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E2F and Its Developmental Regulation in *Xenopus laevis*

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The transcription factor E2F has been implicated in cell cycle control by virtue of its association with cyclins, cyclin-dependent kinases, and pRb-related tumor suppressor gene products. Eggs and embryos from the frog *Xenopus laevis* have been used to investigate the characteristics of E2F-like molecules in the *Xenopus* cell cycle and throughout early development. We find multiple E2F species in *Xenopus* eggs, at least one of which is modified by phosphorylation. The vast majority of E2F remains in the free form throughout the very early embryonic cell cycle, and it also remains predominantly free until some time after the mid-blastula transition, the onset of zygotic transcription. At this time, E2F complexes significantly to pRb but not to cdk2, although cdk2 binding is found in tissue culture cells from a very advanced stage in embryogenesis. This suggests that the complexing of E2F to cyclins, cyclin-dependent kinases, and tumor suppressor gene products may be controlled separately in early *Xenopus* development. Thus, the association of E2F with other molecules may not result solely from processes affecting cell cycle progression but may also reflect developmental and differentiation cues.

Recently, there has been much interest centered on the protein E2F, a factor which is linking the fields of transcription and cell cycle control. E2F was first identified as a cellular activity which was upregulated after adenovirus infection and which binds to the adenoviral E2 promoter (37). E2F and a related factor, DRTF1, which has similar DNA sequence recognition characteristics, become downregulated on the experimentally induced differentiation of F9 embryonal carcinoma cells in culture (38, 39, 54), raising the possibility of an interesting role in development and differentiation.

E2F is found in a variety of complexes with cellular proteins and can be dissociated from these by the adenovirus E1A gene product. The retinoblastoma protein pRB is one of these associated cellular factors (3, 6, 10, 11). Moreover, it is the underphosphorylated and putatively active form of pRB (for a review, see reference 14), predominantly found in G_1 of the cell cycle, which preferentially complexes with E2F (10, 55). E2F has been found in association with the pRB-related protein p107, along with the cyclin-dependent kinase cdk2 and cyclins E and A (4, 9, 19, 40, 43, 51). Complexes between E2F and these different components are cell cycle regulated, with pRB-containing E2F complexes predominantly appearing in G_1 of the cell cycle (55, 58). Complexes between cyclin E, cdk2, p107, and E2F appear in G_1 (40), while cyclin A, cdk2, p107, and E2F complexes appear in S phase (19, 40, 51). Free E2F is found predominantly at the G_1/S boundary and is thought to be transcriptionally active, participating in the cell cycle-regulated transcription of proteins required for DNA synthesis, e.g., dihydrofolate reductase (for a review, see reference 47). E2F has also recently been found associated with the pRb-related protein p130 (12).

Such results clearly implicate E2F in cell cycle control. In cotransfection experiments, both pRB and p107 have been shown to repress transcriptional activation by E2F (15, 24, 29, 55, 60, 61). E2F binds to cyclins and a cyclin-dependent kinase, which have a proposed role in cell cycle progression (21), and it is interesting to speculate on the function of sequence-specific binding of cyclin or cdks to DNA at G_1/S of the cell cycle (also see reference 19 for a discussion). Provocative results by Johnson et al. (33) show that microinjection of E2F into quiescent cells causes them to reenter S phase, indicating a potential causative role in cell cycle progression.

Recently, a protein with some of the characteristics of E2F (designated E2F1) has been cloned by virtue of its association with the pocket region of pRB (27, 34, 56). E2F1 is able to bind to the E2F consensus sequence and activate transcription from it. Another E2F-related molecule, DP1, has been cloned which shows some homology to E2F1 in the DNA binding region and is able to bind weakly to the E2F consensus (22). Huber et al. (31) have used protein purification to show that up to five polypeptides exhibit E2F consensus DNA binding activity. Moreover, these can be separated into two groups on the basis of apparent molecular weight, and mixing of two proteins from different groups results in the substantial enhancement of the E2F DNA binding activity. This has been confirmed by studies of Helin et al. (28) and Bandara et al. (5), who show that E2F1 and DP1 heterodimerize and bind cooperatively to the E2F binding site, resulting in greatly enhanced transcription compared with that seen with each individual protein. Moreover, Helin et al. (28) have shown that such heterodimerization is important for pRb binding to E2F. This has led to the conclusion that E2F is, in fact, a heterodimeric complex of two E2F-related molecules, one from the E2F group and one from the DP1 group.

We have chosen to investigate the role of E2F during *Xenopus* development for a number of reasons. First, there has been a recent surge of interest in the possibility that transcription factors play a more direct role in the initiation of DNA replication (for reviews, see references 17 and 25), possibly by altering chromatin structure or by recruiting replication factors to the origin of replication. The unexplained DNA binding of cyclin-cdk complexes, via p107 and E2F around the time of DNA synthesis, raises the possibility of a direct role for E2F in S phase control independent from its transcriptional activation function, potentially by providing sequence-specific DNA binding of a kinase active in the G_1 and S phases. There exist cell extracts from *Xenopus* eggs which perform many of the major cell cycle events in vitro in the absence of transcription, and these provide a good system in which to study such a function. However, if the role of E2F is solely in the cell cycle regulation of transcription, there is a major developmentally

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regulated activation of transcription at the mid-blastula transition (MBT) in *Xenopus* embryos which can be used to characterize this function. Finally, E2F has been shown to be downregulated on cell differentiation in the F9 embryonal carcinoma cell system (see above). Development of *Xenopus* embryos is well documented and so provides a system to allow the physiological role of E2F in development and differentiation in a whole organism to be investigated.

MATERIALS AND METHODS

Extracts. Egg low-speed supernatants (LSS) were prepared as described by Blow and Laskey (7), and high-speed supernatants (HSS) were prepared by the method of Sheehan et al. (57); both were supplemented by leupeptin, pepstatin A, and chymostatin at 10 μg/ml before freezing. Cycling *Xenopus* egg extracts were prepared and assayed microscopically according to the method of Murray (44). *Xenopus* embryos were classified according to the developmental stage timing of Nieuwkoop and Faber (50). Embryo extracts for gel retardation analysis were prepared according to the method of Taylor et al. (59).

Extracts of *Xenopus* tissue culture (XTC) cells were prepared for gel retardation analysis as follows. Cells which were approaching confluence were removed by scraping and washed three times in phosphate-buffered saline. The cell pellet was resuspended in 150 μl of buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.9], 1.5 mM $MgCl_2$, 0.2 mM EDTA–0.5 mM NaF, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The suspension was made up to 0.42 M NaCl and frozen rapidly. After thawing and incubation at 4°C for 20 min, the suspension was centrifuged at 40,000 rpm for 20 min in a Beckman TL 100 benchtop ultracentrifuge. The clear supernatant was removed and frozen in aliquots.

Gel retardation. Gel retardation assays were performed by the method of Lees et al. (40). Ten micrograms of total protein was used per lane unless otherwise stated. Competitor oligonucleotides were added at approximately 100-fold excess. Where indicated in the text, sodium deoxycholate (DOC) was added to whole extract to a final concentration of 0.8%. After 10 min of incubation, extracts were made up to 1.5% Nonidet P-40 and then used in gel retardation assays as described. When antibodies or glutathione *S*-transferase (GST) fusion proteins were used, they were preincubated for 15 min at room temperature with the extract before addition of the binding buffer.

The E2F oligonucleotide probe sequences were ATTTAAG TTTCGCGCCCTTTCTCAA (wild type) and ATTTAAGTT TCGATCCCTTTCTCAA (mutant [mutated portion underlined]).

Preparation of oligonucleotide beads. One hundred twenty-five micrograms of wild-type or mutant sequence E2F oligonucleotides was annealed with their complementary strands and treated with 40 U of T4 polynucleotide kinase for 2 h at 37°C. After enzyme inactivation and ethanol precipitation, the oligonucleotides were ligated with 500 U of DNA ligase (New England Biolabs) overnight at 14°C, extracted with phenol-chloroform, precipitated, and redissolved in 50 μl of water.

To prepare cyanogen bromide-activated Sepharose 4B (Pierce), 0.75 g of powder was washed with 1 mM HCl and 10 mM K_2HPO_4 and resuspended as a thick slurry in 10 mM K_2HPO_4. Wild-type or mutant ligated oligonucleotides were added to the activated beads and mixed at room temperature for 16 h. The beads were then washed in water, and the remaining groups were inactivated by incubation in 1 M ethanolamine (pH 8.0) with mixing. The slurry was washed in 10 mM and then 1 M potassium phosphate (pH 8.0), and then it was washed in 1 M KCl and stored in 10 mM Tris-Cl (pH 7.6)–0.3 M NaCl–1 mM EDTA.

Oligonucleotide bead precipitations. Egg HSS or extracts from *Xenopus* embryos or XTC cells (52) were supplemented with sonicated salmon sperm DNA to 0.15 μg/μl. Next, one-fifth volume of 5× binding buffer (40) was added and the mixture was incubated for 15 min at 4°C with rocking. Wild-type or mutant oligonucleotide beads (100 μl of packed volume per lane in Fig. 2, 150 μl of packed volume per lane in Fig. 7) were washed in 1× binding buffer and spun to dryness. Preincubated extracts were added to the beads, and the mixture was incubated at 4°C for 1 h. The beads were washed extensively in 1× binding buffer, and then proteins were eluted into sodium dodecyl sulfate (SDS)-polyacrylamide gel sample buffer. Proteins were separated on an SDS–10% polyacrylamide gel electrophoresis (PAGE) gel, transferred to a nylon membrane by Western blotting (immunoblotting), and probed with antibodies as described, with either undiluted hybridoma culture supernatants or polyclonal antibodies diluted 1:1,000. Detection was by enhanced chemiluminescence (Amersham). Stripping of Western blots was performed in 2% SDS–100 mM β-mercaptoethanol–62.5 mM Tris-HCl (pH 6.8) at 70°C for 30 min.

For treatment with lambda phosphatase, wild-type or mutant oligonucleotide beads were incubated in HSS as described above. After two washes twice in binding buffer, 50 μl of lambda phosphatase buffer and 2.5 μl of lambda phosphatase (see Fig. 3, lane 1 only) (New England Biolabs [13]) were added and the mixture was incubated for 3 h at 30°C with frequent mixing. Proteins were separated by SDS-PAGE and Western blotted as described above.

Other reagents. Antibodies raised against the C-terminal 40 amino acids of human E2F1 fused to GST and affinity purified were kindly provided by J. Schwarz and D. Johnson. Anti-DP-1 antibodies (anti-peptide a [22]) were kindly provided by N. B. LaThangue and coworkers. Anti-*Xenopus* cdk2 antibody, raised against the whole bacterially expressed protein, was kindly provided by T. Hunt. GST-human pRb (GST-Rb) (amino acids 379 to 928 [35]) and a fusion protein of human p107 and GST (GST-107) (amino acids 252 to 816 [20]) were kindly provided by K. Helin and N. Dyson, respectively.

RESULTS

E2F DNA Binding activity is present in *Xenopus* eggs. Initially, we wished to identify and characterize any activities from *Xenopus* eggs which complex to the binding site identified for mammalian E2Fs. Gel retardation assays were performed with interphase *Xenopus* egg extract (LSS) and an oligonucleotide containing an E2F binding sequence (9 [Fig. 1]). In *Xenopus* egg extract, only a single band of E2F DNA binding activity is evident (lane 2). This activity can be competitively inhibited by excess unlabelled oligonucleotide containing the wild-type E2F binding site (lane 3) but not by an oligonucleotide containing a mutant site (lane 4), which is unable to bind to human E2F (9), indicating that binding is specific for the E2F site. Thus, we designate this activity *Xenopus* E2F, which is consistent with the initial definition of mammalian E2F on the basis of its DNA binding activity. Treatment with DOC (1) has frequently been used to release E2F DNA binding activity from complexes with other proteins (2). The fastest-migrating form of E2F DNA binding activity after treatment with DOC is thought to be the smallest protein unit required to bind to the E2F consensus sequence, called free E2F here. To see whether this single bandshift activity in *Xenopus* eggs was

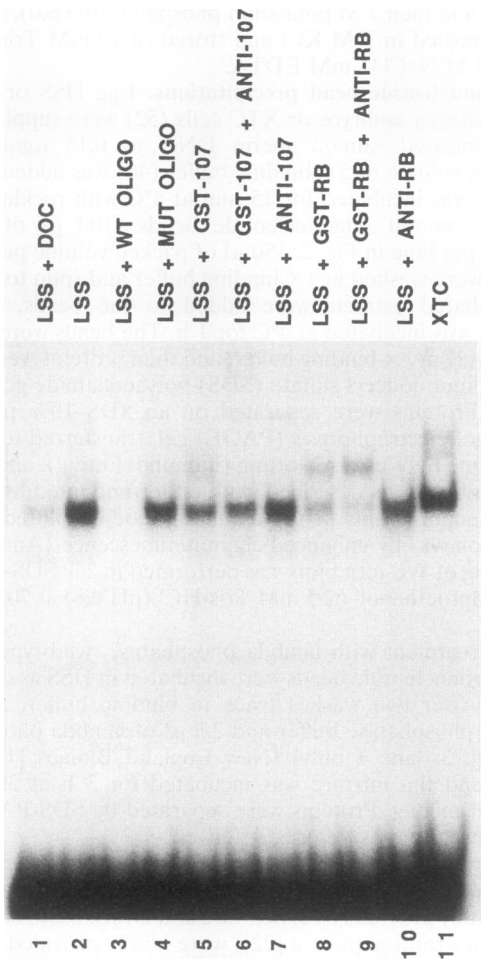

FIG. 1. Characterization of E2F DNA binding activity in *Xenopus* development. Ten micrograms of *Xenopus* egg extract (LSS) was used in gel retardation analysis with a radiolabelled oligonucleotide containing the E2F DNA binding sequence. LSS was incubated with DOC (lane 1 [see Materials and Methods]) or used alone (lane 2) or was preincubated with 800 ng of unlabelled wild-type (WT) E2F DNA binding sequence (lane 3), 800 ng of unlabelled mutant (MUT) E2F DNA binding sequence (lane 4) [see Materials and Methods]), 500 ng of GST-107 (lanes 5 and 6), 0.25 μl of anti-p107 polyclonal antiserum (lanes 6 and 7), 500 ng of GST-Rb (lanes 8 and 9), or 1 μl of anti-pRb monoclonal antibody culture supernatant XZ77 (30) (lanes 9 and 10). Lane 11 contains 10 μg of XTC cell extract.

composed of free E2F, egg extracts were treated with 0.8% DOC (see Materials and Methods) (lane 1). The single retarded band did not increase in mobility after DOC treatment, suggesting that the activity seen in *Xenopus* eggs is free E2F. However, after this treatment, the intensity of the retarded complex is greatly decreased, indicating that protein-protein interactions are required for strong binding of even free E2F to the E2F recognition sequence. Such interactions could be within heterodimeric complexes of E2F homologs which, only together, bind strongly to the site (5, 22, 28, 31). Sensitivity to DOC by free E2F may be peculiar to the *Xenopus* factor; binding of free E2F from human and mouse cells to DNA is not affected by DOC treatment (2, 58).

The tumor suppressor gene products pRb and p107 have been shown to complex with human E2F (see Introduction). Addition of GST-107 to LSS results in retardation of some E2F activity with a concomitant reduction in the amount of free E2F (lane 5). Addition of anti-p107 antibody further supershifts this retarded band (lane 6). Addition of GST-Rb also leads to a retardation of E2F bandshift activity and a reduction in the amount of free E2F (lane 8). Comparison of the intensities of the combined bands in lane 8 (LSS plus GST-Rb) with that of the band in lane 2 (LSS) shows that addition of GST-Rb not only produces a supershifted band but also results in a decrease in overall E2F DNA binding activity, indicating that these pRb-E2F complexes may only bind DNA weakly. GST alone was unable to shift free E2F (data not shown). Addition of an anti-Rb antibody, XZ77, does not produce a supershift but does decrease further the intensity of the free E2F band (lane 9), possibly by stabilizing the weakly binding pRb-E2F complex. Ray et al. (53) have described a 60-kDa protein required for pRb-E2F complexes to bind to DNA. If so, this protein may be absent or present at only low levels in *Xenopus* eggs. The single free E2F band from *Xenopus* eggs is not affected by these antibodies against pRb and p107. Thus, unlike mammalian tissue culture cells, E2F is present in only the free form in *Xenopus* eggs (as detected by gel retardation assay, but also as described later), although this form is capable of complexing to the tumor suppressor gene products pRb and p107.

We wished to investigate whether E2F DNA binding activity in XTC cells from a much later developmental stage (metamorphosing frogs) differed significantly from that found in eggs. Extracts were prepared from asynchronously growing XTC cells (52) and used in the same E2F gel retardation assay (lane 11). In addition to an intense band running close to that of free E2F from *Xenopus* eggs, two more-slowly-migrating bands were visible. It has not been possible to demonstrate pRb as a component of these more-slowly-migrating bands. This could be due to real but low levels of pRb binding. Another complication is that, under certain circumstances, some nonspecific antibodies have produced supershifts when included in bandshift assays. In addition, specific immunoprecipitation followed by DOC release of free E2F, subsequently assayed by gel retardation, is not possible because, as stated above, incubation of E2F with DOC sufficient to dissociate it from other complexing molecules results in free E2F which binds to DNA only very poorly and is barely detectable by gel retardation assay. Therefore, another very sensitive method of identifying E2F-associated proteins was used, namely elution and Western blotting of proteins bound to beads coupled to the E2F consensus DNA binding sequence.

Beads coupled to wild-type or mutant E2F DNA binding sequence (see Materials and Methods) were incubated in extracts from XTC cells and washed extensively, and then the bound proteins were eluted and separated by SDS-PAGE. After Western blotting, membranes were probed with the anti-pRb XZ160 monoclonal antibody (30) or polyclonal antibodies raised against *Xenopus* cdk2. Both pRb and cdk2 were found bound to wild-type E2F sequence beads but not mutant beads (Fig. 2). However, no cyclin A was found associated with E2F consensus sequence beads with the antibodies available (data not shown). Antibodies which recognize *Xenopus* p107 were unavailable for this study. XTC cells contain more-slowly-migrating forms of complexed E2F. Because E2F is complexed with pRb and cdk2 in XTC cells as shown by Western blotting in Fig. 2, we propose that pRb and cdk2 bind E2F, resulting in the two more-slowly-migrating species. These run with retarded mobility similar to that of the E2F–GST-Rb and E2F–GST-107 complexes seen in lanes 5 and 8 of Fig. 1, which contain fusion proteins of approximately the same molecular weight (see Materials and Methods for fusion proteins used).

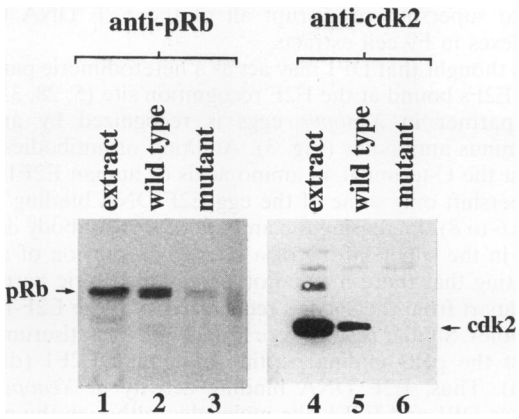

FIG. 2. E2F is complexed to pRb and cdk2 in XTC cells. Sepharose beads coupled to oligonucleotides containing the wild-type E2F DNA binding sequence (lanes 2 and 5) or the mutant E2F DNA binding sequence (lanes 3 and 6) were prepared. Specific DNA binding proteins were precipitated from 75 μl of XTC cell extract (25 μg of total protein per μl) and separated by SDS PAGE on a 10% polyacrylamide gel. Lanes 1 and 4 show 65 μg of total protein of XTC cell lysate. Proteins were Western blotted and were detected with XZ160 (30), a monoclonal antibody known to cross-react with *Xenopus* pRb, or were detected with an antibody raised against *Xenopus* cdk2.

In addition, treatment of XTC cell lysates with DOC results in a gel-retarded band running with the migration of the free form of E2F found in *Xenopus* eggs (data not shown), indicating that these more-slowly-migrating bands are composed of free E2F complexed to other molecules. Thus, while *Xenopus* eggs contain only a single gel-retarded band of E2F activity, E2F is found in two more-slowly-migrating forms in XTC cells, in which it complexes with pRb and cdk2.

Two distinct E2F-like molecules bind to the E2F consensus sequence. Recently, it has been proposed that E2F-like molecules bind to DNA as a heterodimer (5, 28, 31), and several E2F-like proteins have been identified (27, 31, 34, 56). Another E2F-like molecule, DP1, has recently been cloned (22) and has been shown to bind cooperatively to E2F1 (5, 28). An antiserum raised against the N terminus of DP1, which shares no homology with E2F1 (anti-peptide a [22]; gift of N. B. La Thangue), recognized a major band of 50 KDa in *Xenopus* egg extract on Western blotting, which was of similar size to the mouse protein and which we assume to be the *Xenopus* homolog of DP1 (data not shown). Beads coupled to wild-type and mutant E2F oligonucleotide consensus sequences were used to purify proteins from *Xenopus* egg lysate which bind specifically to the E2F recognition site. Proteins eluted from these beads were separated by SDS-PAGE and then Western blotted and probed with antibodies raised against mouse DP-1 (antibody a [22]) or antibodies raised against the C-terminal 40 amino acids of human E2F1 (anti-E2F [Fig. 3]). A protein of approximately 50 kDa was recognized by the antibody against DP-1, bound to the wild-type but not mutant oligonucleotide beads, which is of a size similar to the mouse protein (22). In addition, a doublet of between 54 and 57 kDa specifically bound to the wild-type E2F sequence was detected by the anti-E2F antibodies described above. This same doublet is recognized by a rabbit antiserum raised against the pRB-binding peptide of human E2F1 (data not shown).

To determine whether this doublet represented two distinct proteins or a single species which had been secondarily modified by phosphorylation, proteins were bound to wild-type

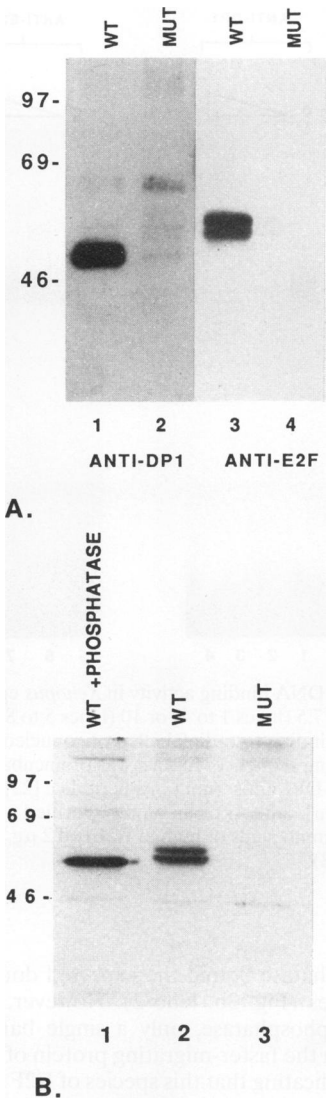

FIG. 3. Identification of E2F DNA binding proteins. (A) Wild-type (WT) sequence beads (lanes 1 and 3) (see Fig. 2) or mutant (MUT) sequence beads (lanes 2 and 4) were used to precipitate specific DNA binding proteins from 50 μl of egg LSS, which were separated by SDS-PAGE on a 10% polyacrylamide gel and Western blotted. Proteins were detected with an anti-DP1 antiserum (anti-peptide a [22]; lanes 1 and 2) or an affinity-purified antibody against the C-terminal 40 amino acids of human E2F1 (lanes 3 and 4). The positions of migration of the molecular mass markers are shown in kilodaltons (Rainbow markers; Amersham). (B) Proteins were precipitated with wild-type (lanes 1 and 2) or mutant (lane 3) sequence beads. The beads were washed extensively, spun to dryness, and resuspended in lambda phosphatase buffer. The beads were incubated in the presence of lambda phosphatase (lane 1 [see Materials and Methods]) or in its absence (lanes 2 and 3). Bound proteins were separated by SDS-PAGE, Western blotted, and then probed with an antibody against the C-terminal 40 amino acids of human E2F1.

oligonucleotide beads and then treated with lambda phosphatase, an enzyme capable of removing phosphates from serine, threonine, and tyrosine residues (13). Proteins bound to the beads were then separated by SDS-PAGE, Western blotted, and probed with antibodies raised against the C terminus of E2F1 (see above). Beads incubated in the absence of

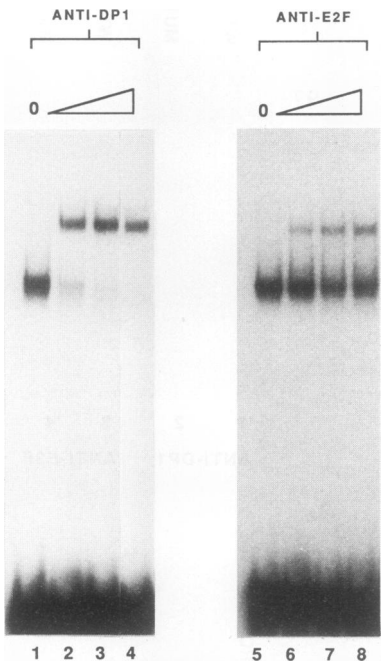

FIG. 4. E2F DNA binding activity in *Xenopus* eggs contains several proteins. Either 7.5 (lanes 1 to 4) or 10 (lanes 5 to 8) μg of LSS protein was tested for binding to radiolabelled oligonucleotide containing the E2F DNA binding sequence. Extract was preincubated with increasing amounts of anti-DP1 antiserum (anti-peptide a [22]; 0.25 μl, lane 2; 0.5 μl, lane 3; 0.75 μl, lane 4) or an affinity-purified antibody against the C-terminal 40 amino acids of human E2F1 (0.2 μg, lane 6; 0.4 μg, lane 7; 0.6 μg, lane 8).

lambda phosphatase bound the expected doublet of immunoreactive species (Fig. 3b, lane 2). However, after incubation with lambda phosphatase, only a single band was detected, which ran with the faster-migrating protein of the doublet (Fig. 3b, lane 1), indicating that this species of E2F is present in both phosphorylated and unphosphorylated or underphosphorylated forms in *Xenopus* eggs.

Thus, in *Xenopus* egg extracts, at least two E2F-like proteins are found bound specifically to the E2F consensus sequence. One of these appears to show homology to DP1, a molecule originally identified as part of a DNA binding complex which becomes downregulated on cellular differentiation of F9 cultured mouse cells (38, 39). The other protein shows epitope homology to the C terminus of E2F1 and can be modified by phosphorylation.

E2F DNA binding activity contains several E2F-like molecules. To investigate whether all of the DNA binding activity can be accounted for by the E2F and DP1 homologs shown in Fig. 2, gel retardation supershifting assays were performed with increasing amounts of anti-DP1 or anti-E2F1 (C terminus) antibodies (Fig. 4). On the addition of anti-DP1 antiserum, the single retarded band in LSS (lane 1) becomes substantially supershifted with a concomitant decrease in the faster-migrating E2F form (lanes 2 to 4). Increasing the amount of anti-DP1 antibody added results in essentially all of the free E2F being supershifted, indicating that a DP1 homolog is a component of all E2F activity in *Xenopus* eggs. Anti-DP1 antiserum has a similar effect on both upper and lower retarded bands in XTC cell lysates (data not shown). Girling et al. (22) showed that this anti-DP1 antiserum was able to supershift or disrupt all of the E2F DNA binding complexes in F9 cell extracts.

It is thought that DP1 may act as a heterodimeric partner of other E2Fs bound at the E2F recognition site (5, 28, 31). One such partner in *Xenopus* eggs is recognized by anti-E2F C-terminus antibodies (Fig. 3). Addition of antibodies raised against the C-terminal 40 amino acids of human E2F1 is able to supershift only some of the egg E2F DNA binding activity (lanes 6 to 8). Increasing the amount of this antibody does not result in the supershifting of a greater proportion of activity, indicating that there may be other heterodimeric partners of DP1, apart from the species recognized by these E2F-reactive antibodies. Similar results were found with an antiserum raised against the pRb-binding peptide of human E2F1 (data not shown). Thus, E2F DNA binding activity in *Xenopus* eggs contains DP1 and E2F1-like molecules, although the proteins recognized by the C-terminal E2F1 antibodies account for only some of the DNA binding activity observed. This gives an indication of the complexity of E2F DNA binding proteins in *Xenopus* eggs.

***Xenopus* E2F DNA binding activity does not change through the cell cycle.** E2F has been implicated in controlling the G_1-to-S phase transition by complexing, in a cell cycle-dependent manner, to the products of tumor suppressor genes, to cyclins, and to their dependent kinases (see the introduction). LSS, used for Fig. 1, is an interphase extract. However, extracts of *Xenopus* eggs have been developed which can perform multiple cell cycles in vitro incorporating alternating S and M phases (45, 46). Timing of mitosis can easily be monitored by morphological changes occurring in added sperm nuclei, including chromosome condensation and nuclear envelope breakdown, and such extracts were used to investigate potential changes in E2F DNA binding activity on progression through the cell cycle in this early embryonic system.

A cycling extract was prepared in which sperm nuclei entered mitosis at 60 min and had returned to interphase by 80 min (Fig. 5, a and a′ [uniform interphase nucleus with intact nuclear membrane], b and b′ [condensed chromosome arms indicative of mitosis and absence of a nuclear envelope], and c and c′ [uniform interphase nucleus with intact nuclear membrane]). A single gel-retarded band of E2F DNA binding activity was observed at all stages of the embryonic cell cycle, indicating that the DNA-bound E2F from eggs does not form cell cycle-regulated complexes with other proteins, as has been described for mammalian E2F. Interphase extracts (LSS) were more closely assayed for the onset of DNA replication by monitoring for onset of incorporation of biotinylated dUTP into the sperm template. Again, no more-slowly-migrating E2F DNA binding complexes were observed either before or after DNA replication had begun (data not shown). Thus, unlike E2Fs in cycling mammalian tissue culture cells, the E2F detected here remains essentially free at all points in the simplified *Xenopus* embryonic cell cycle.

Developmental control of E2F DNA binding. E2F DNA binding activity is abundant in extracts of *Xenopus* eggs which are inactive for transcription. Such accumulation of E2F activity may represent stockpiling of the protein to await the onset of zygotic transcription which occurs at MBT (stage 8/9) (36, 48, 49). As described, only a single gel retardation activity is identified in *Xenopus* eggs as opposed to the multiple more-slowly-migrating E2F activities seen at different points in the cell cycle of cultured mammalian cells (see the introduction), which are indicative of the association with other cell cycle molecules (e.g., pRb, etc.). One may expect the onset of zygotic transcription in *Xenopus* embryos to result in the appearance of these complexed E2F activities, which are

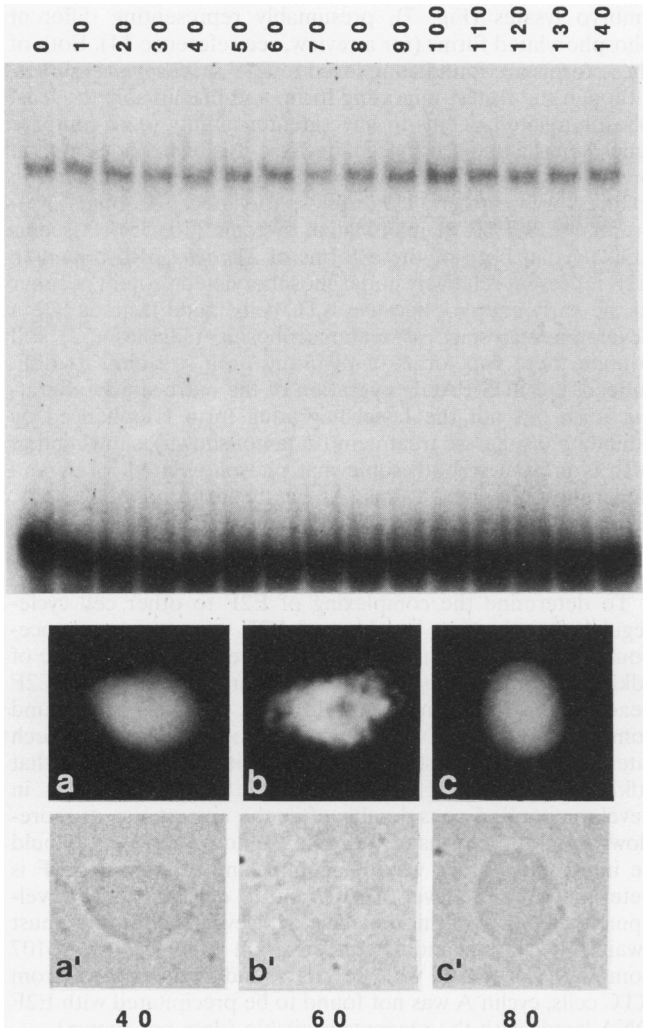

FIG. 5. E2F DNA binding through the cell cycle. Extracts from *Xenopus* eggs were prepared which undergo several cell cycles in vitro. Samples were taken every 10 min (10 to 140 min) and 10 μg of protein from each sample was assayed for binding to a radiolabelled oligonucleotide containing the E2F DNA binding sequence. Panels a, b, and c show total DNA staining with 5 μg of Hoechst 33258 per ml, while panels a′, b′, and c′) show phase-contrast images of *Xenopus* sperm nuclei at 5 ng/μl in cycling extracts when samples were taken at 40 (a and a′), 60 (b and b′), and 80 (c and c′) min.

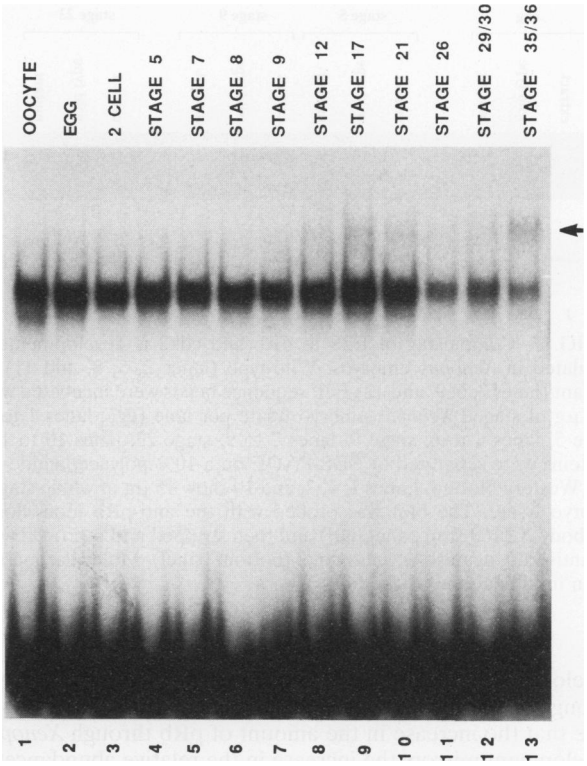

FIG. 6. E2F activity through development. Extracts were prepared from developmentally staged *Xenopus* embryos (see Materials and Methods). Ten micrograms of total protein was assayed for binding to radiolabelled oligonucleotide containing the E2F DNA binding sequence.

thought to control the cell cycle-dependent transcription of E2F-responsive genes (for a review, see reference 26).

To address these questions, extracts were prepared from developmentally staged embryos (see Materials and Methods) and used in a gel retardation assay with the E2F consensus DNA binding sequence (Fig. 6). The single E2F DNA binding activity (free E2F shown in Fig. 1) is present throughout the developmental stages studied. The activity remains at approximately constant levels up to approximately stage 26 (lane 11), when DNA binding activity begins to decrease. This decrease is clearly seen by stage 35/36 (lane 13). In addition to this fastest-migrating activity, around stage 17 (lane 9), a further, prominent, more-slowly-migrating DNA binding activity is observed through stage 35/36. This more-slowly-migrating form can occasionally be resolved into two more-slowly-migrating bands (data not shown) bearing striking similarity to those found in XTC cells (Fig. 1, lane 11). These staged embryo extracts were also used in a gel retardation assay with the serum response factor DNA recognition sequence (42), and DNA binding was constant for all of these developmental stages, showing equal extraction of proteins from these embryos (data not shown). Thus, while overall E2F DNA binding activity decreases through development, the proportion of that activity which is complexed to other proteins, and so migrates more slowly, increases (compare lanes 9 and 13).

It was of considerable interest to determine the protein components of these more-slowly-migrating forms. For reasons stated above, we chose to investigate proteins complexed with E2F by using precipitation with beads coupled to the E2F consensus DNA binding sequence.

pRB but not cdk2 complexes to E2F early in development. To identify the slower components of the more-slowly-migrating forms of E2F DNA binding activity in late stages of *Xenopus* development, extracts were prepared from eggs and developmentally staged embryos (see Materials and Methods). These extracts were incubated with Sepharose beads coupled to wild-type or mutant E2F consensus oligonucleotides (Fig. 2). Proteins eluted from the beads were separated by SDS-PAGE and were Western blotted.

To determine if pRb complexes with E2F, this blot was probed with an antibody known to recognize *Xenopus* pRb, XZ160 (30). Surprisingly, in this assay, *Xenopus* pRb is found complexed to E2F at all developmental stages. However, pRb is expressed only at very low levels in *Xenopus* eggs and early embryos (18 [Fig. 7]), and, concomitantly, only a very small amount is found complexed to E2F in eggs and very early in

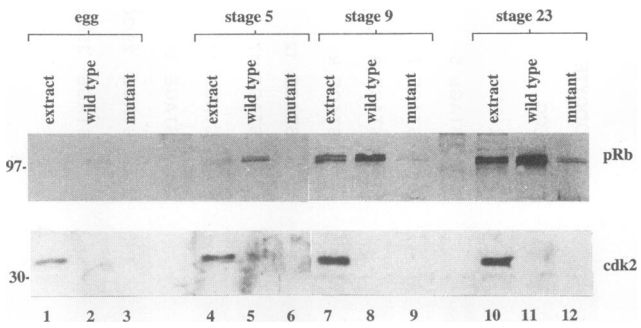

FIG. 7. Complexing of E2F to pRb and cdk2 is developmentally regulated in *Xenopus* embryos. Wild-type (lanes 2, 5, 8, and 11) or mutant (lanes 3, 6, 9, and 12) E2F sequence beads were incubated with 500 μg of staged *Xenopus* embryo lysate per lane (egg, lanes 1 to 3; stage 5, lanes 4 to 6; stage 9, lanes 7 to 9; stage 23, lanes 10 to 12). Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and Western blotted. Lanes 1, 4, 7, and 10 show 15 μg of whole staged embryo lysate. The blot was probed with the anti-pRb monoclonal antibody XZ160 (top panel [30]) and then stripped and reprobed with an antiserum against *Xenopus* cdk2 (bottom panel). Molecular mass is given in kilodaltons on the left.

development. The amount of pRb complexed to E2F increases through development as does its expression. It is important to note that the increase in the amount of pRb through *Xenopus* development mirrors the increase in the relative abundance of the more-slowly-migrating forms of E2F DNA binding activity seen in Fig. 6. However, the lack of a reliable antibody to perform supershifting analysis, or perhaps the fact that pRb may be only a small component of the more-slowly-migrating activities, makes it impossible to show directly that pRb is a component solely of the more-slowly-migrating form of E2F. Nevertheless, it is for the following reasons that we believe this to be the case. First, in eggs and early embryos, where more-slowly-migrating forms of E2F activity are not seen, only a very small amount of pRb is complexed to E2F; the amount of protein detected by Western blot as shown in Fig. 7 is concentrated from 50 times the amount of extract used for a single gel retardation assay. However, by stage 23, when a more-slowly-migrating E2F activity is clearly present, the amount of E2F bound to pRb has increased dramatically (lanes 10 to 12). Second, despite an increase in pRb binding to E2F sequence beads at early points in development (between the egg stage and stage 9), there is no significant change in the fastest-migrating E2F activity, suggesting that this form is not increasingly harboring pRb. Third, DOC treatment (Fig. 1), shown previously to dissociate pRb from E2F to produce a faster-migrating gel-retarded complex in mammalian cells (2), produces no faster-migrating E2F species in *Xenopus* egg extracts, indicating that this is, indeed, free E2F, while extracts from embryos at later stages and XTC cells with much greater amounts of E2F complexed to pRb do have more-slowly-migrating gel-retarded E2F species, which disappear on treatment with DOC (data not shown). Finally, free E2F from *Xenopus* eggs and embryos runs at approximately the position of free E2F from human ML1 cells in gel retardation assays, indicating that they have similar sizes (data not shown).

Two further points about E2F binding to pRb should be noted. Figure 7 shows that only a small proportion of pRb binds strongly to E2F; 30 times as much extract is used for the precipitation on oligonucleotide beads as is used in lanes 1, 4, 7, and 10 to show relative amounts of pRb through development. In addition, at least two forms of pRb are seen in total embryo lysates (Fig. 7), presumably representing different phosphorylated forms (for a review, see reference 14). Both of these forms are found complexed to E2F in *Xenopus* embryos, although the fastest-migrating form, and presumably the least phosphorylated, seems to have greater affinity (e.g., compare lanes 4 and 5 and see Fig. 2), because it is overrepresented in precipitations with E2F beads compared with its abundance in total embryo extracts. Underphosphorylated pRb binds preferentially to E2F in mammalian systems (10, 55). It seems unlikely that both of these forms of *Xenopus* pRb bound to E2F represent relatively underphosphorylated protein peculiar to the early embryo, because XTC cells made from as late a developmental stage as metamorphosing tadpoles (52) still contain these two forms of pRb and both still bind to E2F. Indeed, the SDS-PAGE migration of the more-slowly-migrating form but not the faster-migrating form is enhanced by lambda phosphatase treatment (data not shown), showing that pRb is present in both somewhat phosphorylated forms and underphosphorylated forms. As has been proposed recently, E2F may be able to bind strongly to underphosphorylated pRb but also binds weakly to phosphorylated pRb and that is the phenomenon we are observing here (58).

To determine the complexing of E2F to other cell cycle-regulated molecules, the blot of E2F consensus sequence-bound proteins was stripped and reprobed for the presence of cdk2 (Fig. 7). cdk2 was not detected in precipitations with E2F beads under these conditions. However, cdk2 has been found complexed to E2F in XTC cells made from embryos at a much later developmental stage (Fig. 2), so it seems possible that cdk2 complexes to E2F at detectable levels only later in development and considerably after the appearance of more-slowly-migrating forms of E2F DNA binding activity. It would be interesting to see whether complexing of cdk2 to E2F is determined by the level of p107 and to determine the developmental control of this molecule. However, such studies must await the cloning and characterization of a *Xenopus* p107 homolog. Consistent with results found with extracts from XTC cells, cyclin A was not found to be precipitated with E2F DNA beads with the reagents available (data not shown).

DISCUSSION

E2F is an activity with potentially interesting roles in transcription, cell cycle control, and differentiation. We have characterized an E2F activity in the frog *Xenopus laevis*, which provides well-documented cell cycle and developmental systems. We have found a single E2F DNA binding activity in *Xenopus* eggs with many of the characteristics of mammalian E2F, including the ability to complex with pRb and p107 (Fig. 1). This E2F activity contains homologs of human E2F1 and DP1, as detected by antibodies (Fig. 3 and 4), and one E2F-like component is further modified by phosphorylation (Fig. 3). E2F DNA binding does not change or become significantly complexed during the early embryonic cell cycle (Fig. 5). However, later in development, E2F is found in both free and complexed forms (Fig. 6). The appearance of more-slowly-migrating complexes coincides with the increased binding of E2F to pRb (Fig. 7), an identified component of mammalian E2F complexes (see the introduction), and both pRb and cdk2 can be found complexed to E2F in XTC cells made from metamorphosing tadpoles much later (Fig. 2). However, in addition to these similarities, there are significant differences between the *Xenopus* and mammalian E2F DNA binding activities.

Abundant E2F is found in the uncomplexed free form in eggs at all stages of the cell cycle (Fig. 4). However, E2F

cannot be playing its proposed role in transcriptional activation, because *Xenopus* embryos are transcriptionally inactive until MBT at approximately stage 8.5 (36, 48, 49). If E2F functions solely in transcriptional control through the cell cycle, it may be unnecessary to regulate E2F activity by binding to tumor suppressor gene products at this early stage. pRb is present only at very low levels in *Xenopus* eggs, but this amount increases steadily through development and over the MBT (18 [Fig. 7]). If pRb complexing exists solely to regulate the activity of E2F through the cell cycle, one might expect prominent complexes of E2F with tumor suppressor genes to appear at the onset of zygotic transcription at the MBT. However, although some pRb complexes are present earlier (Fig. 7), we consistently see that retarded complexed forms of E2F activity are barely detectable by gel retardation assay even at stage 12, several hours after MBT, and it is not until stage 17, approximately 12 h after MBT, that prominent retarded forms are seen. We believe these retarded forms represent increasingly abundant pRb complexes with E2F (Fig. 6). Thus, in gastrulating embryos, around stage 10, which have essentially normal cell cycles with short G_1 and G_2 phases (23), the DNA-bound E2F may perform its normal cellular function when complexing relatively small amounts of pRb and without complexing in detectable levels to other cell cycle-regulated molecules, e.g., cdk2. As development progresses, an increasing proportion of E2F is found coupled to pRb. Changes in pRb complexing could represent the interesting possibility of differences in cell cycle control of embryos early in development and just post-MBT with those at a more advanced developmental stage. Indeed, Graham and Morgan (23) have shown that G_1, S, and G_2 phases lengthen considerably as development progresses. Alternatively, such complexing may become increasingly important at later stages of development, when differentiation predominates. In addition to the appearance of more-slowly-migrating complexes, the overall levels of E2F DNA binding activity are downregulated through *Xenopus* development (Fig. 6).

The decrease in E2F DNA binding activity through development in *Xenopus* embryos shows some parallels with the decrease in E2F activity which accompanies the differentiation of F9 embryonal carcinoma cells (38, 39, 54). However, the F9 system uses the experimental chemical induction of differentiation of cultured cells, while we look at E2F activity under physiological conditions during *Xenopus* embryogenesis. In addition, differentiation of F9 cells results in the downregulation of both free and complexed forms of E2F, while progressive development in *Xenopus* embryos results in downregulation of overall E2F activity but shows the appearance and increase in relative abundance of complexed compared with free E2F. The behavior of these E2F forms much later in development has not been carefully investigated here. However, the XTC cell line derived from later metamorphosing embryos contains a substantial amount of E2F activity, both free and complexed (Fig. 1). It is not clear to what extent this represents the embryonic stage from which the cells were derived or the fact that this is a stably maintained cell line.

It is interesting that in both XTC cells (Fig. 2) and *Xenopus* embryo extracts (Fig. 7), a doublet of pRb immunoreactive species is detected, bound to E2F DNA consensus sequence beads. The antibody used for detection (XZ160 [30]) is not thought to cross-react with p107, at least in human cells. pRb is known to exist in multiple phosphorylated forms (8, 16) which vary through the cell cycle, and the underphosphorylated and hence fastest-migrating form found predominantly in G_1 is thought to exert its growth-suppressive effect (for a review, see reference 14). It has been shown that this underphosphorylated pRb preferentially complexes with E2F (10, 55), although Shirodkar et al. (58) have also found E2F-pRb complexes in synchronized S phase cells which presumably contain predominantly phosphorylated pRb. We see that both components of the pRb doublet in *Xenopus* embryos are able to bind to E2F consensus sequence beads, although the faster-migrating, less-phosphorylated form is proportionately overrepresented (Fig. 2, lanes 1 and 2, and Fig. 7, lanes 4 and 5). It is unlikely that both of these bands represent underphosphorylated pRb, because XTC cells and later embryo extracts are made from an asynchronous population of cells with pRb in all states of cell cycle-driven phosphorylation and migration of the more-slowly-migrating form can be enhanced with lambda phosphatase (data not shown). Instead, our results indicate that both phosphorylated pRb and underphosphorylated pRb are able to complex to E2F in *Xenopus* embryos, although the former does so at a lower level of efficiency. This may imply that pRb can exert a transcriptional repression function via E2F (for a review, see reference 26), extending from G_1 phase into S phase.

E2F DNA binding complexes in XTC cells contain the cyclin-dependent kinase cdk2. However, this kinase cannot be detected in E2F DNA binding activity in *Xenopus* eggs or embryos up to stage 23 (Fig. 7), indicating that complexing of pRb and cdk2 to E2F may be regulated separately through development of the *Xenopus* embryo. In addition, other potential components of these complexes, by analogy to mammalian tissue culture cells, have not been identified; cyclin A could not be detected as a component of the late embryonic E2F DNA binding activity. These components may be present but remain undetected, although the antibodies used for detection were raised against and are known to recognize *Xenopus* cyclin A in Western blots. Alternatively, differences in E2F complexes found during *Xenopus* embryogenesis and those in mammalian cultured cells may represent physiologically meaningful and important differences in cell cycle control in these two systems.

E2F DNA binding activity in *Xenopus* eggs seems to be composed of several E2F-like proteins; proteins antigenically related to human E2F1 and mouse DP1 are present but do not seem to account for all of the E2F DNA binding activity observed in *Xenopus* eggs (Fig. 3A and 4). Another level of complexity to add is the existence of an E2F homolog which can exist in different phosphorylated forms (Fig. 3B). The ability of different members of the E2F family to heterodimerize (5, 28) can result in multiple combinations of subunits (31), each with a potentially altered function. In addition, since this work was completed, Lees et al. (41) and Ivey-Hoyle et al. (32) have identified other members of the E2F family. It is not known how these homologs are related to E2F species found in *Xenopus* embryos. It would be interesting to determine whether potential changes in phosphorylation level or heterodimeric pairing affect cell cycle progression or complexing to other molecules or whether they occur in a developmentally regulated fashion.

Thus, E2F activity is abundant in *Xenopus* eggs before the onset of zygotic transcription and shows considerable protein complexity; the E2F DNA binding activity contains at least three proteins, one of which can exist in two different phosphorylated states. E2F activity shows interesting behavior through the simplified embryonic cell cycle, where it remains uncomplexed at all times. However, E2F activity does become complexed to the cell cycle-regulated molecule pRb later in development and also shows a marked developmentally regulated decrease in E2F DNA binding activity. These results indicate that E2F activity in *Xenopus* embryos may be com-

posed of a variety of components which are sensitive not only to the cell cycle but also to developmental signals.

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