Differential regulation of E2F *trans*-activation by cyclin/cdk2 complexes

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The mammalian transcription factor E2F plays a critical role in the expression of genes required for cellular proliferation. To understand how E2F is regulated, we have developed a reconstituted in vitro transcription assay. Using this E2F-responsive assay, we can demonstrate that E2F-mediated transcription can be directly repressed by the tumor suppressor protein pRB. This inhibition is abolished by phosphorylation of pRB with either cyclin A/cdk2 or cyclin E/cdk2. However, these cyclin/kinase complexes exhibit differences in the ability to phosphorylate E2F. Only cyclin A/cdk2 can phosphorylate E2F effectively, and this phosphorylation abolishes its ability to bind DNA and mediate *trans*-activation. Thus, this in vitro transcriptional assay allows activation and inactivation of E2F transcription, and our findings demonstrate how transcriptional regulation of E2F can be linked to cell cycle-dependent activation of kinases.

[Key Words: E2F-1/DP-1; cyclin-kinase complex; in vitro transcription; cell cycle regulation]

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Recent work from several laboratories has linked the regulation of cell cycle progression and gene expression. In mammalian cells much of this work has focused on the transcription factor E2F. This transcription factor has been implicated in the temporal regulation of certain genes required for cellular proliferation, and E2F DNA-binding sites have been identified in the promoters of the many genes whose products play key roles in proliferation control. These include dihydrofolate reductase (DHFR), DNA polymerase α , *cdc2*, *c-myc*, and *b-myc* genes (Blake and Azizkhan 1989; Thalmeier et al. 1989; Pearson et al. 1991; Dalton 1992; Lam and Watson 1993). In the cases of the *c-myc*, *cdc2*, and DHFR promoters, the E2F-binding sites have been shown to be critical for the transcriptional activation of these genes (Blake and Azizkhan 1989; Hiebert et al. 1989; Thalmeier et al. 1989; Dalton 1992). In studies with the DHFR gene, a single E2F-binding site is sufficient to confer the correct temporal expression of this gene (Means et al. 1992; Slansky et al. 1993).

Several critical regulators of growth have been shown to associate with E2F, and their connections with this transcription factor are now being elucidated. For example, the product of the retinoblastoma gene (pRB), a tumor suppressor protein, has been shown to interact with E2F both in vivo and in vitro (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991), and the net result of this association is the inhibition of E2F-mediated *trans*-activation

(Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992; Zamanian and La Thangue 1992; Flemington et al. 1993; Helin et al. 1993a). In addition, the pRB-related protein p107 has been shown to associate with E2F in the form of complexes containing cyclin E/cdk2 or cyclin A/cdk2 (Bandara et al. 1991; Cao et al. 1992; Devoto et al. 1992; Lees et al. 1992; Shirodkar et al. 1992). As shown for pRB, transient expression of p107 decreases E2F-mediated transcriptional activation (Schwarz et al. 1993; Zamanian and Thangue 1993; Zhu et al. 1993). It is not known how another pRB-related factor, p130, which has also been found in E2F complexes, affects the activity of E2F (Cobrinik et al. 1993).

Based primarily on transient expression experiments and cell cycle synchronization, a number of details of E2F regulation have emerged. For example, in addition to the transfection experiments with pRB and p107 showing repression of E2F-mediated transcription, it has been shown that E2F preferentially associates with the underphosphorylated form of pRB both in vivo and in vitro (Chellappan et al. 1991; Helin et al. 1992; Kato et al. 1993). Furthermore, certain E2F-1 mutants that no longer bind pRB are capable of *trans*-activation but are not inhibited by overexpression of pRB (Helin et al. 1993a). Taken together, these experiments suggest that pRB negatively regulates E2F activity. Moreover, it is known that the adenovirus E1A proteins can associate with pRB, p107, and p130 (Yee and Branton 1985; Harlow et al. 1986), resulting in the release of "free" E2F and an increase in transcription from E2F-responsive promoters (Yee et al. 1989; Bagchi et al. 1990). These results

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suggest that uncomplexed E2F is the active transcription factor and that E2F activity is down-regulated, at least in part, through its associations with other proteins.

Further complicating our knowledge of E2F regulation is the fact that this transcription factor constitutes a family of related proteins, several of which have been recently cloned. This family includes the proteins E2F-1, E2F-2, and E2F-3 (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993). These proteins do not bind strongly to DNA by themselves; rather, they heterodimerize with a related protein DP-1, which has recently been cloned and shown to enhance dramatically the DNA-binding and *trans*-activation properties of the E2F members (Bandara et al. 1993; Girling et al. 1993; Helin et al. 1993b; Huber et al. 1993; Krek et al. 1993; Dynlacht et al. 1994). Moreover, it is likely that additional E2F family members exist (Helin et al. 1992; Lees et al. 1993), and a second DP-1-related protein has recently been identified in human cells (C.-L. Wu and E. Harlow, unpubl.). Although it has been shown that the form of E2F-1 found in association with pRB differs from the related proteins bound to p107 (Dyson et al. 1993), it is not yet clear what the functional differences are, if any, between these E2F-related proteins. To avoid confusion over nomenclature, we will refer to the E2F-1/DP-1 heterodimer as E2F and reserve the names E2F-1 and DP-1 to designate the polypeptide components of E2F.

The interactions between E2F and pRB are themselves regulated in complex ways during the course of the cell cycle. For example, an underphosphorylated form of pRB is present during the G₁ phase of the cell cycle, and it is this form of pRB that associates with E2F (Chellappan et al. 1991). Furthermore, it is known that pRB becomes hyperphosphorylated as cells enter S phase (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989). Although it is not known which kinases phosphorylate pRB in the cell, it has been shown that pRB can be phosphorylated in vitro by several cyclin-dependent kinases (cdks), including the cyclin A-, cyclin D-, and cyclin E-associated protein kinases (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993; Meyerson and Harlow 1994). Despite this knowledge, the functional consequences of pRB phosphorylation by these kinases are not yet fully known, nor is it known whether phosphorylation is the only regulatory control on pRB function.

The expression and activation of each of these cyclin/kinase complexes are also temporally regulated. Thus, cyclin E/cdk2 kinase activity appears during late G₁, whereas the cyclin A/cdk2 kinase appears near the onset of S phase (Giordano et al. 1989; Pines and Hunter 1990; Dulic et al. 1992; Pagano et al. 1992; Tsai et al. 1993). The idea that these kinase complexes represent good candidates for regulators of these cell cycle transitions is strengthened by the fact that both cyclin A/cdk2 and cyclin E/cdk2 complexes can associate with E2F (Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Koff et al. 1992; Lees et al. 1992; Pagano et al. 1992; Shirodkar et al. 1992). In addition, these E2F/cyclin/cdk2 complexes

also contain the p107 protein (Cao et al. 1992; Lees et al. 1992; Shirodkar et al. 1992). Interestingly, the p107 complexes found in G₁-phase cells contain predominantly cyclin E/cdk2, whereas those found in S phase contain primarily cyclin A/cdk2, suggesting a specific role for each of these cyclins during cell cycle progression (Lees et al. 1992). Yet the function of each of the individual proteins associated with E2F in these p107 complexes is currently unknown. Moreover, such functional questions are difficult to address in the context of an intact cell. Hence, it is possible that the proteins that make up these complexes may have opposing regulatory activities, acting both positively and negatively.

To clarify how E2F activity is regulated through the course of the cell cycle, we have established an in vitro transcriptional assay for E2F activity. This assay faithfully recapitulates E2F activation as mediated by E2F-1 and DP-1, and activation is fully repressed by the stoichiometric binding of pRB to E2F. The assay enabled us to test several hypotheses regarding pRB repression of E2F activity, as well as the functional relationships between E2F activity and various cyclin/cdk2 complexes. By virtue of this assay, we have shown that phosphorylation of pRB by the cyclin/cdk complex is sufficient to abrogate E2F transcriptional repression. Furthermore, phosphorylation by cyclin A/cdk2, but not by cyclin E/cdk2, down-regulates E2F DNA-binding and transcriptional activity. These data suggest how activation of various cyclin/cdk complexes at appropriate points in the cell cycle is linked to both the activation and inactivation of specific E2F transcriptional events.

Results

Transcriptional activation by E2F in vitro

To study regulation of E2F transcriptional activity, we have established a cell-free system with purified components. To this end, we expressed human E2F-1 and its dimeric partner DP-1, as well as potential regulators of this transcription factor, in bacteria and insect cells and purified them to near homogeneity (Fig. 1). The DNA-binding activity of recombinant E2F-1 and DP-1 was tested in gel mobility shift assays. As expected, E2F-1 alone was unable to bind DNA efficiently, but its activity was dramatically enhanced in the presence of purified DP-1 (Fig. 2A).

We then asked whether the E2F-1/DP-1 heterodimer could activate transcription in vitro using HeLa cell nuclear extracts. While these proteins functioned as potent activators in this assay, activation was not dependent on exogenous DP-1, although it was clearly dependent on the addition of E2F-1 (data not shown). We believe this lack of dependence on DP-1 was attributable to excess DP-1 in the HeLa extracts. To circumvent this possible complication, we decided to use a well-defined set of partially purified and recombinant basal transcription factors consisting of HeLa cell TFIIA, TFIID, TFIIF/H, RNA polymerase II, and recombinant TFIIB and TFIIE. Each of these factors is required for efficient basal tran-

Figure 1. Purified proteins used in this study. Recombinant proteins purified as described in Materials and methods were separated on a 9% SDS-PAGE gel and stained with silver. Numbers at left refer to the sizes (in kD) of molecular mass markers in lane 1. Subsequent lanes contain the following proteins: (lane 2) GST-DP-1; (lane 3) E2F-1, tagged at the carboxyl terminus with the three-amino-acid tubulin epitope; (lane 4) histidine-tagged pRB; (lane 5) cyclin A/cdk2 complex; (lane 6) cyclin A/cdk2-dn complex with a dominant-negative version of cdk2; (lane 7) cyclin E/cdk2 complex. The pRB in lane 4 is in a hypophosphorylated state (Dowdy et al. 1993), and minor bands directly below full-length pRB represent proteolytic products of pRB. The two proteins of ~33 and 32 kD in lanes 5 and 7 represent cdk2 polypeptides, and the faster migrating band (resulting from phosphorylation of Thr-160; Desai et al. 1992) is the activated form of the enzyme. This activated form of cdk2 represents ~20% of the total cdk2 in the cyclin A/cdk2 complex and ~50% of cdk2 in the cyclin E/cdk2 complex. The multiple cdk2-dn bands in lane 6 are observed reproducibly; their identity was confirmed by specific retention on p13 *suc1*-agarose beads (data not shown). The reason for the heterogeneity of forms is unknown.

scription activity (Flores et al. 1992). Using these basal factors, we could obtain significant levels of E2F *trans*-activation with a template containing four E2F sites upstream of the E1B TATA box (E2F₄BCAT; Helin et al. 1993b). The E2F-1/DP-1 heterodimer consistently stimulated transcription from this template at least 12-fold (Fig. 2B, cf. lanes 1 and 3 and 4). Moreover, this activation was specific and required the presence of E2F-binding sites, as reactions directed by a template lacking E2F sites (BCAT, the parent plasmid of E2F₄BCAT; Lillie and Green 1989) failed to respond to addition of E2F-1/DP-1 (lanes 8,9). Both E2F-1 and DP-1 were required for maximal activation, because the addition of either factor alone did not significantly increase transcription (lanes 1,2 and 5,6). In addition, although we could show that E2F-1 strongly interacted with the TATA-binding protein (TBP) in vitro, activation by E2F required the TFIID

fraction, suggesting the involvement of TBP-associated coactivators found in this fraction (data not shown). The TFIIA fraction also appeared to be important for maximal E2F activation (B.D. Dynlacht and E. Harlow, unpubl.). Thus, we satisfied the requirements for an in vitro transcription assay dependent on recombinant E2F-1 and DP-1. Next, we asked if inhibition of E2F-mediated *trans*-activation could be reconstituted in vitro using a protein defined as a repressor in transfection experiments.

pRB represses E2F transcriptional activity

As a starting point, we asked whether the retinoblastoma gene product (pRB) could effectively repress E2F-mediated *trans*-activation in this assay. Using gel mobility shift analysis as a guide, we determined the minimal amount of pRB required to convert the E2F heterodimer quantitatively into the slower-migrating E2F-1/DP-1/pRB complex. For these purposes, we made use of recom-

Figure 2. In vitro transcriptional activation by E2F-1/DP-1. (A) Gel mobility shift analysis of recombinant E2F-1 and GST-DP-1 polypeptides showing that both are required for efficient DNA binding. ³²P-labeled E2F oligonucleotide was incubated with 5 ng of E2F-1 alone (lane 1) or in combination with 10 ng of GST-DP-1 (lane 2). (B) Optimal transcription from an E2F-responsive promoter requires both E2F-1 and DP-1. In vitro transcription reactions reconstituted with HeLa cell and recombinant basal factors were performed as described in Materials and methods using 200 ng of either the E2F₄BCAT template containing E2F sites (lanes 1–7) or the BCAT template (lanes 8,9), which lacks these sites. Additions to each reaction are indicated at the top. Lanes 1, 5, and 8 contain no activator, and lanes 2–4 contain a constant amount of E2F-1 (100 ng) and 50 ng (lanes 3,8,9) or 125 ng (lanes 4,6,7) of GST-DP-1. Subsequent experiments (data not shown) indicated that ~50 ng each of E2F-1 and GST-DP-1 gave optimal levels of activation. Levels of activation similar to, or greater than, those shown here (12-fold as determined by excising gel slices and scintillation counting) were obtained routinely.

binant histidine-tagged pRB purified from insect cells. This protein, which has been shown to be predominantly hypophosphorylated (Dowdy et al. 1993), was purified by affinity chromatography over a column to which a human papilloma virus E7 peptide had been linked. Here, a slight molar excess of pRB could quantitatively convert the E2F heterodimer to a slower migrating species (Fig. 3A). We then performed in vitro transcription reactions in parallel using comparable ratios of pRB to E2F employed in gel mobility shift experiments. By titrating increasing amounts of recombinant pRB into these reactions, we found that E2F activation could be efficiently repressed to basal transcription levels (i.e., levels obtained in the absence of E2F) by a similar amount of pRB required to "supershift" the E2F heterodimer quantitatively (Fig. 3B, lanes 3,4). Based on the following criteria, we were able to show that pRB repression of E2F was specific and that there were no effects from possible contaminating proteins in the pRB preparation. First, basal

transcription levels were not affected by the addition of pRB (lanes 5,6). Second, a control template (E4CAT) that had been shown previously to be unaffected by transient expression of the 12S E1A protein [and, hence, which should not be E2F responsive; (Phelps et al. 1991)] was tested and failed to respond to pRB (lanes 7,8). Third, in parallel experiments, pRB also failed to inhibit *trans*-activation by an unrelated transcriptional activator, GAL4-VP16 (Fig. 3C). As a final test, we have also purified the insect cell-derived pRB to homogeneity using affinity chromatography over glutathione *S*-transferase (GST)-E2F columns, and pRB purified in this way appeared to have a similar or slightly higher specific activity, perhaps in part because it was purified using more gentle, ionic conditions (data not shown). These experiments suggest that pRB can form a ternary complex with the E2F heterodimer in vitro and specifically and directly repress E2F transcriptional activation without a requirement for additional factors. To our knowledge, this rep-

Figure 3. pRB repression of E2F activation. (A) Gel mobility shift analysis of E2F-pRB complexes. Reactions were performed as described in Fig. 2 except that increasing amounts of recombinant pRB (lanes 2–6, containing 1, 2.5, 5, 10, and 25 ng of pRB in that order) were added to 10 ng each of E2F-1 and GST-DP-1. pRB alone fails to bind the E2F oligonucleotide even when the highest amount of pRB was used (data not shown). (B) Transcriptional activity of pRB-E2F complexes generated in vitro. In vitro transcription reactions were performed as described in Fig. 2. Lanes 1–6 contained 100 ng of E2F₁BCAT template; lanes 7 and 8 contained 500 ng of the control template E4CAT, which should not be subject to repression by pRB. Lanes 2–4 were supplemented with E2F-1 and DP-1. In addition, reactions contained 100 ng (lanes 3,5) or 225 ng (lanes 4,6,8) of pRB. Addition of <100 ng of pRB failed to repress E2F activation significantly (data not shown). (C) *Trans*-activation by GAL4-VP16 is not repressed by pRB. Transcription reactions were reconstituted using G₅BCAT (Lillie and Greene 1989) as described in B except that GAL4-VP16 was used instead of E2F. This activator protein consists of the DNA-binding domain of GAL4 fused to the activation domain of VP16 and has been described (Chasman et al. 1989). Reactions contained purified GAL4-VP16 (lanes 2,3) and 225 ng of pRB (lane 3).

resents the first demonstration of *in vitro* repression of a transcriptional activator by an associated protein that does not directly contact DNA.

Phosphorylation of pRB reverses its ability to repress E2F

It has been proposed that phosphorylation of pRB plays a critical role as a cell cycle switch. It is believed that such an event is closely linked with the transition through the G₁ phase of the cycle and is believed to abolish the growth inhibitory properties of pRB (Chellappan et al. 1991 and references therein; Hinds et al. 1992). One explanation resulting from these studies is that hyperphosphorylated pRB can no longer associate with E2F and is thereby unable to repress E2F-mediated gene expression required for cellular proliferation (Chellappan et al. 1991). The evidence suggesting such regulation is indirect and relies primarily on correlative data. To address the hypothesis that modification of pRB can act directly as a switch to regulate the cell cycle, we tested the activity of phosphorylated pRB in our *in vitro* assays.

Several cyclin-cdk2 combinations were tested for the ability to abrogate the association between pRB and E2F, including GST-cyclin A/cdk2 and cyclin E/cdk2, both of which have been shown to phosphorylate pRB *in vitro* (Ewen et al. 1993). Cyclin A/cdk2 and cyclin E/cdk2 complexes were generated by coinfection of insect cells and purification to apparent homogeneity (Fig. 1, lanes 5,7). In addition, complexes were prepared that contained GST-cyclin A and a dominant-negative cdk2 mutant (cdk2-dn; van den Heuvel and Harlow 1993) that is enzymatically inactive yet still capable of binding to cyclins (lane 6; the cdk2-dn virus was kindly provided by E. Lees, this paper). Such a mutant should serve as a negative control, because it is incapable of phosphorylating pRB. Both wild-type and mutant forms of cdk2 were tagged with the hemagglutinin (HA) epitope to facilitate purification; such a tag does not affect the activity of this kinase (Desai et al. 1992; van den Heuvel and Harlow 1993). It should be noted that the activated form of cdk2 (the faster migrating species; Desai et al. 1992) represents ~20% and 50% of the total cdk2 protein present in purified preparations of cyclin A/cdk2 and cyclin E/cdk2, respectively (Fig. 1). To confirm the activity of each of these purified cyclin/cdk2 complexes, kinase assays were performed. As expected, both cyclin A/cdk2 and cyclin E/cdk2 complexes possessed a potent histone H1 kinase activity, whereas the complex containing cdk2-dn was inactive (Fig. 4A, left). In addition, in accordance with previous findings, both cyclin A/cdk2 and cyclin E/cdk2, but not cyclin A/cdk2-dn, were able to phosphorylate recombinant pRB efficiently (Fig. 4A, right).

Initially, cyclin A/cdk2, cyclin E/cdk2, and cyclin A/cdk2-dn complexes were each incubated with purified pRB (enough to shift E2F quantitatively) either in the presence or absence of ATP, and phosphorylation was allowed to proceed. This modified pRB was then added to

E2F, and complexes were resolved by gel mobility shift analysis. Treatment by cyclin A/cdk2 or cyclin E/cdk2 abolished the pRB-E2F complex in an ATP-dependent fashion (Fig. 4B, lanes 3,4,8–15), and the composition of complexes before and after treatment was verified by antibody supershift experiments (data not shown). Treatment with increased amounts of the cyclin A/cdk2 complex also inhibited E2F DNA binding (lanes 3,10; see below). In contrast, similar amounts of the cyclin A/cdk2-dn complex failed to abrogate pRB binding to E2F; rather, it was capable of binding to the E2F-pRB complex, resulting in a complex of slightly reduced mobility (Fig. 4B; lanes 16–20). Furthermore, we could show that the effect of cdk2 on pRB binding was not attributable to nonspecific inhibition (by possible trace contaminants of cdk2-cyclin preparations), as it was reversible by the addition of the cdk2 inhibitor, p21 (also termed Cip1, WAF-1, Sdi1, and CAP20; El-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994). Addition of near-equimolar amounts of purified p21 could completely override the ability of this kinase to prevent association between pRB and E2F (lanes 5,6). Time-course experiments indicated that the E2F-binding ability of pRB was completely abolished by active kinases within 30 min at room temperature (data not shown). In addition, the activity of the cyclin A/cdk2 complex could disrupt RB-E2F complexes prebound to DNA with similar kinetics (data not shown). We have also asked whether these active kinases could abolish endogenous pRB-E2F complexes present in HeLa cell nuclear extracts. As observed in the reconstituted assay, active cyclin A/cdk2 and cyclin E/cdk2 complexes eliminated the endogenous pRB-E2F complex, and cyclin A/cdk2-dn promoted the formation of a higher order complex containing pRB (data not shown).

We then asked whether phosphorylation of pRB abolished its ability to repress E2F-mediated transcriptional activation in our reconstituted *in vitro* assay. pRB was preincubated in the presence of cyclin A/cdk2 or cyclin A/cdk2-dn under conditions promoting phosphorylation. We used the minimal amount of cyclin A/cdk2 complex required to convert completely the pRB-E2F supershift to free E2F (as determined above) without affecting E2F DNA-binding activity and a similar amount of the cyclin A/cdk2-dn complex, as judged by SDS-PAGE and silver staining. In addition, the ratios of cyclin/kinase to pRB and E2F were identical to those used in gel mobility shift experiments. After a 1-hr incubation at room temperature, aliquots of each reaction were removed and tested in both gel mobility shift and transcription assays (Fig. 5A,B). Under these conditions, pRB otherwise untreated retained the ability to supershift the E2F complex, and addition of cyclin A/cdk2 alone did not perturb the DNA-binding activity of the E2F heterodimer (see below). As before, cyclin A/cdk2 activity abolished the pRB supershift, and the dominant-negative version of the complex promoted a reduced mobility complex (Fig. 5A). Presumably, this results from a stable interaction between cyclin A/cdk2-dn and a component (or components) of the DNA/E2F/pRB complex.

Figure 4. pRB-E2F association in the presence of cyclin/kinase complexes. (A) Kinase assays with cyclin/cdk2 complexes. Histone H1 kinase assays were performed in the presence of 3 ng of cyclin A/cdk2 (lane 1), 10 ng of cyclin A/cdk2-dn (lane 2), and 2 ng of cyclin E/cdk2 (lane 3). These active kinases also phosphorylate recombinant pRB. Kinase assays were performed using pRB and either 10 ng of cyclin A/cdk2, 8 ng of cyclin E/cdk2, or 10 ng of cyclin A/cdk2-dn. (B) Gel mobility shift reactions performed with the same cyclin/cdk2 complexes. All gel mobility shift reactions contained E2F-1/GST-DP-1 (10 ng each) and 15 ng of pRB (except lanes 1 and 16, which lacked pRB). The effect of phosphorylation on the E2F-pRB complex was tested by performing the reactions in the presence of cyclin A/cdk2 (lanes 3–6, 8–11), cyclin E/cdk2 (lanes 12–15), or cyclin A/cdk2-dn (lanes 18–20) in the presence (lanes 1–3, 5–14, 16–20) or absence (lanes 4, 11, 15) of ATP. The amount and identity of each complex added was as follows: cyclin A/cdk2, 0.5 ng (lane 8), 3 ng (lanes 3–6, 9, 11), and 10 ng (lane 10); cyclin E/cdk2, 0.5 ng (lane 12), 2 ng (lanes 13, 15), and 8 ng (lane 14); and cyclin A/cdk2-dn, 0.5, 3, and 10 ng (lanes 18–20, in that order). In addition, lanes 5 and 6 contained 7 and 20 ng, respectively, of purified p21/Cip1.

These results were reflected in a transcriptional analysis as well. While preincubation of pRB alone was without effect, the addition of cyclin A/cdk2 was able to reverse this transcriptional repression (Fig. 5B, lanes 3–7), and the level of transcription was comparable to nonrepressed levels observed with E2F alone (lanes 2, 10). Importantly, addition of cyclin A/cdk2-dn did not alter repression by pRB (lane 8). In other experiments, cyclin E/cdk2 was also able to alleviate pRB repression of E2F *trans*-activation, albeit slightly less efficiently (data not shown). This is consistent with the gel mobility shift experiments, in which somewhat higher levels of cyclin

E/cdk2 than cyclin A/cdk2 were required to abrogate the E2F-pRB complex (Fig. 4B). Moreover, the levels of cyclin A/cdk2 used here failed to affect E2F-mediated activation in the absence of pRB (lanes 6, 7), although higher levels did have an effect (see below). Thus, phosphorylated pRB is no longer able to bind E2F efficiently and, more importantly, this modification directly abrogates its ability to repress E2F activation without a requirement for additional factors. To our knowledge, this represents the first direct evidence linking the phosphorylation of pRB (an event known to be regulated during the cell cycle) with activation of E2F transcription.

Figure 5. pRB repression is reversed by active cyclin A/cdk2 kinase. pRB was incubated in transcription buffer (containing $MgCl_2$ and all four nucleoside triphosphates) either alone or in the presence of cyclin A/cdk2 or cyclin A/cdk2-dn for 1 hr at room temperature. In some cases, pRB was left out of the reactions. Reactions were then split into aliquots and tested in gel mobility shift (A) and transcription (B) reactions. (A) Gel mobility shift reactions were performed as in Fig. 4B by adding these aliquots to E2F-1/GST-DP-1. Lanes 2–4 and 7 contain the equivalent of 15 ng of pRB, and lanes 3–6 contain the equivalent of 1 ng (lanes 3,5) or 2 ng of cyclin A/cdk2 (lanes 4,6); lane 7 contains the equivalent of 2 ng of cyclin A/cdk2-dn. (B) Transcriptional analysis of the reactions in A. Additions to each reaction are indicated at the top. Lanes 2–8 and 10 contained 50 ng each of E2F-1 and GST-DP-1, and the indicated reactions contain the equivalent of 200 ng of pRB. The equivalent of 10 ng of cyclin A/cdk2 was added in lanes 4 and 6 while 20 ng of this complex was added in lanes 5 and 7. Lane 8 had the equivalent of 20 ng of cyclin A/cdk2-dn.

Phosphorylation of E2F by cyclin A/cdk2, but not cyclin E/cdk2, inhibits E2F activity

As noted earlier, phosphorylation by cyclin A/cdk2 reduced the DNA-binding activity of the E2F heterodimer as well as the pRB/E2F interaction in gel mobility shift experiments when the cyclin A/cdk2 complex and E2F were present at near stoichiometric amounts (Fig. 4). This prompted us to ask whether this effect was specific and to determine the functional consequences of this activity. In particular, we wished to determine whether this decreased DNA-binding affinity was attributable to phosphorylation of E2F-1, DP-1, or both and whether both cyclin A- and cyclin E-containing complexes could promote this effect.

First, we performed histone H1 kinase assays with our purified cyclin A/cdk2, cyclin A/cdk2-dn, and cyclin E/cdk2 complexes to determine the specific activities of each of the complexes (Figs. 4A and 6C, lanes 1–4 and 9–11). Next, similar amounts of each complex were titrated into gel mobility shift reactions containing E2F-1 and DP-1 (Fig. 6A). We noticed a striking difference in the activity of each of these cyclin/kinase complexes. As expected, addition of the dominant-negative cdk2/cyclin A complex failed to reduce E2F DNA-binding activity; instead, it promoted a slower mobility complex (lanes 8–11). In contrast, E2F DNA-binding activity was nearly abolished by the addition of wild-type cyclin A/cdk2 (lanes 2–4). Interestingly, addition of the cyclin E/cdk2 complex did not affect E2F DNA binding, even at the highest level tested (lane 7), although amounts of

histone H1 kinase activity equivalent to that of cyclin A/cdk2 were added to both reactions (Fig. 6A, cf. lanes 4 and 7).

As an additional functional test of this inhibitory activity, *in vitro* transcription reactions were performed with similar amounts of cyclin A/cdk2 and cyclin E/cdk2, normalized to histone H1 kinase units. As a control, similar amounts of cyclin A/cdk2-dn (as determined by comparison with cyclin A/cdk2 on silver-stained gels) were used. The ratios of cyclin/cdk2 to E2F were identical to those in the gel mobility shift assays. In accordance with the gel mobility shift analysis, increasing amounts of cyclin A/cdk2 significantly inhibited activation by E2F (Fig. 6B, lanes 3,4). Importantly, *trans*-activation was not diminished by similar amounts of the dominant-negative cdk2/cyclin A complex; this complex actually increased activation slightly (lanes 13,14). Moreover, consistent with the gel mobility shift analysis, cyclin E/cdk2 was also without effect in this assay (lanes 7,8). The effect of cyclin A/cdk2 was specific in this assay, as the basal transcription reaction was only slightly affected by the active cyclin A/cdk2 kinase (lanes 5,6) and unaffected by the two other complexes (lanes 9,10,15). As a further control for the specificity of inhibition of E2F *trans*-activation by cyclin A/cdk2, we have shown that activation by the GAL4-VP16 protein was not significantly reduced by the addition of cyclin A/cdk2 to transcription reactions reconstituted with this activator (B.D. Dynlacht and E. Harlow, unpubl.).

To circumvent the possible complication of having cyclin/kinases present during the transcription reactions

Next, it was important to ascertain whether E2F-1, DP-1, or both could serve as substrates for cyclin A/cdk2 and cyclin E/cdk2 complexes in kinase assays. To avoid possible nonspecific effects due to high protein concentrations, we limited the amounts of E2F-1 and DP-1 in the reaction to those used in the gel mobility shift reac-

Consistent with these observations, when we examined the amino acid sequence of the E2F-1 and DP-1 proteins, we noted several potential cdk2 consensus sites [Moreno and Nurse 1990] in E2F-1 and DP-1. Further-

more, it has been noted that endogenous E2F-1 and DP-1 are phosphoproteins (Dyson et al. 1993; J.A. Lees, C.-L. Wu, and E. Harlow, unpubl.). To pursue this issue further, we asked whether cyclin A/cdk2 might be the kinase responsible for the phosphorylation of E2F in vivo. To address this question, we attempted to label cells with [32 P]orthophosphate and immunoprecipitate endogenous E2F-1 and DP-1. However, despite several attempts, we have not been successful, perhaps because of the low abundance of these proteins and possibly the lack of more suitable antibodies against DP-1. To circumvent this problem, E2F-1 and HA-tagged DP-1 were expressed in cells by transient transfection, and 32 P-labeled proteins were recovered by immunoprecipitation with anti-HA antibodies. After digestion of the purified proteins with trypsin, the resulting peptides were resolved by two dimensional electrophoresis and chromatography. In parallel, we analyzed E2F-1 and DP-1 phosphorylated in vitro by purified cyclin A/cdk2. The results of this experiment are shown in Figure 7. Interestingly, the tryptic maps of in vitro- and in vivo-phosphorylated DP-1 were identical, and only a single major phosphopeptide was evident. In addition, the two sources of labeled E2F-1 also shared a major phosphopeptide species, although phosphorylation of E2F-1 in vitro produced two additional labeled peptides.

These experiments suggest that E2F may be a genuine substrate for cyclin A/cdk2 in vivo, and taken together with the experiments in Figure 6, we conclude that phosphorylation of E2F-1/DP-1 by cyclin A/cdk2 results in a decrease in its DNA-binding ability. Although we were concerned that the additional phosphopeptides of E2F-1 produced by cyclin A/cdk2 in vitro might not reflect

events in vivo, several recent observations suggest that it is the phosphorylation of DP-1 that is most critical for this down-regulation of DNA-binding activity. First, gel mobility shift experiments with GST-E2F-1 (which is capable of DNA-binding on its own) and DP-1 suggest that the DP-1 component is required for cyclin A/cdk2-mediated inhibitory activity (data not shown). Second, in preliminary experiments, either E2F-1 or DP-1 was first treated with cyclin A/cdk2, followed by inactivation of this kinase activity and subsequent addition of the other member of the heterodimer. In these experiments, prior phosphorylation of DP-1, but not E2F-1, dramatically reduced DNA-binding activity of the heterodimer (data not shown). Lastly, Krek and colleagues have shown that phosphorylation of DP-1 present in the E2F complex appears to be S phase-specific, coinciding with the activation of a cyclin A/kinase capable of binding to E2F-1, and this phosphorylation correlates with inhibition of DNA binding (Krek et al. 1994). Additional experiments will be required to understand the further role of E2F-1 phosphorylation, if any, in this down-regulation.

Discussion

An important current issue in the study of cell division is how cyclin-dependent kinases control passage through various cell cycle transitions. An essential step of this analysis is the identification of key kinase targets whose functions are modified by cdk phosphorylation. One important class of downstream targets includes transcription factors whose activity is temporally controlled by cdk phosphorylation. In mammalian cells much of this interest has been focused on the E2F transcription factor.

Figure 7. Comparison of in vitro and in vivo phosphorylated E2F-1 and DP-1. E2F-1 and DP-1 were labeled either in vitro using purified cyclin A/cdk2, or in vivo, by transient transfection into C-33A cells, and then purified and digested with trypsin. Phosphopeptide species were then resolved by electrophoresis (toward cathode) and ascending chromatography either separately or as a mixture of the in vitro and in vivo samples as indicated. The direction of electrophoresis and chromatography is indicated.

Many of our views of the cell cycle regulation of E2F transcription have been based on transient transfection studies. Although these experiments have been essential in the identification of proteins involved in these regulatory complexes, transient transfections have several disadvantages. They rely on high level expression of these proteins, and it is impossible to measure the contributions that endogenous proteins make to the response. To circumvent these complications, we have developed an assay containing defined components to study the direct effects of these proteins. We have used an in vitro transcription assay that recapitulates *trans*-activation through E2F DNA-binding sites. E2F-binding sites have been identified in the promoters of several growth regulatory genes that show dramatic cell cycle activation near the G₁-to-S transition. Our studies have identified three stages in which E2F *trans*-activation oscillates between inactive and active states. The changes from OFF to ON and from ON to OFF can be regulated by the sequential activation of different cyclin-dependent kinases, thus providing a compelling model (Fig. 8) for how activation and inhibition of E2F-regulated promoters could be temporally tied to changes in cell cycle position.

A model for switching E2F activity on and off

Recent work has established connections between several known cell cycle regulators and the transcription factor E2F. A number of studies have shown that critical cell cycle regulatory proteins, including pRB, p107, cyclin A, cyclin E, and cdk2, can associate with E2F in various complexes in the cell. The demonstration of these complexes suggested that these proteins might interact productively with E2F to regulate its transcriptional activity. The mechanisms of this regulation are now becoming clear. E2F is a specific DNA-binding factor with a strong *trans*-activation domain and is composed of one polypeptide from the E2F family and one from the DP family. The results presented here show that purified E2F-1 and DP-1 bind to each other, interact with specific binding sites on DNA, and are sufficient to mediate E2F *trans*-activation dependent on basal transcription factors. This *trans*-activation can be inhibited by the physical interaction of pRB with E2F. The ability

of pRB to bind E2F can be overcome by phosphorylation of pRB with various cyclin/cdk2 complexes. In vitro, both cyclin E/cdk2 and cyclin A/cdk2 are efficient in blocking the ability of pRB to bind to E2F, thereby alleviating pRB inhibition. Because the kinase activity of cyclin E/cdk2 occurs several hours earlier in the cell cycle than that of cyclin A/cdk2, it is a more attractive candidate for an early signal to activate E2F-mediated transcription. However, we wish to emphasize that other kinases may participate in this control in vivo. The cyclin D/cdk4 and cyclin D/cdk6 complexes are potential candidates for other kinases that might participate in this inhibition of pRB function (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993; Meyerson and Harlow 1994). Because this in vivo question is difficult to resolve, we can only state at this time that the cyclin E/cdk2 and cyclin A/cdk2 complexes are capable of reversing the ability of pRB to bind E2F and inhibit its *trans*-activation potential.

The results presented here constitute the first example of an in vitro transcription system that responds to regulatory events acting upstream of the binding of a *trans*-activator. Other in vitro systems have demonstrated transcriptional inhibition by proteins that compete for DNA binding or reduce basal transcription levels. The pRB/E2F regulation pathway also has been extended farther to include upstream regulatory events by using kinases that block pRB's ability to bind to E2F, and we expect that this regulation can be extended to earlier events in this pathway. For example, the observation that p21 blocks this pathway (Hunter 1993) suggests that such upstream regulation is possible, in this case providing a means by which E2F activity could be inhibited by cdk inactivation. Experiments that address the role of p21 in the regulation of cyclin/kinase and E2F activity are currently in progress. The inhibition of E2F *trans*-activation by pRB demonstrated in these in vitro transcription assays is consistent with previously reported findings from transfection experiments. Given that the two assays are quite different, it is encouraging that both of these approaches lead to similar conclusions about the regulation of E2F activation.

In addition to providing clear indications of how E2F transcription is activated, the in vitro transcription results have led to unexpected findings about how E2F

Figure 8. A model summarizing the regulation of E2F activity during the cell cycle. When pRB and E2F-1/DP-1 are hypophosphorylated (unshaded), they form a stable complex that is transcriptionally repressed. Later, as the levels of cyclin E/cdk2 and other cyclin/cdk complexes increase, pRB becomes phosphorylated (denoted by shading), releasing it from E2F. Activation of the cyclin A/cdk2 complex results in the phosphorylation of E2F-1/DP-1 (shaded as for pRB phosphorylation), which down-regulates DNA-binding and transcriptional activation by the heterodimer.

trans-activation is down-regulated. Whereas both cyclin E/cdk2 and cyclin A/cdk2 were able to alleviate pRB repression, cyclin A/cdk2 was uniquely able to reverse E2F *trans*-activation. This down-regulation was dependent on the ability of cyclin A/cdk2 to phosphorylate E2F. The phosphorylation of either or both of the E2F component polypeptides, E2F-1 and DP-1, leads to a loss of DNA-binding activity. Cyclin E/cdk2 is activated prior to the initiation of DNA synthesis, but cyclin A/cdk2 is not activated until S phase, and this provides a method for timing the inactivation of E2F *trans*-activation. This cyclin/cdk distinction is also the first example of a single cdk catalytic subunit acquiring a different functional activity by simple exchange of cyclin regulatory subunits.

Together, activation of the E2F transcription factor by release of pRB and the down-regulation of E2F by reducing DNA binding provide a mechanism for the temporal regulation of E2F-mediated transcription (Fig. 8). This window of *trans*-activation is controlled by specific cell cycle phosphorylation events. As different cyclin/cdk complexes are activated, E2F transcription is cycled from the OFF to ON to OFF states. This model is consistent with the proposed role of E2F in cell cycle control and may explain how cell cycle position can be translated into transcriptional control. Moreover, our work is corroborated and complemented by the findings of another independent group. Livingston and colleagues have shown that cyclin A/E2F, but not cyclin E/E2F, complexes can be readily detected in vivo and in vitro; furthermore, the appearance of this cyclin A/cdk2/E2F complex correlates with the phosphorylation of DP-1 and the loss of E2F DNA-binding activity (Krek et al. 1994).

Nevertheless, many questions remain to be answered. For example, it will be interesting to ask why cyclin E/cdk2 is unable to phosphorylate E2F. Also, can substrate preference be redirected by the p107 protein, which is present in E2F complexes containing cyclin E/cdk2, or artificially by creating cyclin A/cyclin E hybrid proteins? Substrate specificity dictated by cyclin subunits in mammalian cells may be a prevalent theme in cell cycle regulation, as judged by two recent examples. p107 is phosphorylated by cdk2 complexes containing cyclin A but not cyclin B (Peeper et al. 1993), and pRB is phosphorylated by certain cyclin/cdk2 complexes but not by others (Kato et al. 1993). In addition, it has been documented that the cyclin D/cdk6 exhibits substrate specificity, preferring pRB to histone H1 in kinase assays (Meyerson and Harlow 1994).

Recently, the effects of cyclin/kinase and related protein complexes on transcription factors have been documented in both mammalian cells and yeast, and in both cases, the net result is transcriptional inhibition. For example, the cyclin B/cdc2 complex can negatively regulate transcription by RNA polymerase III, and the target of this inhibition is likely to be the TFIIB TBP-TAF complex (Gottesfeld et al. 1994). A second example is the phosphorylation of the PHO4 transcription factor by the PHO80/PHO85 complex, proteins that appear to be ho-

mologous to cyclin/cdk complexes, and PHO4 modification correlates with its inactivation (Kaffman et al. 1994). It remains to be determined whether the various cyclin/kinase complexes recognize additional substrates involved in polymerase II transcription, including both site-specific activators and basal transcription factors. This appears to be likely, as judged by the above-mentioned examples, and it can be readily tested using an in vitro transcription assay.

In addition, the E2F-responsive assay will permit us to look at the functions of other members of the E2F family of transcription factors, which includes at least three E2F members (Lees et al. 1993) and two DP-1 type proteins (C.-L. Wu and E. Harlow, unpubl.). For example, do these different family members have different promoter specificities, and are they regulated by the same factors as E2F-1? It will also be important to determine the regulatory functions of other purified E2F-containing complexes, such as those associated with p107/cyclin A/cdk2, p107/cyclin E/cdk2, and the recently characterized p130 protein (Cobrinik et al. 1993). In addition, it has been proposed that pRB may regulate the activity of numerous transcription factors, including Sp1, ATF-2, c-Myc, and Elf-1 (Rustgi et al. 1991; Kim et al. 1992a,b; Wang et al. 1993). The in vitro transcription assay described here will be useful for defining the proteins required for the regulation of these transcription factors.

Materials and methods

Expression and purification of recombinant proteins

Full-length E2F-1 containing a carboxy-terminal tubulin epitope (Glu-Glu-Phe) was expressed from plasmid pT5T-Rbp3 (a gift of H. Huber and M. Ivey-Hoyle) in *Escherichia coli* strain BL21-DE3 and purified as described (Huber et al. 1993), with the following modifications. Bacteria were lysed by sonication in HEMGN buffer (25 mM HEPES at pH 7.6, 0.1 mM EDTA, 12.5 mM MgCl₂, 10% glycerol, 0.1% NP-40) containing 100 mM KCl, 1 mM DTT, 0.2 mM AEBSF (Calbiochem), 5 µg/ml of leupeptin, and 10 µg/ml of aprotinin. The lysate was applied to a column containing the YL1/2 (Harlan Bioproducts) antibody coupled to CNBr-activated Sepharose that was equilibrated in lysis buffer and eluted with 5 mM Asp-Phe dipeptide (Sigma) in the same column buffer. At this point, E2F-1 is ~90% pure as judged by silver staining. To enrich for protein capable of binding DNA, the eluate was loaded directly onto a heparin-Sepharose CL-6B (Pharmacia) column, washed with lysis buffer, and eluted with the same buffer containing 500 mM KCl. The eluate was then dialyzed against HEMGN containing 100 mM KCl and 1 mM DTT. Full length DP-1 was produced as a GST fusion protein and has been described (Helin et al. 1993b). Bacteria expressing GST-DP-1 were lysed as described for E2F-1, and after spinning down debris, the lysate was loaded directly onto glutathione-agarose beads equilibrated in lysis buffer. After several washes in lysis buffer, protein was eluted with 20 mM reduced glutathione in HEMGN containing 100 mM KCl, 1 mM DTT, and 0.2 mM AEBSF. The protein was subsequently dialyzed against lysis buffer containing 1 mM DTT.

All other proteins used in this study were produced in insect cells (High Five cells; Invitrogen) as follows. In general, infected cells were harvested 40–48 hr postinfection. Full-length pRB was produced using a previously described baculovirus-express-

ing pRB as a histidine-tagged fusion protein [Dowdy et al. 1993]. Cells infected with this virus were harvested, washed once with PBS, and disrupted by sonication in 0.25 M HMGN (the same buffer as HEMG except that it lacks EDTA) containing 250 mM NaCl, 5 mM β -mercaptoethanol, and the protease inhibitors listed above. The crude extract was then chromatographed on a column containing a human papillomavirus (HPV) E7 peptide (amino acids 20–29) linked to CNBr–Sepharose as described [Edwards et al. 1992] with the following modifications. The lysate was loaded by batch incubation at 4°C onto resin equilibrated in 0.25 M HMGN, and after extensive washes in this buffer, the column was eluted with unbuffered 50 mM Na_2CO_3 containing 150 mM NaCl, 1 mM DTT, and 0.2 mM AEBSF directly into 1 M HEPES (pH 7.0), to neutralize the eluate. NP-40 (to 0.01%) and carrier protein (0.2 mg/ml of insulin) were added to the eluate, which was then dialyzed against 0.1 M HEG (the same buffer as HEMGN except that it lacks MgCl_2 and contains 0.01% NP-40). The pRB preparation appeared to contain largely full-length protein with some slightly smaller proteolytic products evident.

The GST–cyclin A and HA-tagged cdk2 baculoviruses have been described previously [Peeper et al. 1993; Desai et al. 1992]. The cyclin E virus was a gift from Pharmingen, and the cdk2–dn–HA virus was a gift of E. Lees (unpubl.). GST/cyclin A/cdk2–HA, GST/cyclin A/cdk2–dn–HA, and cyclin E/cdk2/HA complexes were produced and purified as follows. Each complex was generated by coinfection, and cells were harvested, washed in PBS, and lysed by sonication in 0.1 M HEMGN containing 100 mM KCl, 1 mM DTT, the protease inhibitors listed above, 10 mM NaF, and 50 mM β -glycerophosphate. The GST–cyclin A/cdk2–HA and GST–cyclin A/cdk2–dn–HA complexes were purified on glutathione–agarose resin and dialyzed as described for GST–DP-1 above. Cyclin E/cdk2–HA complexes were purified by batch loading cell lysates onto columns containing the anti-HA monoclonal antibody 12CA5 [Field et al. 1988] cross-linked to protein A–Sepharose. After 4- to 5-hr incubations, the resin was washed with lysis buffer and eluted with 2 mg/ml of HA peptide (Berkeley Antibody Co.) in 0.25 M HEMGN containing 250 mM KCl, 0.01% NP-40, 1 mM DTT, and 0.2 mM AEBSF; carrier protein (0.2 mg/ml) was added as described above. Cyclin A-containing complexes could be purified with mAb 12CA5 resin as well, giving the same complex obtained with glutathione–agarose purification.

The purity and concentration of each of these protein preparations were assessed by SDS–PAGE followed by silver staining. Purified bacterial native Cip1 (a single band on Coomassie-stained gels) was a kind gift of W. Harper and has been described [Harper et al. 1993]. Purified GAL4–VP16 was provided by G. Peterson [Tularik, Inc.]; purification of this recombinant protein has been described [Chasman et al. 1989].

Generation of affinity chromatography resins

The anti-tubulin antibody YL1/2 and E7 peptide were coupled to CNBr-activated Sepharose (Pharmacia) following the manufacturer's instructions. mAb 12CA5 was cross-linked to protein A–Sepharose as described [Harlow and Lane 1988].

In vitro transcription analysis

The basal transcription factors used in this study were purified as follows. RNA polymerase II was purified from HeLa cell nuclear pellets (2.5×10^{11} cells). Enzyme extraction and fractionation on DEAE–cellulose was as described [Reinberg et al. 1987]. Further purification on heparin–Sepharose, TSK phenyl, and TSK DEAE 5PW was as described [Lu et al. 1991]. Factors TFIIA, IID, IIF, and IIH were purified from 500 ml of HeLa nuclear extracts (2.5×10^{11} cells). TFIIA and TFIID were purified on

phosphocellulose and DEAE-52 columns as described [Reinberg and Roeder 1987]. A chromatographic fraction containing factors IIF and IIH was obtained after fractionation of the phosphocellulose 1.0 M fraction on DEAE-52, SP-Sepharose, and S-Sepharose using conditions described previously [Flores et al. 1992]. S-Sepharose column fractions were tested in an *in vitro* transcription complementation assay for TFIIF and TFIID activities; active fractions were pooled. Recombinant TFIIB and TFIIE were expressed in, and purified from, *E. coli* as described [Ha et al. 1991; Peterson et al. 1991], respectively. A single preparation of each of these factors was used for all of the transcription assays to ensure reproducibility. Transcription reactions of 50 μ l containing the above factors were performed at 30°C for 45 min as described previously [Flores et al. 1992] with the following modifications. Reaction mixtures contained 3–4 mM MgCl_2 , 40–45 mM KCl, 5 mM ammonium sulfate, and 0.5 mM NTPs, 0.1 mg/ml of BSA and 8 units of RNasin (Promega) were included in the reactions. Reactions contained 5 μ g of TFIIA, 1.5 μ g of TFIID, 1.4 μ g of the TFIIF/H fraction, 35 ng of polymerase II, 30 ng of TFIIB, and 200 ng of TFIIE. Reactions contained 50 or 100 ng of template, except where noted. Transcription templates BCAT, E2F₁BCAT, and E4CAT have been described previously [Lillie and Green 1989; Phelps et al. 1991; Helin et al. 1993b]. For activated transcription, ~50 or 100 ng each of E2F-1 and DP-1 (50 ng of each gives saturating amounts of activated transcription) was preincubated on ice for 10–15 min prior to the addition of other proteins (as noted) and basal transcription factors. *In vitro* transcription products were analyzed by primer extension using a chloramphenicol acetyltransferase (CAT) gene oligonucleotide as described previously [Dynlacht et al. 1991]. All transcription reactions were performed a minimum of three times; in most cases, they were performed more than five times, and representative data are shown.

Gel mobility shift assays

Gel mobility shift assays were performed essentially as described [Yee et al. 1987] with the following modifications. In cases where crude HeLa nuclear extracts (1 μ l) were used, 1 μ g of denatured salmon sperm DNA was included as nonspecific competitor; it was omitted from reactions performed with purified recombinant proteins. Assays with recombinant E2F contained 5–10 ng each of E2F-1 and GST–DP-1. For pRB or cyclin/kinase addition experiments, E2F was incubated with pRB or a specific complex for 15 min on ice. Cyclin/kinase complexes were diluted in gel mobility shift buffer containing 100 mM KCl, 0.1% NP-40, and 0.2 mg/ml of BSA. For experiments in which pRB was first phosphorylated by cyclin/kinase complexes, 20-min room temperature (25°C) incubations were performed in the presence of 10 mM MgCl_2 and ATP. This preincubation was not required, as pRB–E2F complexes were also abolished by adding cyclin A/cdk2 or cyclin E/cdk 2 complexes to gel mobility shift reactions containing pRB and E2F in the presence of ATP (not shown). Where added, the final concentration of ATP was 0.5 mM (as for transcription assays). After the addition of ^{32}P -labeled oligonucleotide, reactions were incubated further at room temperature for 20–25 min, loaded onto 4% acrylamide gels, and electrophoresed in $0.25 \times$ TBE for 2 hr at 4°C at 180 V.

Kinase assays

Kinase assays were performed in 50- μ l reaction volumes containing 50 mM HEPES (pH 7.0), 10 mM MgCl_2 , 5 mM MnCl_2 , 1 mM DTT, and 0.2 mg/ml of BSA. Reactions also contained 2.5 μ Ci of [γ - ^{32}P]ATP (3000 Ci/mmol). In some assays, 2.5 μ g of histone H1 or 100 ng of purified full-length pRB was used as a substrate. Reactions were incubated at 30°C for 30–60 min and

were stopped by the addition of 2× sample buffer. Proteins were resolved on 10% polyacrylamide gels, dried, and visualized by autoradiography.

Transient transfections and cell labeling

The human cervical carcinoma cell line C-33A was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Six 10-cm² dishes of cells were transiently transfected with 8 µg each of the expression vectors pCMVE2F-1 and pCMVHADP-1 (Helin et al. 1993b) and 8 µg of pBluescript SK+ according to the method of Graham and van der Eb (1973). Transfected cells were then incubated at 37°C for 5 hr in 2 ml of phosphate-free DMEM containing 20 mCi of [³²P]orthophosphate (ICN) per plate.

Immunoprecipitations

Cells were lysed in E1A lysis buffer (250 mM NaCl, 50 mM HEPES at pH 7.0, 0.1% NP-40) containing 50 mM NaF, 5 mM EDTA, 50 µg/ml of PMSF, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin, 1 mM DTT, and immunoprecipitated using the anti-HA mAb 12CA5 (Field et al. 1988) as described previously (Harlow et al. 1985). The precipitated proteins were then resolved on 8% SDS-polyacrylamide gels and visualized by autoradiography.

Two-dimensional electrophoresis and chromatography

E2F-1 and HA-DP-1 were excised from unfixed gels and eluted, oxidized, and digested with trypsin as described previously (Lees et al. 1991). Between 200–500 cpm of the resulting peptides were resolved on cellulose thin-layer plates in two dimensions. Electrophoresis was performed at pH 1.9 [88% formic acid/acetic acid/water, 25:78:879 (by volume)] for 40 min at 800 V followed by ascending chromatography in 1-butanol/acetic acid/pyridine/water, 75:15:50:60 (by volume).

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