserved region 2). We wished to determine whether any of the E2F complexes X, Y, and Z contained Rb, p107, or a related E1A-binding protein. T-cell extracts were incubated with two different bacterially expressed GST-E1A fusion proteins. GST-E1A 976 contains wild-type E1A sequences (residues 1 to 139) which are sufficient for Rb and p107 binding and disruption of Rb-E2F and p107-E2F complexes (38). GST-E1A 922 harbors a mutation within E1A conserved region 2 (residues 122 to 129 deleted) which greatly reduces binding to Rb and p107 as well as the ability to dissociate Rb-E2F and p107-E2F complexes (38). To test the biological activity of these E1A fusion proteins, a T-cell extract prepared 40 h after mitogen stimulation was incubated with equal quantities of GST-E1A 976 and 922 and analyzed by EMSA (Fig. 2B). As has been demonstrated previously (38), the wild-type (976) but not the mutant (922) E1A protein dissociated both Rb-E2F and p107-E2F complexes (bands B and C, respectively), resulting in an increase in free E2F (consisting largely of A).

X predominates in unstimulated T-cell extracts (Fig. 1) and continues to be the most abundant E2F activity until a significant number of cells have entered S phase (>24 h after

stimulation; see below). A T-cell extract prepared 20 h (late G_1) after mitogen stimulation was incubated with either purified wild-type GST-E1A 976 or mutant GST-E1A 922 protein and then analyzed by EMSA (Fig. 2A). Treatment with wild-type GST-E1A 976 diminished X with a corresponding increase in A, a species of free E2F (Fig. 2A). The mutant GST-E1A 922 protein did not have this effect. The degree to which X was diminished by wild-type E1A varied between experiments, with typically a 30 to 50% decrease. In experiments in which the E1A sensitivity of X was less pronounced than in Fig. 2A and B, there was a correspondingly less dramatic increase in band A intensity (Fig. 2C). These results imply that X contains a species of E2F (corresponding to A) associated with a cellular E1A-binding protein. The inability of GST-E1A 922 to dissociate X suggests that this protein may be Rb, p107, or another protein which binds to E1A conserved region 2.

The minor E2F activities, Y and Z, could be detected in the experiment shown in Fig. 2A and B but only after longer exposures. The E1A sensitivity of Y and Z was examined by similar experiments using unstimulated T-cell extracts, in which these complexes were easier to detect (Fig. 2C). In all experiments, Z appeared to be unaffected by incubation with E1A. However, the second minor E2F species, Y, was entirely sensitive to wild-type but not mutant E1A (Fig. 2C). The E1A-induced dissociation of Y correlated with the appearance of a new E2F DNA-binding activity, labeled A″, characterized by a gel mobility faster than that of the previously defined E2F species (Fig. 2C). This result suggests that Y is composed of a biochemically distinct form of E2F (A″) associated with a cellular E1A-binding protein. As in the case of X, E1A conserved region 2 sequences were required for E1A dissociation of Y, again implicating Rb, p107, or a related E1A-binding protein as an E2F-binding protein in this complex.

Additional experiments were performed to characterize A″. Typically, A″ was either not detectable or present in very low levels in untreated T-cell extracts and was prominent only following incubation with E1A. To determine whether A″ is indeed a form of free E2F, we tested the ability of A″ to bind a bacterially synthesized GST-E4 protein. An unstimulated T-cell extract was treated with wild-type GST-E1A 976 to generate sufficient levels of A″ for analysis. The E1A-treated extracts were also incubated with either wild-type (GST-E4), or mutant (GST-E4Δ14-15) fusion proteins (Fig. 3). GST-E4Δ14-15 contains a small C-terminal deletion that abolishes the ability to interact with E2F (29). Addition of wild-type E4 (but not the mutant form) resulted in an apparent supershift of A″ (Fig. 3). The small amount of A generated by E1A (presumably from the partial dissociation of X) was also supershifted by wild-type GST-E4. Thus, by the criteria of adenovirus E4-binding capacity, A″ is a third species of free E2F (in addition to A and A′).

Y is a novel Rb-E2F complex. The observed dissociation of X and Y by E1A with a requirement for conserved region 2 raised the question of whether either of these E2F complexes contained Rb or p107. We tested whether a variety of Rb and p107 antibodies could perturb X or Y in an unstimulated T-cell extract by EMSA. The addition of several different Rb monoclonal antibodies (XZ140, XZ55, and C36) or a polyclonal anti-Rb antiserum (N9) supershifted or ablated Y (Fig. 4A), indicating that Rb is a component of complex Y. These antibodies have also been shown to perturb Rb-E2F complex B (reference 38 and data not shown); however, they had no effect on complex X. Incubation with several p107 monoclonal antibodies (SD4, SD6, and SD15) which recognize different epitopes within p107 (10a) or with polyclonal anti-p107 antiserum had no discernible effect on either X or Y (Fig. 4A). In an experiment performed in parallel, these p107 antibodies were able to supershift C, the S-phase-specific p107 complex (Fig. 4B). As noted previously (38), a nonspecific DNA-binding activity appeared upon the addition of all polyclonal antisera, including preimmune serum (Fig. 4A, lane P.I.), migrating

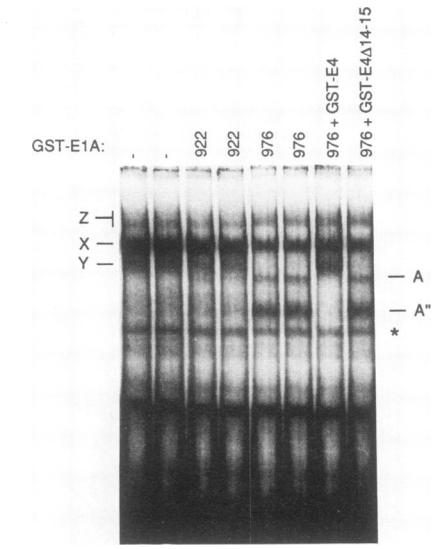

FIG. 3. Interaction of the E2F species A″ with adenovirus E4 protein. An unstimulated T-cell extract was incubated with wild-type (976) or mutant (922) GST-E1A fusion protein (− indicates no fusion protein added). Where indicated, a GST-E4 fusion protein representing either a wild-type (GST-E4) or a mutant E4 protein unable to interact with E2F (GST-E4Δ14-15) was also added to the binding reactions. E2F activities were then analyzed by EMSA.

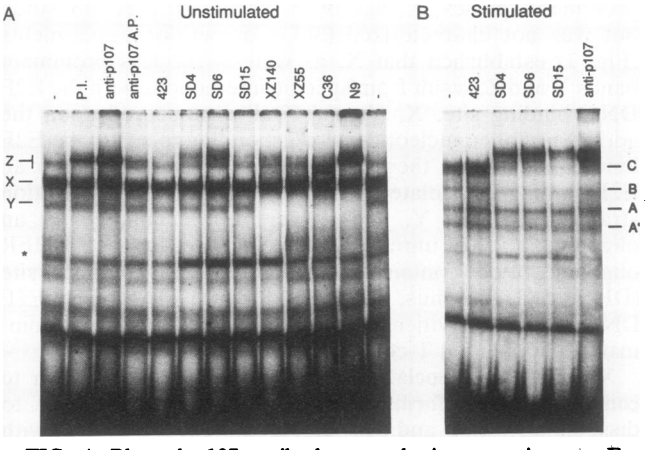

FIG. 4. Rb and p107 antibody perturbation experiments. Extracts were prepared from either unstimulated (A) or mitogen-stimulated (B) T cells. They were then incubated with the indicated antibodies and analyzed by EMSA. XZ140, XZ55, and C36 are monoclonal antibodies directed against Rb; SD4, SD6, and SD15 are monoclonal antibodies against p107; 423 is a monoclonal antibody raised against simian virus 40 large T antigen; N9 is a rabbit anti-Rb polyclonal antiserum. A rabbit anti-p107 polyclonal antiserum and an affinity-purified preparation (anti-p107 A.P.) were also tested. −, no antibody; P.I., preimmune rabbit antiserum.

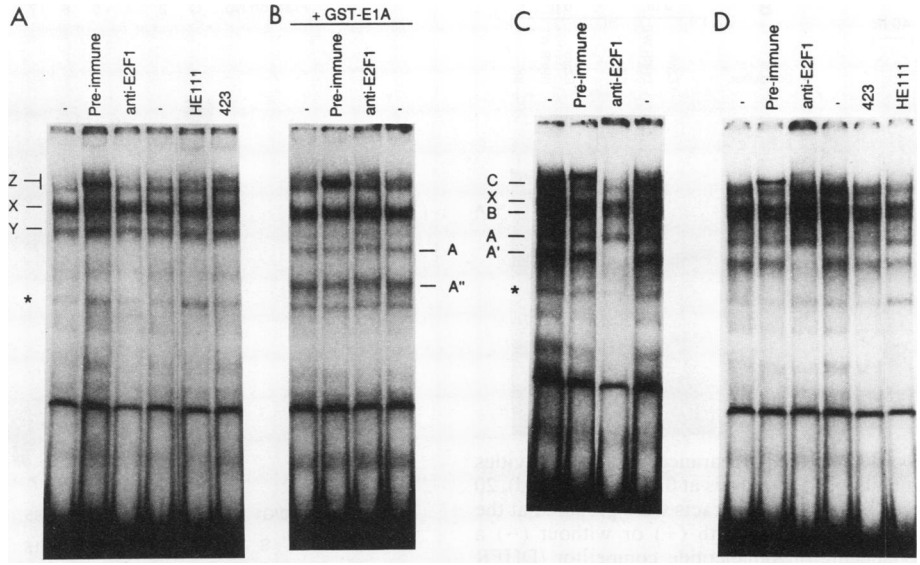

FIG. 5. Effects of antibodies against E2F-1 and cyclin E on E2F complexes. (A and B) Unstimulated T cells. (A) Extracts were prepared from resting T cells, incubated with the indicated antibodies, and analyzed by EMSA. Anti-E2F-1 is an affinity-purified polyclonal rabbit antibody directed against E2F-1, HE111 is a cyclin E monoclonal antibody, and 423 recognizes simian virus 40 large T antigen. (B) Extracts were also incubated with the wild-type E1A fusion protein GST-E1A 976 along with the indicated antibodies. (C and D) Stimulated T cells. Extracts were prepared 48 h after mitogen stimulation, incubated with the indicated antibodies (see above), and analyzed by EMSA. Two experiments are shown. −, no antibody.

just above complex Z. Although it remains possible that the epitopes for these antibodies are masked specifically in complex X, we postulate that X contains an E1A-binding protein other than Rb or p107.

E2F-1 is a component of free E2F species A′. A cDNA encoding a protein with the expected properties of E2F (designated E2F-1) has recently been cloned (13, 19, 37). To determine whether E2F-1 is present in any of the E2F complexes identified here, we tested whether a polyclonal antibody raised against E2F-1 could perturb any of the T-cell E2F activities (Fig. 5). None of the E2F complexes detected in unstimulated T-cell extracts reacted with the anti-E2F-1 antiserum (Fig. 5A and B). Free E2F species A″, generated by E1A treatment of an unstimulated T-cell extract, also was unaffected by the anti-E2F-1 antibody (Fig. 5B). In stimulated T-cell extracts, the anti-E2F-1 antiserum consistently eliminated free E2F species A′ (Fig. 5C and D), consistent with previous results (19). In some experiments, complex B also appeared to be at least partly affected by the anti-E2F-1 antibody (19); however, this effect appeared to vary between different cell extracts. Nevertheless, these results suggest that E2F-1 is a component of only one (A′) of the three free E2F species identified here.

A cell cycle-dependent interaction between cyclin E and E2F has recently been described (22), raising the question of whether cyclin E is present in any of the T-cell E2F complexes. A monoclonal antibody against cyclin E (HE111) failed to supershift or alter any of the E2F species detected by EMSA in unstimulated (Fig. 5A) or stimulated (Fig. 5D) T-cell extracts. The He111 antibody can supershift an E2F activity in other cell extracts (21a). Immunoprecipitation with HE111, followed by EMSA analysis of detergent-released associated proteins, revealed a small amount of associated E2F in stimulated T-cell extracts (data not shown), indicating the antibody can react with cyclin E in complex with E2F. Thus, while we were able to detect an interaction between E2F and cyclin E in T cells, our results suggest that this may be a complex of very low abundance.

Different E2F complexes and free E2F species exhibit distinct cell cycle behaviors. We examined whether the levels of the various E2F species described above vary throughout the cell cycle. In one approach, resting T cells were stimulated with mitogens and E2F complexes were analyzed by EMSA at various times later as the cells exited G_0 and entered the cell cycle. Figure 6A shows an experiment in which resting T cells (0 h) were stimulated with mitogens and extracts were prepared at 20 or 40 h later. Consistent with previous results (38), the p107 complex C appeared only after cells had entered S phase (40 h). Also, as previously reported (38), the Rb-E2F complex B was not detected in unstimulated cells (0 h, G_0), was present at low levels at 20 h (predominantly G_1), and was prominent at the 40-h time point (entry into S). The minor Rb-E2F complex, Y, was present in unstimulated cells (0 h; Fig. 6A [seen better in Fig. 1 and 2C]); thus, the two Rb-E2F complexes display somewhat different temporal regulation. Because of the abundance of other complexes migrating nearby, it was difficult to determine whether Y and Z persisted through G_1 and/or into S.

The two major species of free E2F, A and A′, displayed quite different cell cycle kinetics. A′ was not detected until cells entered S phase (Fig. 6A, 40 h). By contrast to A′, A was readily detectable at earlier times (20 h). Indeed, we were able to detect A as early as 6 h after mitogen stimulation (Fig. 6B). While A was not detected at 0 h, it could be generated in unstimulated cell extracts by incubation with E1A (Fig. 2C), whereas E1A treatments of G_0 cell extracts never generated detectable levels of A′. One interpretation of these results is that the form of E2F responsible for A is present in G_0, although not in its free form (A) but instead sequestered in complex X (see above). Thus, the two major forms of free E2F behaved differently; A first appeared

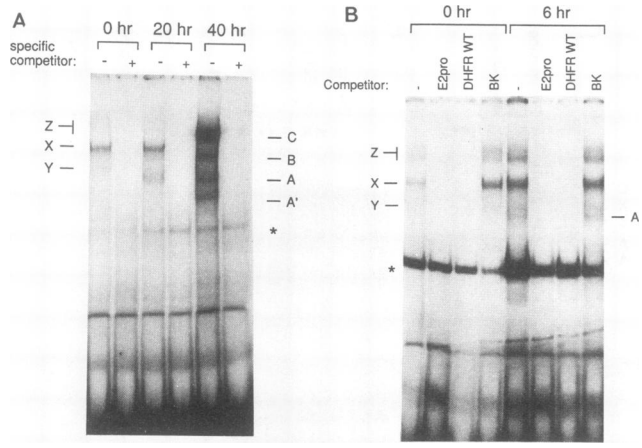

FIG. 6. Temporal order in the appearance of E2F activities following mitogen stimulation. (A) Analysis at 0 h (unstimulated), 20 h, and 40 h after mitogen stimulation. Extracts were prepared at the indicated times and incubated either with (+) or without (−) a 100-fold molar excess specific oligonucleotide competitor (DHFR WT). Reactions were then analyzed by EMSA. (B) Analysis at 0 h (unstimulated) and 6 h after mitogen addition. Extracts were incubated with a 100-fold molar excess of the indicated oligonucleotide (− denotes no competitor added). E2pro and DHFR WT contain E2F binding sites. BK consists of an unrelated DNA sequence.

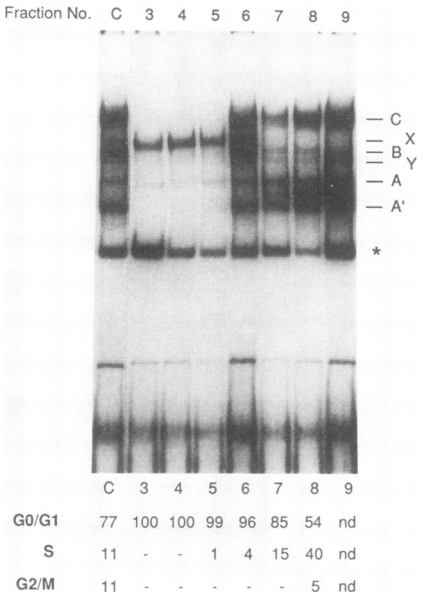

FIG. 7. EMSA analysis of elutriated T cells. Stimulated T cells (43 h after mitogen addition) were fractionated by centrifugal elutriation, and fractions that were enriched for G_1- and S-phase cells were analyzed by EMSA. Fraction C was prepared from the asynchronous culture prior to elutriation. The gel mobilities of the various E2F species are indicated. The cell cycle profile of the fractions was determined by FACS analysis of cellular DNA content and is listed at the bottom. nd, not determined.

shortly after mitogen stimulation (early G_1), whereas A′ emerged at the onset of S (or possibly late G_1).

Elutriation experiments yielded analogous findings regarding the temporal regulation of different E2F species. A preparation of mitogen-stimulated T cells (43 h poststimulation), was fractionated by centrifugal elutriation. Extracts were prepared from G_1- and S-phase-enriched cell fractions (as determined by fluorescence-activated cell sorting [FACS] analysis), and E2F DNA-binding activities were monitored by EMSA (Fig. 7). The p107-cyclin A complex (C) appeared only in fractions containing S-phase cells (Fig. 7, fractions 6 through 9). The major Rb-E2F complex (B), while present in G_1 (seen more clearly on longer exposures), persisted in all S-phase fractions (Fig. 7, fractions 6 through 9), consistent with previous findings (38). The behavior of the minor Rb-E2F complex, Y, appeared to parallel that of B. By contrast to Rb-E2F complexes, X was restricted almost completely to G_0/G_1-enriched fractions (Fig. 7, fractions 3 through 6). Z could be detected only after long exposures, and it was not possible to determine whether its abundance varied in the cell cycle.

Levels of the two major free E2F species A and A′ again displayed differences in cell cycle dependency (Fig. 7). A was detected in both G_1 and S fractions but was less abundant in G_1. A′ was nearly absent in early G_1 fractions and increased substantially in late G_1/early S phase (Fig. 7, fractions 6 through 9). This finding is consistent with the results described above (Fig. 6) showing that A appeared shortly after stimulation of resting T cells (early G_1), whereas A′ was not detected until the G_1/S transition. It was not possible to decipher the kinetics of A″, given its relatively low abundance in the absence of exposure to E1A.

UV cross-linking provides additional evidence for biochemically distinct forms of E2F. The wide variety of E2F activities observed in these experiments may result from a single transcription factor interacting with multiple different cellular proteins. The diversity of E2F complexes may also result from the presence of multiple E2F species (i.e., a family of related transcription factors with E2F DNA-binding activity), at least some of which form complexes with a variety of cellular proteins. As one approach to investigating whether different E2F species are present in various E2F complexes, we performed UV cross-linking experiments.

Gel shift experiments were carried out with a BuDR-substituted ^{32}P-labeled E2F binding site probe. Following native gel electrophoresis, the gel was irradiated with UV light in order to covalently cross-link the probe to the DNA-binding protein(s). Individual bands corresponding to E2F complexes A, B, C, and X were excised from the gel, and the ^{32}P photoaffinity-labeled protein within these bands was fractionated by SDS-gel electrophoresis (Fig. 8). As a control, a nonspecific band (lane NS) was also excised and analyzed in parallel. Cross-linking experiments with the minor E2F species (Y, Z, and A″) proved difficult because of the low abundance of these species and were not informative.

A protein(s) with an approximate molecular mass of 56 kDa (relative to prestained markers) was cross-linked in each E2F complex (Fig. 8A), which is in agreement with previous reports on the molecular weight of E2F (43). The 56-kDa band in lane B was faint in the exposure shown. Proteins with small differences in molecular weight might not be resolved on this gel. However, even at this resolution, it is clear that two proteins (approximately 56 and 60 kDa) emerged from the cross-linking of C, the p107-cyclin A-E2F complex. The 60-kDa protein was specific to complex C and was not cross-linked in any of the other E2F complexes examined, whereas the faster-migrating band was indistinguishable by gel migration from that emerging from complexes X, A, and B. Identical results were obtained upon

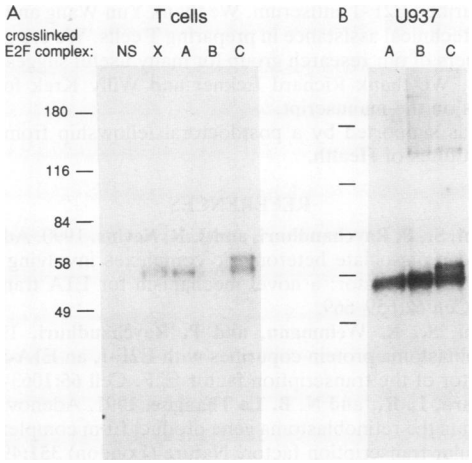

FIG. 8. UV cross-linking analysis of E2F complexes. Gel shift experiments were performed by using a ^{32}P-labeled, BUdR-substituted E2F binding site probe, with extracts prepared from either T cells (A) or U937 cells (B). Following native gel electrophoresis, the gel was irradiated with UV light, and the indicated E2F complexes (X, A, B, and C) were excised from the gel. A nonspecific band (NS) was also excised. Labeled proteins within the excised bands were then separated by electrophoresis in an SDS–8% polyacrylamide gel. The gel mobilities of prestained protein molecular weight markers are indicated in kilodaltons.

analysis of E2F complexes in the human promonocytic leukemia cell line U937. Complex C in U937 cells yielded two cross-linked proteins with approximate molecular masses of 56 and 60 kDa (Fig. 8B). These findings suggest that the S-phase p107-E2F complex contains an E2F DNA-binding protein of greater molecular weight than those found in other E2F complexes and imply that p107 interacts with at least two different E2F species. The 60-kDa protein might be an E2F species of different primary sequence, the same primary but different covalent modification structure, or both.

DISCUSSION

An increasingly complex picture of E2F and its cell cycle regulation has emerged from the analysis of E2F in primary human T cells. We wish to summarize here the main findings obtained in this study. First, several new E2F DNA-binding activities were identified and characterized. These include an additional Rb-E2F complex (Y) distinct from the previously identified complex (B), a novel form of free E2F (A″), a more slowly migrating species of completely unknown composition (Z), and an E2F complex (X) restricted to G_0/G_1 that appears to involve an interaction with an E1A-binding protein possibly different from Rb or p107 (discussed below). Second, the results provide evidence for significant biochemical diversity in the DNA-binding activities that have collectively been termed E2F. Three forms of free E2F (A, A′, and A″) with distinctly different gel mobilities were resolved in EMSAs, and at least two proteins of different molecular weights were photoaffinity labeled in UV cross-linking experiments. Third, experiments with synchronized cells demonstrated that the two major free E2F species (A and A′) displayed distinct behavior within the cell cycle. These observations raise the possibility (see below) that different species of free E2F perform different biological functions.

Of the additional E2F complexes described in this report, X is of particular interest. X is by far the most abundant G_0/G_1 E2F DNA-binding activity. The temporal pattern of X is tightly restricted to G_0 and G_1, unlike the case for Rb-E2F complexes, which are also found in S phase. In this regard, the behavior of X more closely fits what might be predicted for a negative regulator of E2F function, since cells must prevent E2F from activating growth-responsive genes at inappropriate times (i.e., in G_0). The E1A-binding protein that is a component of X may function to sequester E2F and prevent its function as a transcriptional activation. Alternatively, since X binds E2F sites, X may actively function as a repressor by binding to E2F-responsive promoters. Such a function has already been postulated for Rb-E2F complexes (41). If so, it is possible that X represents an important target for E1A in naturally occurring adenovirus infections of resting cells.

What is the composition of X? The results presented here suggest that one component of X is an E1A-binding protein other than Rb or p107. Antibodies to Rb and p107 which successfully supershifted Rb-E2F and p107-E2F complexes failed to alter X, yet E1A dissociation of X required E1A conserved region 2. At least three cellular proteins in addition to Rb and p107 bind E1A with a requirement for this sequence: p80, p90, and p130 (11, 42). The gel mobility of X, migrating slightly more slowly than the major Rb-E2F complex, makes p130 an attractive candidate. However, it should be noted that a G_1-restricted E2F complex detected in NIH 3T3 cells (27) reacts with a polyclonal p107 antiserum (cited in reference 30 as J. K. Schwarz et al., unpublished data [36a]). A current question is whether it is the murine equivalent of X. Presently, our conclusions are limited to stating that the p107 antibodies used in our study failed to react with X, although they did supershift the S-phase p107-cyclin A-E2F complex.

It is interesting to note that X was largely absent from many transformed cell lines tested to date, including U937, Rb27, U2OS, SAOS2, and HeLa (reference 38 and data not shown). The failure to detect X may not simply be due to insufficient G_1 cells in transformed cell cultures, since X was not detected even in G_1-enriched U937 cell fractions following elutriation (38). X does not appear to be specific to primary T cells, since we have detected a complex with electrophoretic mobility identical to that of X in the non-transformed diploid human fibroblast line MRC-5 (data not shown). Furthermore, Pagano et al. (32) detected a G_0/G_1-specific complex in primary human fibroblasts with a similar gel mobility. An interesting possibility is that X is frequently lost or disrupted in transformed cells. If X is a negative regulator of E2F function, perhaps its loss contributes to deregulation of growth-responsive promoters and the transformation process.

It has been reported that free E2F is the form active in stimulating transcription (1). As demonstrated in this work, there are at least three forms of free E2F that can be resolved in EMSAs (A, A′, and A″). If free E2F is the form responsible for activation of cellular promoters (a possibility that remains to be proven), it is of particular interest to note the different cell cycle kinetics of the two predominant species of free E2F. As might be expected, no free E2F was detected in unstimulated T cells (G_0). Shortly after mitogen stimulation (between 0 and 6 h), free E2F species A appeared, and it was not until the G_1/S transition (24 to 36 h poststimulation) that A′ was first detected. This temporal regulation suggests a scenario wherein A activates immediate-early E2F-responsive promoters such as the c-*myc* promoter,

whereas A′ functions primarily at promoters activated at G_1/S, such as the DHFR promoter. In elutriated cells, A′ was most abundant in late G_1/early S-phase fractions, again consistent with a role for A′ in activating genes at the G_1/S transition.

Several explanations could account for the observed diversity of free E2F species. It seems unlikely that the different forms of free E2F described here simply reflect in vitro proteolysis of a single progenitor species. The relative levels of A and A′ do not vary following repeated freeze-thawings of a cell extract and appeared and disappeared in a reproducible cell cycle-dependent manner. Furthermore, since A and A′ exhibit different cell cycle behaviors, if proteolysis accounted for their existence, it too would have to be cell cycle dependent. More to the point, if A′ and A″ are indeed degradation products, they have been proteolyzed in such a way as to retain DNA-binding activity and to have the potential to associate with E4 and Rb. It is possible that other posttranslational modifications such as dimerization or phosphorylation account for some heterogeneity of E2F activities. A′, for example, could be generated by a posttranslational modification of A, such as phosphorylation, occurring in late G_1/early S. UV cross-linking of E2F complex C yielded two proteins of 56 and 60 kDa. The larger of these could be a phosphorylated form of the smaller, and if so, E2F could conceivably be a substrate of the cyclin A/cdk2 kinase present in C.

An alternative, but not mutually exclusive, possibility is that heterogeneity of E2F activities may be explained by the existence of a family of related E2F DNA-binding proteins. If so, the different free E2F species (A, A′, and A″) may be the products of several related genes. There is ample precedence for families of related transcription factors all recognizing similar DNA sequences (18). One cDNA for E2F (designated E2F-1) has been cloned (13, 19, 37). However, additional clones with related structure have recently been isolated (22a). A polyclonal antiserum raised against E2F-1 reacted with A′ but did not perturb either A or A″ (Fig. 5), providing evidence that A and A″ are immunologically distinct from A′ and thus may be gene products different from E2F-1. The conclusion that E2F-1 is solely a component of A′ is consistent with Northern (RNA) analysis of E2F-1 gene expression (19). The E2F-1 mRNA was not detected in unstimulated T cells, and the temporal appearance of the E2F-1 message following mitogen stimulation correlates with the appearance of A′ by EMSA analysis.

If these various species of free E2F are different members of an E2F gene family, the data presented here suggest that they may, in part, function at different points in the cell cycle. In addition, one could speculate that different E2F family members selectively interact with different cellular proteins, such as Rb, p107, and perhaps other nuclear pocket proteins. A′, for example, does not seem to be a component of X. The 60-kDa protein identified by UV cross-linking appeared specifically in the p107 complex, implying that this form may interact exclusively with p107, not with Rb or the E2F-binding protein in complex X. A major challenge for the future will be to determine in detail the nature of the E2F gene product(s) present in the various complexes and when, where, and how these complexes operate during the cell cycle.

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