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# p27$^{KIP1}$ Blocks Cyclin E-Dependent Transactivation of Cyclin A Gene Expression

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**Cyclin E is necessary and rate limiting for the passage of mammalian cells through the $G_1$ phase of the cell cycle. Control of cell cycle progression by cyclin E involves cdk2 kinase, which requires cyclin E for catalytic activity. Expression of cyclin E/cdk2 leads to an activation of cyclin A gene expression, as monitored by reporter gene constructs derived from the human cyclin A promoter. Promoter activation by cyclin E/cdk2 requires an E2F binding site in the cyclin A promoter. We show here that cyclin E/cdk2 kinase can directly bind to E2F/p107 complexes formed on the cyclin A promoter-derived E2F binding site, and this association is controlled by p27$^{KIP1}$, most likely through direct protein-protein interaction. These observations suggest that cyclin E/cdk2 associates with E2F/p107 complexes in late $G_1$ phase, once p27$^{KIP1}$ has decreased below a critical threshold level. Since a kinase-negative mutant of cdk2 prevents promoter activation, it appears that transcriptional activation of the cyclin A gene requires an active cdk2 kinase tethered to its promoter region.**

Progression through the mammalian cell cycle is controlled by cyclins and cyclin-dependent kinases (cdk) (for a review, see reference 35). Cyclin gene expression is tightly regulated at the transcriptional level in a phase-specific manner. Cyclin E (23, 28) is expressed in mid $G_1$ phase; it associates with and activates cdk2 kinase (7, 24). Conditional overexpression of cyclin E results in a limited decrease in the length of the $G_1$ interval, consistent with an acceleration of the $G_1$/S transition (39, 53). Microinjection of anti-cyclin E antibodies during $G_1$ inhibits entry into S phase in various mammalian cell types (31), and genetic evidence suggests that cyclin E controls S phase entry during early embryogenesis in *Drosophila melanogaster* (22). These findings strongly suggest that cyclin E, and its associated kinase, is centrally involved in the decision to enter S phase in most if not all cells. Nevertheless, cyclin E protein accumulates in physiological situations where cells do not enter S phase (13, 47); most likely, this reflects posttranslational control of cyclin E activity. The enzymatic activity of cyclin/cdk complexes is modulated by cdk inhibitors, including p27$^{KIP1}$ (37, 48), p21$^{WAF\text{-}1/Cip1}$, and p16$^{INK4}$. In the case of cyclin E, the associated kinase is controlled by binding of cdi proteins to the cyclin E/cdk2 complex (reviewed in reference 45). p27$^{KIP1}$ is linked to $G_1$ arrest in contact-inhibited cells (36), and p27$^{KIP1}$ was shown to accumulate to high levels in $G_0$-arrested cells (16, 30, 34).

Cyclin A is first expressed at the $G_1$/S transition; it is required for S phase and passage through $G_2$ (11, 33, 58). Whereas cyclin A gene expression is tightly controlled at transcriptional level in most cells (15), posttranslational inactivation of cyclin A appears restricted to $G_2$/M, where the protein is degraded (for a review, see reference 27); although cyclin A/cdk2 kinase can be inhibited by cdi in vitro, posttranslational control of cyclin A function was not described as a rate-limiting step involved in $G_1$ arrest so far. This observation emphasizes the importance of transcriptional regulation of cyclin A gene expression to control S phase entry. Constitutive expression of cyclin A from a viral promoter has been associated with tumorigenesis (51); inversely, abolishment of cyclin A gene expression is involved in the $G_1$ arrest of adhesion-dependent cells grown in suspension (13).

We have shown previously that ectopic expression of D-type cyclins in mammalian cells leads to transcriptional activation of the cyclin A gene (42). Since overexpression of cyclin D1 is linked to the altered growth properties of certain tumor cells (1), the observation that cyclin D1 can induce cyclin A gene expression provides a potential molecular basis for the suspected role of cyclin D1 overexpression in tumorigenesis (18). However, D-type cyclins are not required for the progression through $G_1$ in all cell types: in virally transformed cells and RB-deficient cells S phase entry occurs in the absence of any D-type cyclins but still requires cyclin A (33); consequently, in these cell types activation of cyclin A gene expression must be driven by a different mechanism, independent of cyclin D1. Unlike D type cyclins, cyclin E is required for S phase entry in all mammalian cells tested so far (31). Activation of cyclin A gene expression by the human papillomavirus E7 protein requires synthesis of an as yet unidentified cellular protein(s) (54). Since cyclin D1 gene expression is not affected by E7, it appears possible that cyclin E, which is induced by E7 directly before the onset of cyclin A gene expression, is involved in transcriptional activation of the cyclin A gene. Consistent with this interpretation, we show here that cyclin E and its associated kinase cdk2 activate cyclin A transcription, and this involves the cell cycle regulatory element of the cyclin A promoter, consisting of a variant E2F binding site (42) and an adjacent element, referred to as CHR (59). Cyclin E/cdk2 complexes physically associate with an E2F multiprotein complex bound to this regulatory element of the cyclin A promoter. Binding of cyclin E/cdk2 complexes to E2F is regulated during the cell cycle and inversely correlated with p27$^{KIP1}$ levels. Expression of p27$^{KIP1}$ leads to disappearance of this complex in vivo, and addition of recombinant p27$^{KIP1}$ dissociates cyclin E/cdk2 from E2F complexes in cellular extracts. By using a dominant negative mutant of cdk2, it is shown that binding of cyclin E to the cyclin A promoter is not sufficient for activation

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but that binding of an active kinase is required. The data support a model for activation of the cyclin A promoter by cyclin E, in which an active cdk2 kinase is tethered to the promoter and subsequently triggers transcriptional activation.

## MATERIALS AND METHODS

**Cell culture and transfection.** NIH 3T3 cells were propagated in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% bovine calf serum. C33A cells were grown in D-MEM–10% fetal calf serum. Whole-cell extracts were produced as described previously (19).

Cells were transfected by calcium phosphate precipitation as described elsewhere (4). At 16 h postincubation, cells were washed and placed in D-MEM containing 10% fetal calf serum. Luciferase and β-galactosidase assays were performed on cell extracts prepared 36 h after transfection as described previously (42). Variations in transfection efficiency were accounted for by normalizing to a cotransfected cytomegalovirus (CMV) promoter-driven β-galactosidase control plasmid.

**FACS analysis of transfected cells.** U2-OS cells were transfected with CMV-driven expression vectors for cyclin E or cyclin A, together with an expression vector for the CD20 surface marker (50). At 36 h posttransfection, cells were harvested and DNA content was determined by fluorescence-activated cell sorter (FACS) analysis. Transfected cells were identified by staining with a CD20-specific fluorescein isothiocyanate-conjugated antibody (Becton-Dickinson, San Jose, Calif.).

**Reporter constructs and expression plasmids.** cDNAs encoding human cyclin E (23), cyclin A (52), $p16^{INK4}$ (44), DP-1 (12, 14a), and E2F-4 (3) have been cloned into the CMV-driven expression vector pX (33) by standard techniques. Expression vectors coding for human cdk2 (49), cdk2 DN (50), p107, HA-p107 (2), cyclin D1 (42), and $p27^{KIP1}$ (37) have been described elsewhere. Human cyclin A promoter/luciferase reporter plasmids and mutants thereof have been described previously (42).

**Western blot (immunoblot) analysis.** Cell extracts were separated on 7 or 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (DuPont de Nemours). Immunoblot analysis was performed as described previously (54) by using the chemiluminescence Western blotting detection system (DuPont de Nemours). Polyclonal antibodies recognizing p107 and $p27^{KIP1}$ were obtained from Santa Cruz Biotechnology, Inc. (Alameda, Calif.). Human cyclin E was detected by using monoclonal antibody HE 12 (a gift from E. Lees); anti-HA antibody 12CA5 was purchased from Boehringer (Mannheim, Germany). Monoclonal antibody TFD10 was raised to recombinant DP-1 protein by standard procedures.

**Immunoprecipitation.** Cells were extracted in buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 100 μM sodium orthovanadate ($Na_3VO_4$), 10 mM β-glycerophosphate, 1 mM sodium fluoride, and 10 μg of aprotinin per ml, as described previously (54). Precleared lysates were subjected to immunoprecipitation with polyclonal antibodies to $p27^{KIP1}$, p107 (both purchased from Santa Cruz Biotechnology, Inc.), monoclonal anti-cyclin E antibody (HE 111, kindly provided by E. Lees), or monoclonal anti-DP-1 antibody TFD10.

**Electrophoretic mobility shift assay.** Band shift experiments were performed as described previously (32). The double-stranded probe cycA wt (5′-TTCAAT AGTCGCGGGATACTT-3′) used represents the wild-type E2F binding site from the human cyclin A promoter. E2F-associated proteins were analyzed by incubation of the band shift reaction with specific antibodies for 50 min on ice, prior to electrophoresis. Antibodies to p107, p130, cyclin E, and cdk2 were purchased from Santa Cruz Biotechnology, Inc. Recombinant histidine-tagged $p27^{KIP1}$ was expressed in *Escherichia coli* and purified by $Ni^{2+}$ agarose chromatography, as described previously (37).

## RESULTS

**Transactivation of the cyclin A promoter by cyclin E/cdk2.** Both cyclin E (31) and cyclin A (33) are required for S phase entry in all mammalian cells studied so far. Since activation of cyclin E gene expression precedes activation of the cyclin A gene in most if not all cases, we asked if cyclin E may contribute to transcriptional activation of the cyclin A gene, as a prerequisite for the cell to enter S phase. Asynchronously growing NIH 3T3 cells were cotransfected with reporter gene constructs derived from the human cyclin A promoter and an expression vector for cyclin E. Ectopic expression of cyclin E led to an eightfold increase of transcription from a reporter gene, containing 7.3 kb of 5′ flanking sequence of the human cyclin A gene (data not shown), and responsiveness to cyclin E was retained in the −89/+11 cyclin A minimal promoter (Fig. 1A). Activation was specific for cyclin E in that ectopic expression of cyclin A did not significantly affect activity of the cyclin A promoter. As monitored by immunofluorescence analysis, both cyclin E and cyclin A were expressed to high level in about 5% of the cells after transfection (representative of the average transfection efficiency; data not shown), indicating that the failure of cyclin A to activate transcription of the cyclin A reporter gene construct is not due to insufficient activity of the expression vector.

To study the influence of ectopically expressed cyclins on the cell cycle profile of transfected cells, cyclins E and A were coexpressed with a CD20 surface marker in U2-OS cells, which allows the subsequent FACS analysis of transfected cells (50). We found that expression of both cyclin E or cyclin A in U2-OS cells increased the amount of S phase cells from 23% ± 2% to 28% ± 2%, in keeping with the observation that expression of either cyclin E (39) or cyclin A (40) only marginally advances S phase in rodent fibroblasts. As in NIH 3T3 cells, expression of cyclin E but not cyclin A led to a sixfold ± twofold activation of cyclin A promoter activity in U2-OS cells. Thus, the observed activation of cyclin A transcription by cyclin E cannot be due to premature S phase entry of the transfected cells.

Cell cycle regulation of cyclin A gene expression depends on an E2F binding site located at −33/−37 in the cyclin A promoter (42). While a second element, referred to as CHR (59), cooperates with the E2F site to mediate cell cycle regulation of the cyclin A gene, the identity of cellular transcription factors acting through this element remains to be elucidated. Disruption of either the E2F binding site or the CHR element renders the promoter unresponsive to cyclin E (Fig. 1A), indicating that both elements are required for cyclin E-dependent transactivation in NIH 3T3 cells. The E2F binding site in the cyclin A promoter was shown previously to bind p107 but not pRb (42). To analyze the role of pRb in the process of cyclin E-driven activation of the cyclin A promoter, similar transfection experiments were performed with C33A cells, a pRb-deficient human carcinoma cell line. As in NIH 3T3 cells, the cyclin A promoter was activated by cyclin E in C33A cells (Fig. 1B), indicating that pRb is not required. Transactivation was dependent on the integrity of the E2F binding site also in C33A cells (Fig. 1B), supporting the conclusion that a functional interaction with pRb is not of critical importance for the ability of cyclin E to activate cyclin A gene expression. While cyclin D1 leads to an activation of cyclin A gene expression in pRb-positive cells (Fig. 1A), expression of cyclin D1 did not affect activity of the cyclin A promoter in C33A cells (Fig. 1B), indicating that the pathway of activation might be different for cyclin D1 and cyclin E.

**Requirement of cyclin E-associated kinase for activation of the cyclin A promoter.** While transfection of a cdk2 expression vector failed to activate the cyclin A promoter, cdk2 cooperated to some extent with cyclin E to increase the level of cyclin A transcription upon coexpression (Fig. 1C). Thus, cyclin E but not cdk2 appears rate limiting for activation of cyclin A gene expression. To investigate whether cyclin E-associated kinase is required for activation of cyclin A transcription by cyclin E, we made use of a cdk2 mutant which can efficiently bind to cyclin E but has no enzymatic activity, due to a point mutation in the catalytic domain. This cdk2 mutant has a dominant negative effect over the activity of wild-type cdk2 (50) and is referred to as cdk2DN in this report. Cotransfection of cdk2DN impaired the ability of cyclin E to activate the cyclin A promoter (Fig. 1C). It was shown previously that cyclin E-associated kinase is inhibited by the cdk inhibitor $p27^{KIP1}$, which binds to and inhibits cyclin E/cdk2 complexes (36, 48) in re-

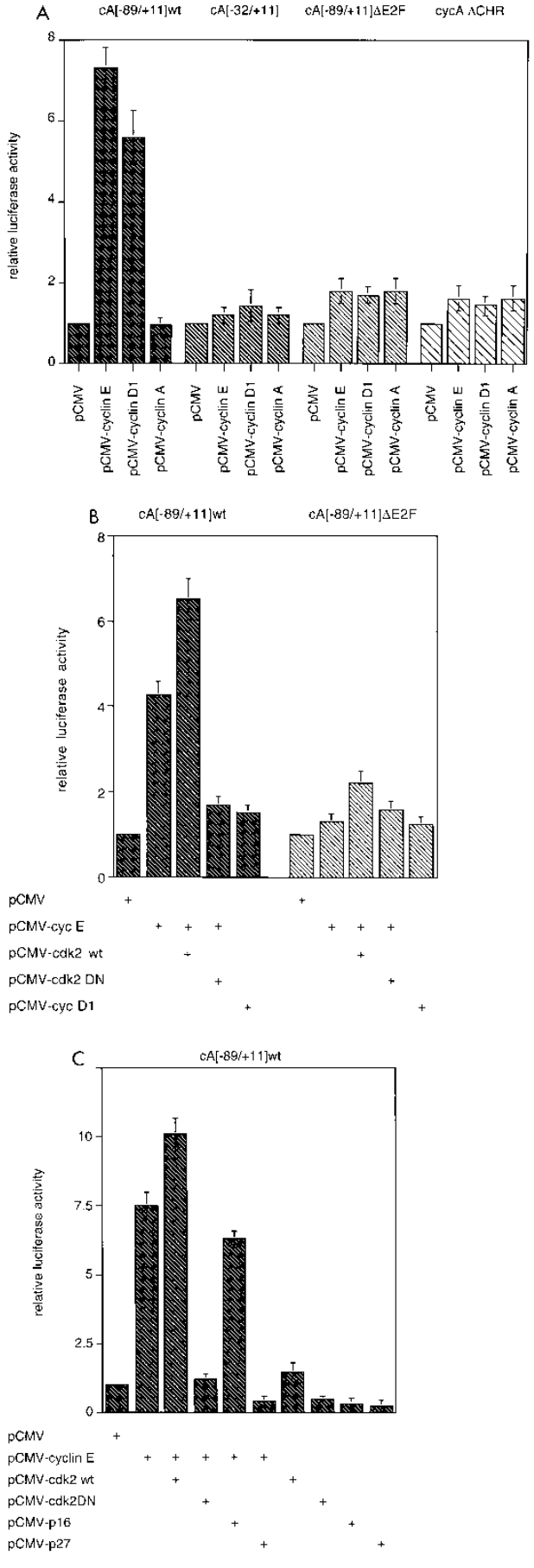


FIG. 1. Transactivation of the human cyclin A promoter by cyclin E. (A) Identification of the cyclin E-responsive element of the cyclin A promoter. Transcriptional activities of the wild-type cyclin A promoter construct and various promoter mutants were tested in cotransfection experiments with CMV-driven expression vectors for cyclin E, cyclin D1, and cyclin A, respectively. The fold induction of luciferase activity in growing NIH 3T3 cells is given. Luciferase activity was normalized to β-galactosidase activity obtained from a cotransfected CMV-driven β-galactosidase vector. The basal activities were set to 1 for all reporter gene constructs. It should be noted that the uninduced activity of both the ΔE2F and ΔCHR constructs was about threefold higher than the activity of the corresponding wild-type constructs, indicating that this part of the promoter confers negative regulation to the cyclin A gene (42). (B) Activation of cyclin A transcription in pRb-deficient C33A cells. Transcriptional activation of cyclin A −89/+11 wt and −89/+11ΔE2F reporter gene constructs by cyclin E, cyclin D1, cdk2 wt, and cdk2 DN was analyzed in transient transfections in C33A cells lacking functional pRb. The fold induction of luciferase activity is shown. Transfections were performed and analyzed as described for panel A. (C) Transactivation of the cyclin A promoter by cyclin E depends on its associated kinase activity. The cyclin A −89/+11 reporter gene construct was cotransfected in NIH 3T3 cells with expression vectors coding for cyclin E and cdk2 wt. Transcriptional activity was repressed by cotransfection of a dominant negative mutant of cdk2 (cdk2DN) or the cdk inhibitor $p27^{KIP1}$, respectively, but not by coexpression of the cdk inhibitor $p16^{INK4}$. Transfection experiments were performed and analyzed as in panel A.

sponse to various growth-suppressive signals (21, 30, 36). To address the ability of $p27^{KIP1}$ to prevent activation of the cyclin A promoter by cyclin E, NIH 3T3 cells were transfected with a cyclin A reporter gene construct, together with expression vectors for cyclin E and $p27^{KIP1}$. Activation of the cyclin A promoter by cyclin E/cdk2 is impaired by ectopic expression of the cdk2 inhibitor $p27^{KIP1}$, whereas $p16^{INK4}$, shown to specifically

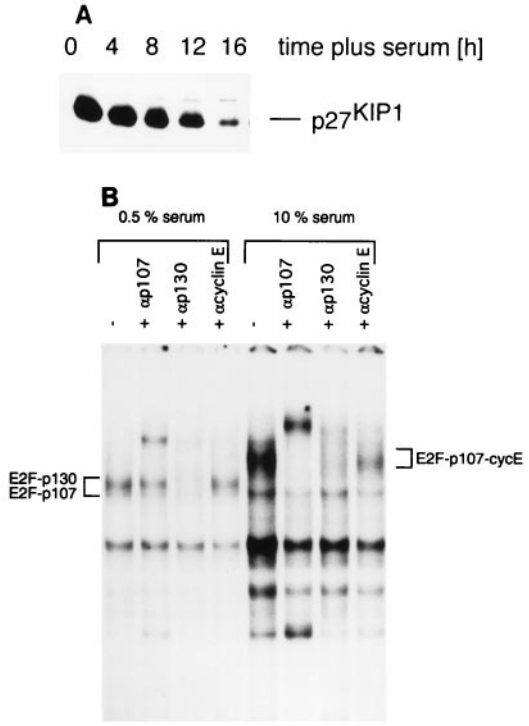


FIG. 2. Changes in $p27^{KIP1}$ levels and E2F complexes in serum-starved NIH 3T3 cells. (A) Abundance of $p27^{KIP1}$ in synchronized NIH 3T3 cells. Whole-cell extracts of NIH 3T3 cells after serum withdrawal for 72 h and serum readdition for the indicated times were analyzed by immunoblotting for $p27^{KIP1}$ expression. (B) Binding of pocket proteins to the cyclin A promoter. Band shift experiments using extracts from serum-starved NIH 3T3 cells were performed in the presence of specific antibodies to p130, p107, and cyclin E. Extracts from asynchronously growing cells were used as controls.

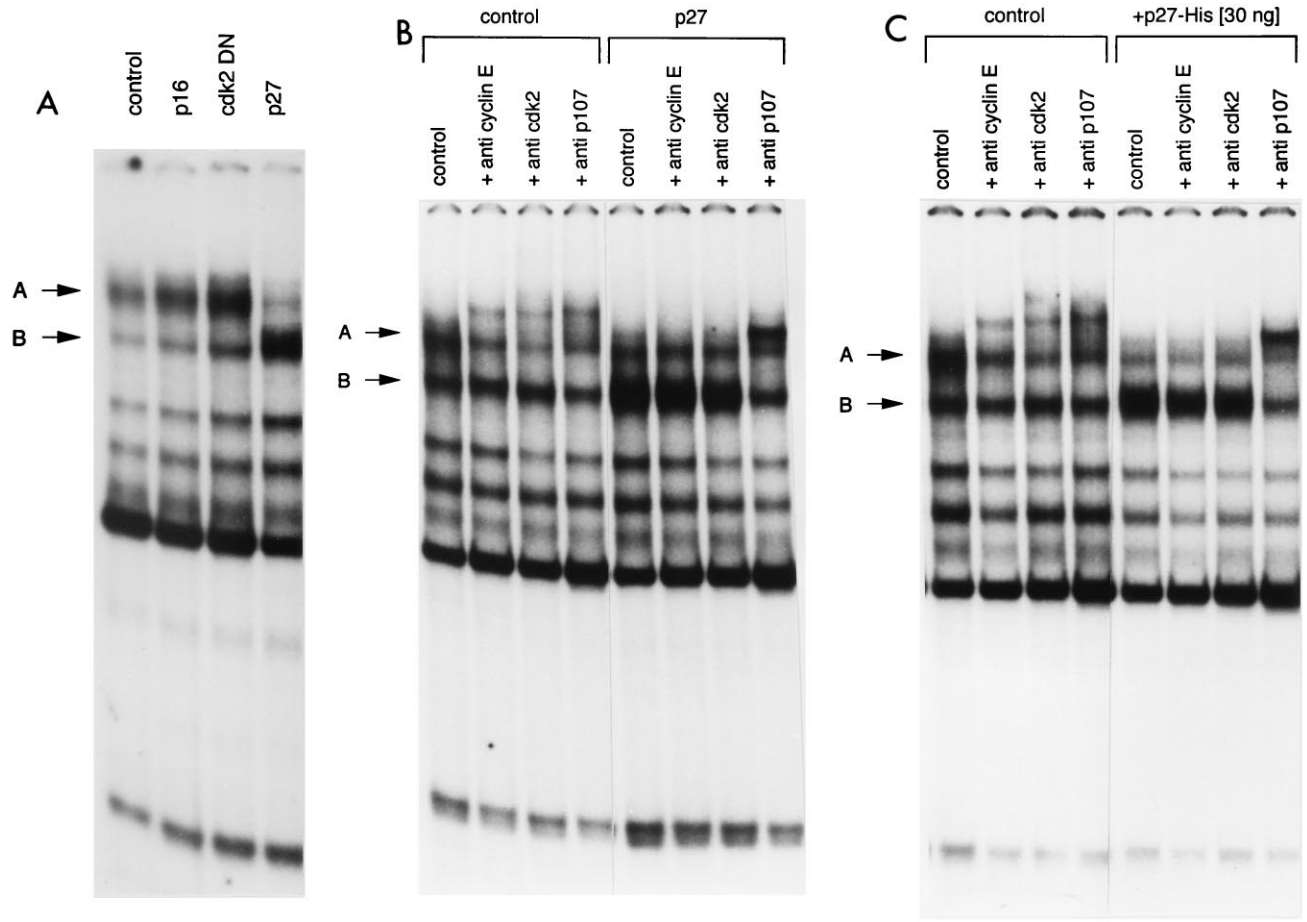


FIG. 3. The cdk inhibitor $p27^{KIP1}$ controls binding of cyclin E to the E2F-binding site of the human cyclin A promoter. (A) Whole-cell extracts obtained after cotransfection of C33A cells with expression vectors for $p16^{INK4}$, cdk2 DN, and $p27^{KIP1}$ were analyzed by electrophoretic mobility shift assay for protein binding to an oligonucleotide representing the E2F-binding site of the human cyclin A promoter (cycA wt). Specific complexes A and B are marked. (B) Oligonucleotide cycA wt was incubated with complexes from extracts of mock-transfected C33A cells and from C33A cells, transfected with $p27^{KIP1}$ expression vector. Complex formation was challenged by polyclonal antibodies to p107, cyclin E, and cdk2, as indicated. (C) Extracts from asynchronously growing C33A cells were incubated with oligonucleotide cycA wt. Addition of recombinant $p27^{KIP1}$ protein to the reaction mixture led to specific disruption of complex A. Complex formation was challenged by polyclonal antibodies to p107, cyclin E, and cdk2, as indicated. (D) Assembly of E2F multiprotein complexes from transfected components. C33A cells were transfected with pCMV-p107, pCMV-cyclin E, pCMV-cdk2 wt, pCMV-DP-1, and pCMV-E2F-4, as indicated. Extracts were prepared from transfected cells and analyzed by electrophoretic mobility shift assay as in panel A. Annotations on the left side of the picture refer to specific complexes endogenous to C33A cells; annotations on the right side of the picture indicate the presumable components of complexes reconstituted from transfected proteins.

inhibit cdk4 and cdk6 (14, 44), was much less active in this assay (Fig. 1C). Thus, two different experimental strategies, described by others to block cyclin E/cdk2 kinase activity, are shown here to prevent activation of the cyclin A promoter, indicating that cyclin E-associated kinase is critical for promoter activation.

**$p27^{KIP1}$ controls binding of cyclin E to the cyclin A promoter.** The results shown in Fig. 1C indicate that $p27^{KIP1}$ has a potential to block cyclin A gene expression. To analyze if this finding might be related to the observed repression of cyclin A transcription in NIH 3T3 cells during $G_1$ (42), we investigated the levels of $p27^{KIP1}$ in synchronized NIH 3T3 cells. In keeping with results reported by others (28), we found that in NIH 3T3 cells high levels of $p27^{KIP1}$ accumulate in serum-starved cells and $p27^{KIP1}$ is virtually absent from S phase cells (Fig. 2A). To

analyze the binding of endogenous E2F complexes to the cyclin A promoter under these conditions, band shift analyses were performed. As was shown before (42), serum starvation of NIH 3T3 cells led to a change in the E2F complexes. When the complexes were analyzed by antibody supershift experiments, it appeared that cyclin E is present in E2F complexes at 14 h after serum addition but is absent from E2F complexes in $G_0$ cells (42) (Fig. 2B). To analyze the pocket proteins present in these complexes, we used monoclonal antibody SD15, which recognizes p107 but not p130 (6, 8a). SD15 supershifted only part of the complex in $G_0$ extracts, while this antibody yielded a complete supershift of the S phase-specific complex (Fig. 2B). In contrast, a polyclonal antibody to p130, which was shown to cross-react with p107 in Western blot experiments (55a), supershifted all of the $G_0$ and most of the S phase complexes. From these results it appears that both p107 and p130 are present in the $G_0$ complexes observed on the E2F site of the cyclin A promoter, while p130 complexes disappear during $G_1$ progression (Fig. 2B). Since the E2F binding site is a target for activation of the cyclin A promoter by cyclin E-associated kinase, we analyzed changes in protein binding to this element in cells which had been transfected by expression vector for p27$^{KIP1}$, i.e., under conditions where transcription from transfected cyclin A promoter constructs is inhibited (Fig. 1C). C33A cells were chosen for this experiment, as such cells can be transfected with high efficiency, allowing for better detection of biochemical alterations caused by a given transfected gene. Similar to the results obtained with NIH 3T3 cells, several specific complexes were obtained on the cyclin A promoter-derived E2F binding site in extracts from mock-transfected asynchronously growing C33A cells. Transfection of C33A cells with the p27$^{KIP1}$ expression vector resulted in a significant change of the band shift pattern: the abundance of one complex, designated A in Fig. 3A, was severely reduced in extracts from p27$^{KIP1}$-transfected cells, and complex A was replaced by a second complex of higher mobility, referred to as complex B. In contrast to the results obtained with p27$^{KIP1}$ overexpression, expression of the dominant negative mutant of cdk2, although capable of blocking cyclin E-mediated activation of the cyclin A promoter (Fig. 1C), did not significantly alter the band shift pattern (Fig. 3A). Similarly, expression of p16$^{INK4}$ did not induce major changes in the band shift pattern (Fig. 3A). High-level expression of all transfected genes, strongly exceeding endogenous levels of the respective proteins, was verified by Western blotting (data not shown). The protein composition of the complexes designated A and B was revealed by the appearance of supershifts upon addition of specific antibodies. Complex A is supershifted by antibodies to p107, cyclin E, and cdk2, and complex B contains p107 but not cyclin E nor cdk2 (Fig. 3B). Antibodies to pRb and p130 failed to recognize any of the bands shown here, indicating that both pRb, which is mutated in C33A cells, and p130 may not be critically involved in control of this promoter element in C33A cells (see also Discussion). As shown for endogenous complex B, the new complex obtained upon p27$^{KIP1}$ expression is devoid of cyclin E and cdk2 but contains p107 (Fig. 3B). Since even under optimal transfection conditions not more than 10 to 30% of C33A cells express a given transfected gene (data not shown), quantitative disruption of complex A indicates that p27$^{KIP1}$ protein, highly expressed in a small subfraction of the cells only, diffuses in the extract and disrupts cyclin E/E2F complexes extracted from untransfected cells. To confirm the ability of p27$^{KIP1}$ to disrupt preexisting cyclin E/cdk2/p107/E2F complexes, recombinant p27$^{KIP1}$ protein, produced in *E. coli*, was added to cellular extracts, and changes in the composition of the E2F complexes were monitored by band shift experiments. Addition of recombinant p27$^{KIP1}$ to extracts from mock-transfected C33A cells also led to a conversion of complex A to complex B (Fig. 3C). Taken together, these observations strongly suggest that p27$^{KIP1}$ promotes dissociation of cyclin E/cdk2 from complex A to generate complex B.

The results presented above allow conclusions concerning the presence of several proteins in complexes A and B. As a further control to study the composition of both complexes, an attempt was made to reconstitute these complexes from their known components. While the E2F family members participating in the complexes native to C33A cells are unknown, the presence of p107 in both complexes A and B prompted us to use for the reconstitution experiments E2F-4, a known member of the p107 binding subset of the E2F family of proteins (3, 10). When C33A cells were transfected with expression vectors for E2F-4 and DP-1, coexpression of both proteins was required to obtain a detectable new band in the band shift assay. Coexpression of p107 virtually abolished this band but led to the appearance of a new, more slowly migrating band, comigrating with complex B. Coexpression of cyclin E and cdk2 then produced a complex with even lower mobility that comigrated with complex A (Fig. 3D). These results are consistent with a sequential reconstitution of E2F complexes containing E2F-4, DP-1, p107, cyclin E, and cdk2 and indicate that complexes A and B differ mainly by the presence of cyclin E/cdk2.

To demonstrate p27$^{KIP1}$-driven disruption of cyclin E/cdk2/p107/E2F complexes by an independent approach, immunoprecipitation experiments were performed. Cells were transfected with expression vectors for E2F-4, DP-1, and p107 to reconstitute a high amount of the ternary complex (complex B in Fig. 3). When DP-1 was immunoprecipitated from extracts of transfected cells, both endogenous cyclin E and the transfected p107 protein were coprecipitated (Fig. 4A, lane 2). Similarly, when cyclin E was immunoprecipitated from extracts of transfected cells, p107 was coprecipitated (Fig. 4B, lane 2). While in the latter case detection of DP-1 was impossible due to comigrating immunoglobulins, these results are consistent with the association of cyclin E to both p107 and DP-1 in this experiment. When cyclin E/cdk2 expression vectors were included in the transfection experiments, a higher amount of cyclin E was coprecipitated with DP-1 (Fig. 4A, lane 3); similarly, a higher amount of p107 could be coprecipitated by antibodies to cyclin E (Fig. 4B, lane 3). It should be noted that two slightly different forms of cyclin E were expressed from the transfected vector. It is possible that the second form represents the product of partial degradation or the occurrence of internal translation starts; this was not further investigated. These findings indicate that also ectopically expressed cyclin E interacts with p107 and DP-1 in cellular extracts. When an expression vector for the dominant negative mutant of cdk2 was included in the transfection experiment (lane 5 in Fig. 4A, B, and C), the interaction between cyclin E, p107, and DP-1 was not disturbed; rather, a slightly higher abundance of cyclin E-bound p107 was detected (Fig. 4B, lane 5). In contrast, the interaction between p107 and cyclin E was completely abolished when p27$^{KIP1}$ was coexpressed (Fig. 4B, lane 4); however, p107 remained bound to DP-1 under these conditions (Fig. 4A, lane 4). Disruption of p107/cyclin E complexes by p27$^{KIP1}$ is accompanied by efficient association of p27$^{KIP1}$ with cyclin E (Fig. 4B, C, lane 4), indicating that p27$^{KIP1}$ may disrupt cyclin E/p107 complexes by blocking the p107 interaction site on cyclin E. Alternatively, it appears possible that the conformational change that is caused by p27$^{KIP1}$ binding to cyclin/cdk complexes (41) triggers the dissociation of p107 from such complexes.

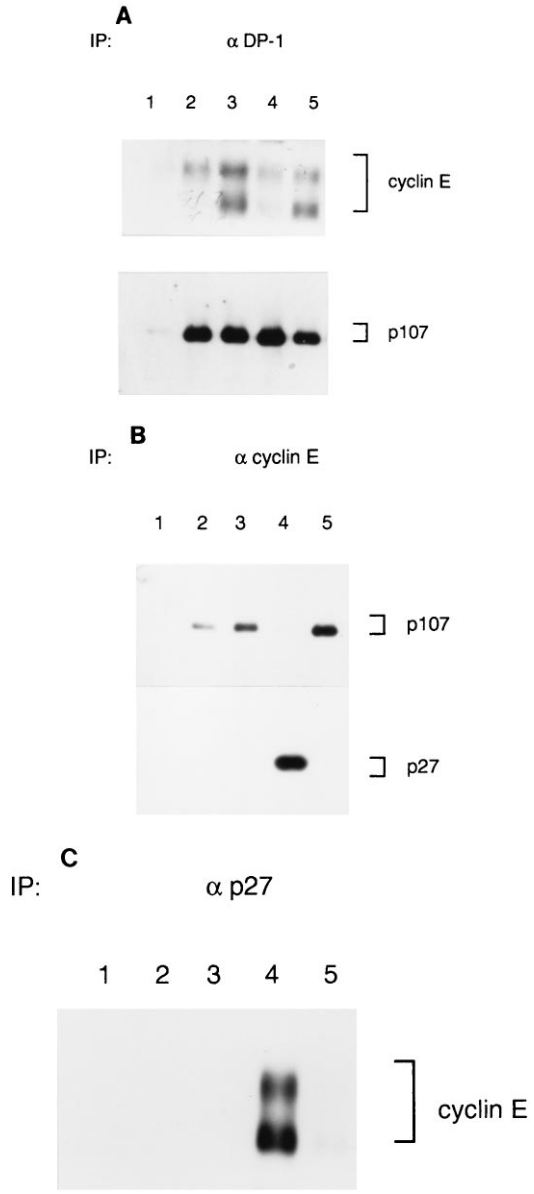


FIG. 4. p27$^{KIP1}$ prevents association of cyclin E with E2F/p107 complexes. C33A cells had been transiently transfected with pCMV (lane 1); pCMV-p107 (lane 2); pCMV-p107, pCMV-cyclin E, pCMV-cdk2wt, pCMV-DP-1, and pCMV-E2F-4 (lane 3); pCMV-p107, pCMV-cyclin E, pCMV-cdk2 wt, pCMV-DP-1, pCMV-E2F-4, and pCMV-p27$^{KIP1}$ (lane 4); and pCMV-p107, pCMV-cyclin E, pCMV-DP-1, pCMV-E2F-4, and pCMV-cdk2DN (lane 5). Extracts from transfected cells were immunoprecipitated by using antibodies to DP-1 (panel A), cyclin E (panel B), and p27$^{KIP1}$ (panel C). Samples of immunoprecipitates were separated on SDS–7 and 10% polyacrylamide gels and analyzed by Western blotting for coprecipitation of cyclin E, p107, and p27$^{KIP1}$, respectively.

**Differential effect of cyclins D1 and E on the stability of E2F/p107 complexes.** We have shown that cyclin D1 (42) and cyclin E (this report) share the ability to activate transcription of the cyclin A gene, and in both cases the associated kinase activity is required. Since ectopic expression of p107 was shown to block cyclin A transcription (55), phosphorylation of p107 by cdk may contribute to activation of cyclin A transcription via E2F. In support of this, it was shown that expression of cyclin D1/cdk4 leads to a hyperphosphorylation of p107, resulting in dissociation of p107 from E2F/DP complexes and the generation of transcriptionally active "free" E2F (2). To investigate if a similar mechanism is involved in the activation of cyclin A gene expression by cyclin E, we analyzed changes in the p107 phosphorylation status upon ectopic expression of cyclin E/cdk2. C33A cells were transfected with expression vectors encoding p107 and either cyclin E/cdk2 or cyclin D1/cdk4; the phosphorylation status of p107 was then investigated by Western blotting. While phosphorylation by cdk4 induces a pronounced shift in mobility of p107 (similar to results reported in reference 2), a weaker shift in p107 mobility is reproducibly obtained with cyclin E/cdk2 (Fig. 5A); in both cases, the alteration of electrophoretic mobility can be reversed by treatment of the extracts with lambda phosphatase (reference 2 and data not shown). This result indicates that p107 is a potential in vivo substrate for cdk2 kinase, in keeping with the observation that cyclin E/cdk2 phosphorylates p107 in vitro (26).

To address the question of whether, like cyclin D1/cdk4, expression of cyclin E/cdk2 can release E2F from p107, we used coimmunoprecipitation experiments. To selectively study p107 interactions occurring in transfected cells, an HA-epitope tagged version of the p107 protein (p107-HA) was used. When cyclin E/cdk2 was coexpressed with p107-HA in C33A cells, the association of p107-HA with DP-1 was not significantly affected. In contrast, DP-1 antibodies failed to coprecipitate p107-HA from extracts of cells cotransfected with cyclin D1/cdk4 (Fig. 5B), consistent with a quantitative disruption of E2F-p107 complexes by cyclin D1/cdk4, as reported by Beijersbergen et al. (2). High-level expression of all transfected proteins was monitored by Western blotting (data not shown). Together the results of these experiments indicate that, in contrast to cyclin D1/cdk4, cyclin E/cdk2 fails to trigger dissociation of p107 form E2F complexes; instead, the available evidence suggests that cyclin E/cdk2 physically associates with E2F/p107 complexes, and this leads to promoter activation.

## DISCUSSION

Results presented in this communication demonstrate that cyclin E, a rate-limiting determinant of $G_1$ progression in mammalian cells, binds to the cell cycle regulatory element of the human cyclin A promoter and triggers activation of cyclin A transcription upon ectopic expression. While the data reported here indicate that the E2F site is essential for promoter activation, our finding that three tandem copies of the E2F site of the cyclin A promoter do not confer inducibility by cyclin E/cdk2 to a heterologous promoter (55a) suggests that activation of the cyclin A promoter by cyclin E requires additional promoter elements. The failure of cyclin E/cdk2 to activate a cyclin A reporter gene construct in which the CHR element (59) is deleted (Fig. 1A) indicates that this element is involved in the response to cyclin E/cdk2. Promoter activation requires cdk2 kinase activity, as activation can be enhanced by cyclin E/cdk2 and blocked by coexpression of cdk2DN and p27$^{KIP1}$, two known specific inhibitors of cyclin E/cdk2 kinase (37, 50). The results shown in this report significantly extend previous findings that E2F-dependent transcription can be augmented by $G_1$ cyclins and their associated kinases (8, 20, 43), by showing that association of cyclin E with E2F complexes on the cyclin A promoter is rate limiting for transcriptional activation. The association of cyclin E/cdk2 with E2F complexes is sensitive to transient expression of p27$^{KIP1}$ in vivo and to addition of recombinant p27$^{KIP1}$ in vitro, indicating that p27$^{KIP1}$ actively disrupts preexisting E2F/DP/p107/cyclin E/cdk2 complexes. A role for p27$^{KIP1}$ in regulation of cyclin A promoter activity in NIH 3T3 cells is further supported by our finding that, in agreement with results reported by others (30), high levels of

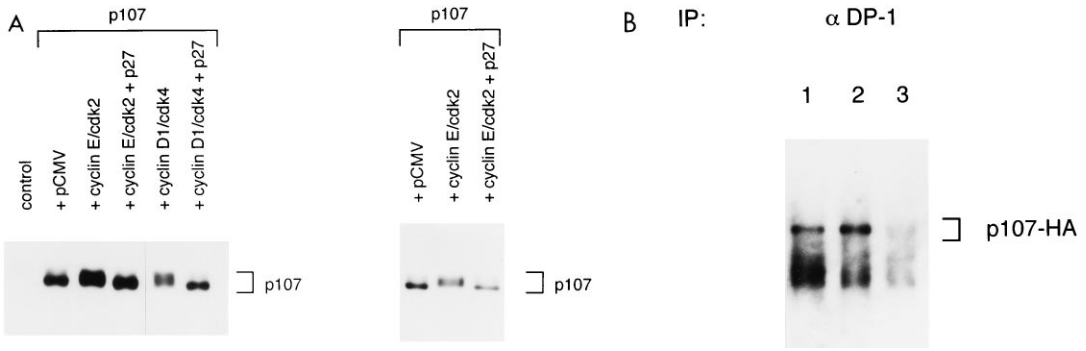


FIG. 5. Differential effects of cyclin E/cdk2 and cyclin D1/cdk4 on p107/E2F complexes. (A) Cyclin E/cdk2 phosphorylates p107 protein in vivo. C33A cells transfected with expression constructs for p107 together with expression plasmids for cyclin D1, cyclin E, cdk4, cdk2, and $p27^{KIP1}$ as indicated. Whole cell extracts were separated on a 7% polyacrylamide gel and analyzed by direct immunoblotting for p107 expression and modification. (B) Analysis of p107/E2F complexes. C33A cells were transiently cotransfected with expression vectors for p107-HA, E2F-4, and DP-1 (lane 1) and in addition cyclin E/cdk2 (lane 2) and cyclin D1/cdk4 (lane 3). Extracts from transfected cells were immunoprecipitated with antibodies to DP-1. Immunoprecipitates were separated on a 7% polyacrylamide gel and analyzed by immunoblotting by using anti-HA antibody for detection of coprecipitated HA-tagged p107.

$p27^{KIP1}$ accumulate in serum-starved NIH 3T3 cells, and $p27^{KIP1}$ is virtually absent from S phase cells (Fig. 2A). In contrast to these observations, Poon et al. reported that in Swiss 3T3 cells $p27^{KIP1}$ levels show only weak fluctuation during the cell cycle, and a large proportion of the cellular $p27^{KIP1}$ is sequestered by cyclin D1 (38), indicating that the actual role of $p27^{KIP1}$ in control of cdk activity may vary between different cell types; this hypothesis is clearly supported by the recent discovery that fibroblasts from $p27^{KIP1}$ knockout mice have no overt defects in cell cycle regulation (29). The easiest assumption to reconcile these data is that in a $p27^{KIP1}$-negative background the function of $p27^{KIP1}$ is taken over by other cdk inhibitors, which remain to be identified. Similar to the findings shown here, it was reported that $p21^{WAF-1/Cip1}$ can disrupt E2F/cyclin A complexes in vitro (57). However, while both cyclin E and cyclin A can bind E2F complexes, cyclin E can activate E2F-driven transcription while cyclin A cannot (Fig. 1); in the case of E2F-1, cyclin A even blocks E2F-driven transcription (25); hence, the consequences of complex dissociation may be quite different in either case.

Recently, Shiyanov et al. (46) have demonstrated disruption by $p21^{WAF-1/Cip1}$ of E2F/p130/cdk2 complexes, the cyclin component of which was not identified. While this observation suggests that p130-containing complexes may also be a target for disruption by $p27^{KIP1}$, our attempts to identify p130 in E2F complexes obtained from C33A cells consistently failed, and this is explained by our observation that in asynchronously growing C33A cells p130 protein levels are very low (data not shown). While p130 can be found as a minor constituent of E2F complexes in serum-starved NIH 3T3 cells, we did not observe any cyclin-containing p130/E2F complex in NIH 3T3 cells; therefore, in these cells the disruption of E2F-p130-cyclin complexes by $p27^{KIP1}$ cannot be the critical event controlling cyclin A promoter activity. Unlike p107-containing complexes, p130 complexes disappear during $G_1$ progression (Fig. 2B), and this coincides with a drastic decrease of p130 abundance (55a), which may at least in part account for derepression of the cyclin A gene. In contrast, p107 remains bound to E2F in serum-stimulated NIH 3T3 cells; these complexes then acquire the new components cyclin E and cdk2, which may be required for transcriptional activation. That p107 and p130 may be able to independently downregulate cyclin A transcription is in accordance with the recent genetic demonstration that the function of the p107 and p130 genes may be largely redundant (5).

Although it was shown before that, similar to cyclin E/cdk2, ectopic expression of cyclin D1 and its associated kinase cdk4 activates cyclin A gene expression (42), there is compelling evidence that cyclin D1 and cyclin E perform distinct functions in the mammalian cell cycle. The data reported here indicate that the mechanism by which cyclin A transcription gets activated is quite different for cyclin D1 and cyclin E. It is shown here that promoter activation by cyclin E/cdk2 does not involve substantial disruption of E2F/p107 complexes, whereas expression of cyclin D1/cdk4 leads to a rapid and efficient dissociation of p107 from E2F complexes (2) (Fig. 5B). In support of a differential mechanism of transactivation by cyclin D1 versus cyclin E, it was shown that cyclin E but not cyclin D1 binds to p107 with high affinity (56). Furthermore, while cyclin E/E2F complexes are easily detectable in cellular extracts, we failed to detect stably bound cyclin D1 in E2F complexes (41a). Results shown in this report indicate that ectopic expression of cyclin E/cdk2 induces phosphorylation of p107 (Fig. 5A), but p107 is not dissociated from E2F complexes under these conditions (Fig. 5B). This result contrasts with the finding that phosphorylation of pRb by $G_1$ cyclins, including cyclin E, leads to a release of free E2F, at least in vitro (8), indicating that the role of cyclin E-mediated phosphorylation may differ considerably between pRb and p107. Thus, it is possible that phosphorylation of p107 by cyclin E/cdk2, instead of releasing free E2F, can modulate the intrinsic ability of p107 to repress transcription, even though the protein remains bound to E2F. Alternatively, other proteins that are associated with the cyclin A promoter could be substrates for cyclin E/cdk2, and their phosphorylation may be required for transcriptional activation. A potential clue to additional cdk2 substrates comes from the recent observation that $G_1$ arrest induced in NIH 3T3 cells by cdk2DN is resistant to coexpression of simian virus 40 T antigen, although in this experiment active E2F was released from negative control by pRb family members (17). This finding suggests that the ability of cdk2 to promote S phase entry may involve the phosphorylation of nonpocket protein substrates, e.g., E2F-1 (9). The dependence of transcriptional activation of the cyclin A promoter on the CHR element (Fig. 1A) raises the possibility that proteins binding to this element might also be critical substrates for promoter-bound cyclin E/cdk2. Identification of these proteins will be required to address this question.

The results presented in this report suggest a two-step model for cyclin A promoter activation by cyclin E (Fig. 6). First, cyclin E/cdk2 complexes assemble with p107 molecules bound

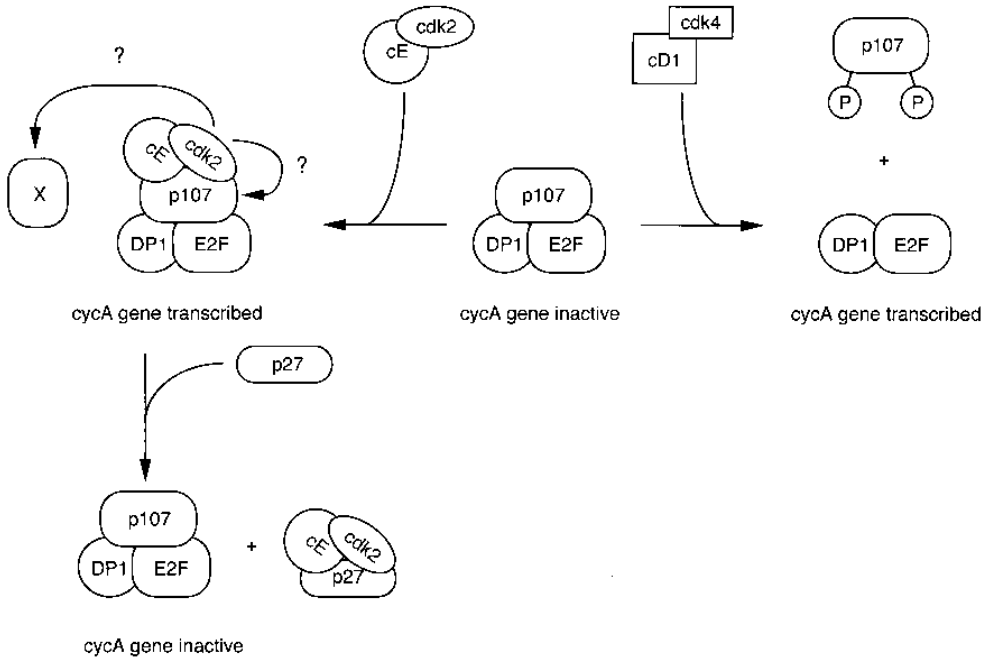


FIG. 6. Model for induction of cyclin A promoter activity by cyclin D1 and cyclin E. A model is shown for the differential activation of cyclin A gene expression by cyclin D1 and cyclin E. Phosphorylation of p107 by cyclin D1/cdk4 leads to a dissociation of the protein from E2F/DP complexes and the subsequent generation of free E2F, which is known to represent a transcriptionally active form of the E2F transcription factor. In contrast to the efficient disruption of E2F-p107 complexes by cyclin D1/cdk4, cyclin E/cdk2 stably associates with these complexes; according to the model, the cdk2 kinase activity, brought to the promoter via p107, is required to phosphorylate some substrate proteins in order to release the promoter from repression. While p107 itself is a potential target for cdk2 phosphorylation, additional substrates for cdk2 (referred to as X) cannot be excluded. Disruption of cyclin E/E2F complexes by excess $p27^{KIP1}$ can revert activation of the cyclin A promoter.

to the promoter through E2F. In support of this, it was shown that activation of the cyclin A promoter in serum-stimulated NIH 3T3 cells is accompanied by the appearance of complexes containing p107, DP-1, cyclin E, and cdk2, around 10 h after serum stimulation (42). Since p107 and DP-1 are major components also of the predominant complex observed in $G_0$ cells, these results suggest that cyclin E/cdk2 associates with preexisting E2F/DP-1/p107 complexes in mid $G_1$ phase and that this interaction results in promoter activation. The observation that cyclin E binds to E2F complexes in the presence of cdk2DN (Fig. 4; see also Fig. 3A), yet fails to activate transcription under these conditions (Fig. 1C), indicates that a functional cyclin E/cdk2 kinase must be tethered to the promoter, in order to activate transcription. The identification of the critical substrates for this kinase will be required to resolve the mechanism involved.

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