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Differential Control of Transcription by DNA-bound Cyclins

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Different cyclins mediate different cell-cycle transitions. Some cyclins, such as cyclin A and cyclin E, form stable complexes with proteins that bind directly or indirectly to DNA and thus might be recruited to certain regions of the genome at specific times in the cell cycle. Furthermore, cyclins contain structural motifs that are also present in known transcriptional modulators. We found that cyclin A is a potent transcriptional repressor and cyclin E is a potent transcriptional activator when bound to DNA via a heterologous DNA binding domain. The former activity was linked to the integrity of the cyclin A cyclin fold, whereas the latter activity related to the ability of cyclin E to activate cdk2 and recognize substrates. Furthermore, we found that cyclin E, but not cyclin A, activated transcription in a cell-cycle–dependent manner when present in physiological concentrations as an unfused protein. These results suggest that cyclin A and cyclin E intrinsically differ with respect to their ability to modulate transcription when tethered to DNA.

INTRODUCTION

Progression through the mammalian cell cycle is linked to the orchestrated appearance and destruction of cyclins. Different cyclins are associated with different cell-cycle transitions. For example, cyclin E is active in late G1- and early S-phase, cyclin A is active in S-phase, and cyclin B is active in mitosis (Weinberg, 1995; Sherr, 1996; Roberts, 1999). Cyclins bind to cyclin-dependent kinases (cdks). In this context, cyclins activate the catalytic activity of their partner cdk(s) and also play roles in substrate recognition (Peeper *et al.*, 1993; Adams *et al.*, 1996, 1999; Chen *et al.*, 1996; Dynlacht *et al.*, 1997; Schulman *et al.*, 1998; Roberts, 1999).

Some transcriptional regulatory proteins, such as the pRB homologues p107 and p130 (Ewen *et al.*, 1992; Faha *et al.*, 1992; Lees *et al.*, 1992; Hannon *et al.*, 1993; Zhu *et al.*, 1995a; Smith *et al.*, 1998), the E2F family members E2F1, E2F2, and E2F3 (Bandara *et al.*, 1991; Mudryj *et al.*, 1991; Devoto *et al.*, 1992; Dynlacht *et al.*, 1994, 1997; Krek *et al.*, 1994; Xu *et al.*, 1994; Adams *et al.*, 1996), the transcriptional coactivator p300 (Perkins *et al.*, 1997; Felzen *et al.*, 1999), and NPAT (nuclear protein mapped to the AT locus; Zhao *et al.*, 1998; Ma *et al.*, 2000; Zhao *et al.*, 2000) form stable complexes with cyclin A/cdk2 and/or cyclin E/cdk2. All of these proteins bind directly or indirectly to DNA. Thus, such complexes might serve as vehicles for increasing the concentration of cyclin A/cdk2 or cyclin E/cdk2 at certain sites within the genome. If true, cyclin A/cdk2 and cyclin E/cdk2 might play relatively direct roles in processes such as transcription and DNA replication.

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MATERIALS AND METHODS

Cell Lines and Transfection

U2OS human osteosarcoma cells were grown in Dulbecco's modified Eagle media (DMEM) supplemented with 10% heat-inactivated fetal clone (Hyclone, Logan, UT), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. SAOS-2 human osteosarcoma cells and NIH 3T3 mouse fibroblast cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin/glutamine (PSG). NIH 3T3 stable subclones transfected with the pCMV-neo and pUHC13-3 reporter plasmid alone or with pSG5-TETr-cdk2 or with pSG5-TETr-cdk2 (N132A) were maintained in 0.7 mg/ml G418. Cells were transfected with the use of 2× 2-(bis[2-hydroxyethyl]amino)ethanesulfonic acid-buffered saline (2× BBS)/calcium phosphate as described by Chen and Okayama, 1987). Where indicated doxycycline (Sigma, St. Louis, MO) was added 24 h after transfection to a final concentration of 2 μg/ml. Cells were maintained in doxycycline for an additional 24 h before harvest.

Plasmids

pRcCMV-cdk2 dominant-negative form (van den Heuvel and Harlow, 1993) was a gift of Dr. Ed Harlow; pVL1393-cdk2 (N132A) (Xu *et al.*, 1994) was a gift of Dr. Helen Piwnica-Worms; pCD19 (Tedder and Isaacs, 1989) was a gift of Dr. Thomas Tedder; pUHC13-3; ptet1-T81-luc, ptet2-T81-luc, ptet3-T81-luc, and ptet7-T81-luc (Gossen and Bujard, 1992) were gifts of Dr. Manfred Gossen. pSG5-TETr-

E2F1, pSG5-TETr-RB, pSG5-HA-RB (Sellers *et al.*, 1995), and pGEX-2TK-cdk2 (Adams *et al.*, 1996) have been described previously. To make pSG5-TETr-cyclin A, a protein phosphatase 1 (PP1) cDNA was first polymerase chain reaction (PCR) amplified with oligonucleotides 5′-GCGCTGATCAGGCGGAGGCGGATCAGGAGGAG-GAGGATCAGGCGGAGGAGGATCAGGATCCATGTCCGACAG-CGAGAA-3′ and 5′-GCGCGAATTCATTTCTTGGCTTTGGCAGA-3′. The PCR product was cut with *Bcl*I and *Eco*RI and subcloned into pSG5-TETr cut with *Bam*HI and *Eco*RI to make pSG5-TETr-(Gly $_4$-Ser) $_3$-PP1. The cyclin A open reading frame (ORF) was PCR amplified with primers that introduced a 5′-*Bam*HI site and a 3′-*Eco*RI site and subcloned into pSP72 (Promega, Madison, WI) cut with these two enzymes to make pSP72-cyclin A. The PP1 "stuffer" from pSG5-TETr-(Gly $_4$-Ser) $_3$-PP1 was then excised by digestion with *Bam*HI and *Eco*RI and replaced with the cyclin A cDNA insert from pSP72-cyclin A. To make pSG5-TETr-cyclin E, the cyclin E ORF in pRcCMV-cyclin E was PCR amplified with primers that introduced a 5′-*Bgl*II and 3′-*Eco*RI site. The PCR product was cut with these two enzymes and ligated into the *Bam*HI-*Eco*RI backbone of pSG5-TETr-PP1. In parallel, these restricted cyclin A and cyclin E PCR products were subcloned into pSG5-HA cut with *Bam*HI and *Eco*RI to make pSG5-HA-cyclin A and pSG5-HA-cyclin E, respectively. Plasmids encoding cyclin A and cyclin E N-terminal and C-terminal deletion mutants were made in an analogous manner by the use of PCR primers that selectively amplified the desired coding regions. To make pSG5-TETr-cdk2 and pSG5-TETr-cdk2 (N132A), the cdk2 ORF in pRcCMV-cdk2 and pVL1393-cdk2 (N132A), respectively, were PCR amplified with primers that introduced a 5′-*Bam*HI and 3′-*Eco*RI site. The PCR products were cut with these two enzymes and ligated into the *Bam*HI-*Eco*RI backbone of pSG5-TETr-PP1. All PCR reactions were performed with Pfu DNA polymerase, and the authenticity of plasmids containing the entire cyclin A, cyclin E, or cdk2 ORF was confirmed by direct DNA sequencing. pSG5-TETr-cyclin A (E220A) and pSG5-TETr-cyclin E (L134A/Q174A) were generated with the use of a Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions with the use of pSG5-TETr-cyclin A and pSG5-TETr-cyclin E as templates, respectively, and confirmed by DNA sequencing.

Antibodies and Immunoblot Analysis

Monoclonal anti-TETr was purchased from Clontech and anti-HA (12CA5) was purchased from Boehringer Mannheim (Indianapolis, IN). Polyclonal anti-cyclin A (SC-751), monoclonal and polyclonal anti-cyclin E (SC-247, SC-481), and polyclonal anti-cdk2 (SC-163) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell extracts were made by lysis in EBC buffer (50 mM Tris [pH 8], 120 mM NaCl, 0.5% Nonidet P-40). For immunoblot analysis, ~100 μg of cell extract were loaded per lane. Nitrocellulose filters were blocked in 4% powdered milk/1% goat serum in TBS-T (10 mM Tris [pH 8], 0.05% Tween, 150 mM NaCl) for 1 h at room temperature before incubation in primary antibody. Anti-HA (12CA5) was used at a concentration of 1.0 μg/ml, anti-TETr antibody was used at 1:500 dilution (vol/vol), anti-cyclin A (SC-751) was used at 1:1000 dilution (vol/vol), anti-cyclin E (SC-247, SC-481) was used at 1:1000 dilution (vol/vol), and anti-cdk2 (SC-163) was used at 1: 1000 dilution (vol/vol). After four washes with TBS/T, bound antibody was detected with the use of alkaline phosphatase-conjugated secondary antibodies.

Glutathione S-transferase (GST) Pull-Down Assay

GST pull-down assays were performed basically as described previously (Kaelin *et al.*, 1991). Binding reactions contained 10 μl of ^{35}S-radiolabeled in vitro translates made with a TNT kit (Promega) and ~1 μg of the indicated GST fusion protein in 1 ml of NETN (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). After 1 h of incubation at 4°C with rocking, the Sepharose was washed five times with NETN. Bound proteins were eluted by boiling in SDS-containing sample buffer and resolved by SDS-PAGE. Comparable loading of GST-fusion proteins was confirmed by Coomassie brilliant blue staining, and ^{35}S-radiolabeled proteins were detected by fluorography.

Fluorescence-activated cell sorting (FACS)/Cell Cycle Analysis

FACS was done essentially as described by Qin *et al.* (1995). Briefly, subconfluent SAOS-2 cells grown in 100-mm dishes were transfected with 2 μg of pCD19 and 10 μg of pSG5-HA-RB together with plasmids encoding the indicated cyclins. Later (72 h) the cells were harvested with trypsin-EDTA and stained with fluorescein isothiocyanate-conjugated anti-CD19 antibody (Caltag, South San Francisco, CA) and propidium iodide. Samples were analyzed by two-color FACS with a FACScan (Becton Dickinson, Mountain View, CA). For cell-cycle synchronization, cells were starved in serum-free DMEM for 72 h before being stimulated with 10% fetal bovine serum.

Luciferase Reporter Gene Assay

For TETr-fusion transcriptional assay, subconfluent U2OS cells were transiently transfected in six-well plates in duplicate with 1 μg of pCMV-βgal, 1 μg of pUHC13-3 reporter plasmid, and 3 μg of the indicated plasmids encoding TETr-fusion proteins. Sufficient parental pSG5-TETr was added so that each reaction mixture contained the same amount of pSG5-TETr backbone. After transfection (48 h) luciferase activity and β-galactosidase activity was determined as described previously (Qin *et al.*, 1995).

In Vitro Kinase Assay

Cell extract (500 μg) was incubated with protein A Sepharose and 1 μg of anti-cyclin E (SC-481) or anti-cyclin A (SC-751) antibody for 1 h at 4°C in a final volume of 0.5 ml. The Sepharose was then washed five times with NETN and three times in immunoprecipitation kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT). The Sepharose was then resuspended in 27 μl of immunoprecipitation kinase buffer to which 2 μl of histone H1 (1 mg/ml) and 1 μl of [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/ml) was added and incubated for 30 min at 30°C. Reactions were stopped by addition of Laemmli sample buffer, boiled, resolved by SDS-PAGE, and subjected to autoradiography.

RESULTS

To ask whether cyclins A and E can directly affect transcription, we made mammalian expression plasmids encoding fusion proteins consisting of the TET repressor DNA-binding domain (TETr) (Gossen and Bujard, 1992) fused to cyclin A or cyclin E with an intervening flexible linker consisting of Gly_4-Ser repeats (Figure 1A). Both of these plasmids gave rise to stable proteins of the expected size after transfection into mammalian cells (Figure 1B). In pilot experiments, we confirmed that TETr-cyclin A and TETr-cyclin E, like their unfused counterparts, bound to cdk2 (Figures 4 and 5) and could phosphorylate p107 in vitro (Kim and Kaelin, unpublished results). Furthermore, both TETr-cyclin A and TETr-cyclin E promoted pRB phosphorylation and bypassed a pRB-induced G1/S block when cointroduced with wild-type pRB into pRB-defective tumor cells (Figure 1C). We therefore concluded that fusion to the TETr domain did not disrupt the hallmark biochemical and biological properties of these cyclins.

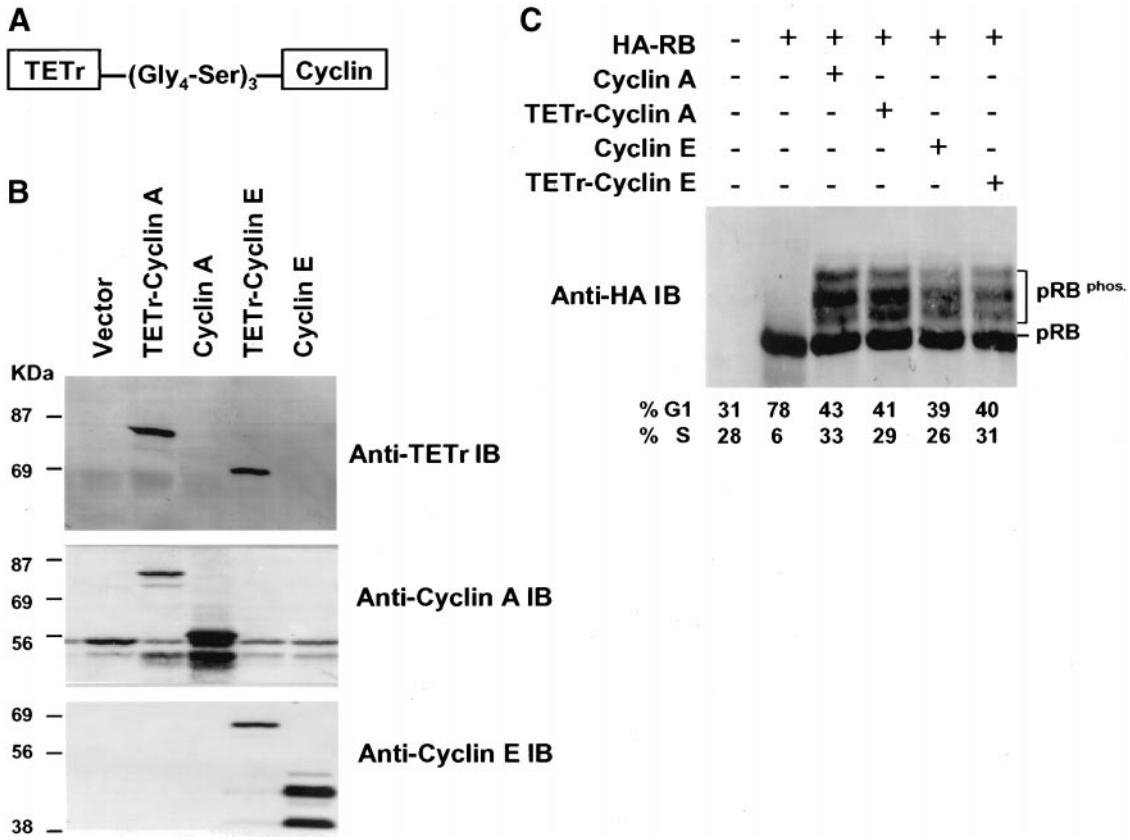

Figure 1. Production of TETr-cyclins A and E. (A) Schematic of TETr-cyclin fusion protein. (B) Production of TETr- cyclin A and TETr-cyclin E. Cells transfected to produce the indicated cyclin A or cyclin E proteins were lysed and immunoblotted (IB) with the indicated antibodies. (C) Phosphorylation of pRB by TETr-cyclins A and E in SAOS-2 cells. pRB-defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated cyclins were lysed and immunoblotted with anti-HA antibody. The percentage of transfected cells in G1- and S-phase was determined by FACS.

U2OS cells were next transiently transfected with plasmids encoding various TETr-fusion proteins and a luciferase reporter plasmid containing seven TETo-binding sites upstream of a TATA box derived from the CMV promoter (Figure 2A). TETr binds specifically to TETo sites. As expected, TETr-RB repressed transcription from this reporter plasmid, whereas TETr-E2F1 activated the reporter (Figure 2, B and C). The basal activity observed with this reporter plasmid presumably reflects the presence of cryptic enhancer sequences. In this and subsequent assays, the TETr domain alone was essentially inert. Surprisingly, TETr-cyclin A and TETr-cyclin E both dramatically affected transcription in this assay and did so in opposite ways. TETr-cyclin A decreased transcription ~80% (fivefold repression), whereas TETr-cyclin E increased transcription 10-fold (Figure 2, B and C).

To ask whether the effects of TETr-cyclin A and TETr-cyclin E were direct, we repeated these experiments in the presence or absence of doxycycline. Doxycycline prevents the binding of TETr to TETo and completely blocked the transcriptional effects of TETr-cyclin A and TETr-cyclin E (Figure 3A). As expected, doxycycline also blocked the transcriptional effects of TETr-RB and TETr-E2F1, which were tested in parallel. Furthermore, unfused cyclin A and E had no effects on the TETo-driven reporter plasmid (Figure 3B). These results suggest that cyclin A and cyclin E directly affect transcription once tethered to DNA. To exclude the possibility that the observed transcriptional effects were peculiar to the presence of seven TETo or the CMV TATA box in the reporter under study, these experiments were repeated with the use of reporters containing one, two, three, or seven TETo in which the CMV-derived TATA box was replaced with a minimal HSV TK promoter (Gossen and Bujard, 1992; Figure 3C). TETr-cyclin E also activated these reporters in a doxycycline-inhibitable manner. The degree of activation observed with the HSV TK series of reporters was lower than with the CMV TATA-based reporter, in keeping with earlier results obtained with these reporters and TETr fused to the HSV VP16 transcriptional activation domain (Gossen and Bujard). The low basal level of transcription from these reporters precluded analysis of repression by cyclin A. These results suggest that a single cyclin E/cdk2 complex might suffice to activate transcription in an appropriate promoter context.

We next made plasmids encoding TETr fused to various colinear fragments of cyclin A and E to determine which regions of these molecules are required for transcriptional regulation (Figures 4 and 5). All of the resulting fusion

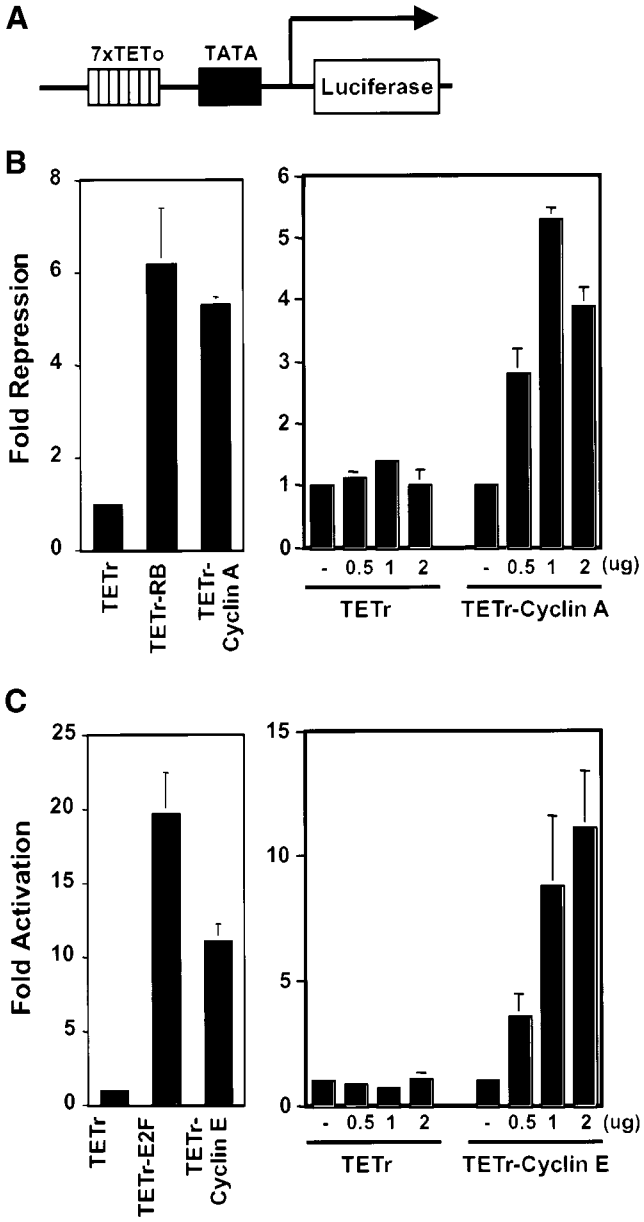

Figure 2. DNA-bound cyclins A and E differentially affect transcription. (A) Schematic of reporter plasmid (pUHC 13-3) that contains seven TETracycline operator sequences (TETo) upstream of a minimal CMV promoter that includes a TATA box. (B and C) U2OS cells were cotransfected with plasmids encoding the indicated TETr-fusion proteins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β-galactosidase. Numbers shown at the bottom of the graph indicate the amount of TETr plasmid (in μg). Forty-eight hours later, luciferase activity, normalized for β-galactosidase, was determined. Fold repression is the corrected luciferase value for TETr alone divided by the corrected luciferase values for the indicated TETr-fusion proteins. Fold activation represents the corrected luciferase values for the indicated TETr-fusion proteins divided by the corrected luciferase value for TETr alone.

proteins were expressed at comparable levels in transient transfection experiments (Kim and Kaelin, unpublished results). Cyclin A (1–310), like wild-type cyclin A, repressed transcription when fused to TETr (Figure 4A). This fragment of cyclin A does not bind to cdk2 (Figure 4B) and cannot direct the phosphorylation of pRB when introduced into cells (Figure 4C). Conversely, a cyclin A point mutant (E220A) (Schulman *et al.*, 1998) that measurably interacts with cdk2 (Figure 4B) and directs the phosphorylation of pRB (Figure 4C) did not repress transcription in these assays (Figure 4A). This mutation maps to the cyclin A cyclin box (Figure 4A). TETr-cyclin A also repressed transcription when tested in p107$-/-$;p130 $-/-$ mouse fibroblasts (Kim and Kaelin, unpublished results), and cyclin A (1–310) does not bind to either p107 or p130 (Kim and Kaelin, unpublished results). Together, these results suggested that transcriptional repression by cyclin A was linked to the integrity of its cyclin box but not to its ability to activate cdk2 or its ability to recruit the known transcriptional repressors p107 and p130.

In contrast to the results obtained with cyclin A, only those cyclin E mutants that could bind to cdk2 (Figure 5B) and could direct the phosphorylation of pRB (Figure 5C) scored as transcriptional activators (Figure 5A). For example, Schulman *et al.* (1998) identified cyclin A residues that are critical for substrate binding and assembly with cdk2. Mutation of analogous residues in cyclin E produced a mutant (cyclin E L134A/Q174A) that likewise failed to bind to cdk2 (Figure 5B) and failed to phosphorylate pRB (Figure 5C). This mutant did not activate transcription (Figure 5A). In keeping with these results, a dominant-negative form of cdk2 blocked transcriptional activation by cyclin E (Figure 6B) but had no effect on transcriptional repression by cyclin A (Figure 6A). Similarly, cyclin E, but not cyclin A, activated transcription in concert with a TETr-cdk2 fusion provided the kinase domain was intact (Figure 6C). Comparable production of TETr-cdk2 and kinase-defective TETr-cdk2 (N132A) was confirmed by immunoblot assay (Kim and Kaelin, unpublished results). *Xenopus* cyclin E (Jackson *et al.*, 1995), like its human counterpart, also activated transcription in these assays (Kim and Kaelin, unpublished results). This activity was specific because *Xenopus* cyclin E variants with point mutations affecting the cyclin box were inert (Kim and Kaelin, unpublished results). Thus, the ability of cyclin E to activate transcription is conserved across divergent species.

To ask whether cyclin E could activate transcription under physiological conditions, we transfected 3T3 cells with a plasmid containing a selectable marker and TETo reporter plasmid with or without a plasmid encoding TETr-cdk2. After drug selection, the stable transfectants were maintained as polyclonal pools and serum starved into quiescence. At various times after serum refeeding, cell lysates were prepared and used in immunoblot, in vitro kinase, and luciferase assays (Figure 7). In parallel, aliquots of the cells were analyzed for DNA content by FACS. In this system, S-phase entry began 18–20 h after the addition of serum. As expected, luciferase activity increased in the TETr-cdk2 producing cells coincident with an increase in cyclin E protein levels and cyclin E-associated kinase activity (Figure 7). No such increase was observed in the cells producing equivalent amounts of TETr-cdk2 (N132A) or transfected with the

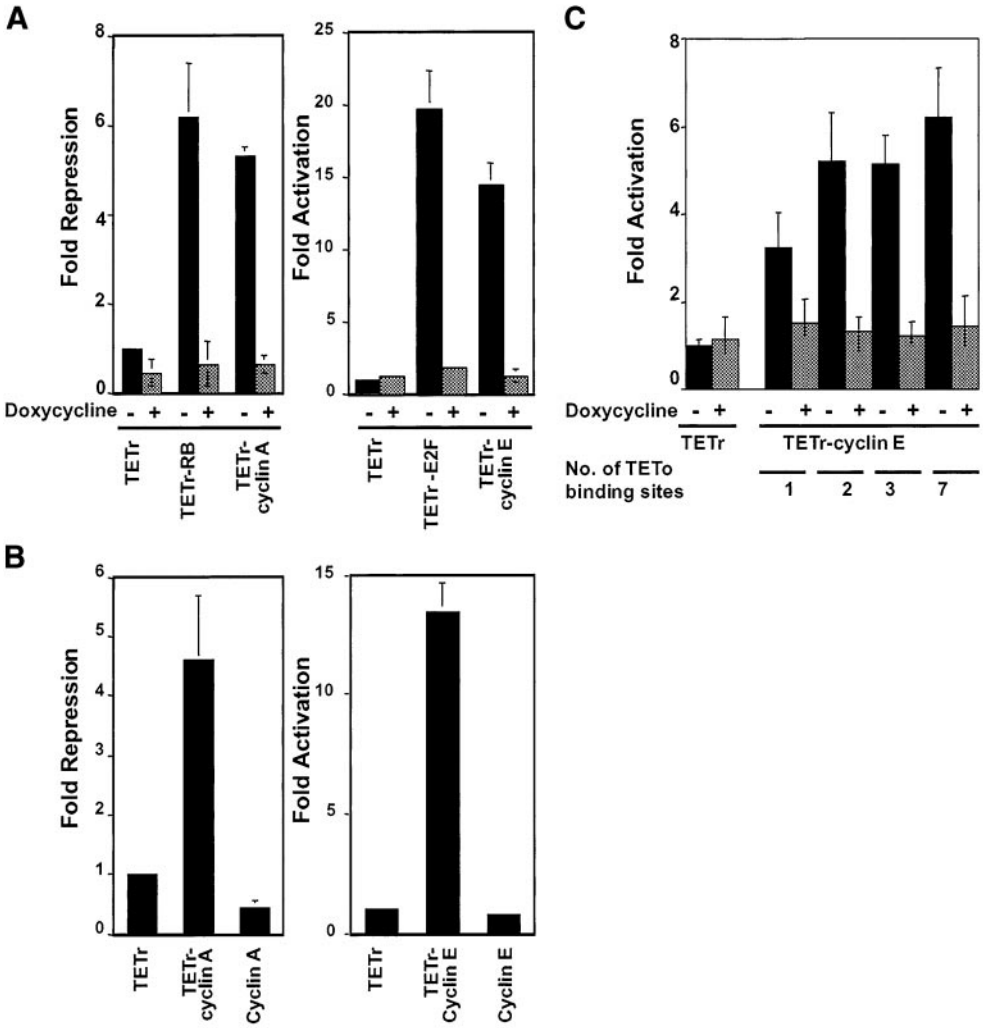

Figure 3. Transcriptional regulation by cyclins A and E dependent on DNA binding. (A) U2OS cells were cotransfected with plasmids encoding the indicated TETr-fusion proteins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β-galactosidase. Doxycycline was added 24 h later to a final concentration of 2 μg/ml where indicated by a "+." After an additional 24 h luciferase activity, corrected for β-galactosidase activity, was determined and expressed as fold repression or activation relative to cells producing TETr alone. (B) U2OS cells were cotransfected with plasmids encoding the indicated cyclins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β-galactosidase. Fold repression and activation was determined as in A. (C) U2OS cells were cotransfected with plasmids encoding TETr or TETr-cyclin E, along with a minimal HSV-TK promoter reporter plasmid containing the indicated number of TETo-binding sites and a plasmid encoding β-galactosidase. Doxycycline was added as in A.

reporter alone (Figure 7C; Kim and Kaelin, unpublished results). Note that the amount of TETr-cdk2 in these cells was less than the amount of endogenous cdk2 (Figure 7B). Thus, the results are unlikely to be an artifact of overproduction. Luciferase values declined as cyclin E levels decreased and cyclin A levels began to increase. Together, these results suggest that cyclin E, but not cyclin A, can activate transcription under physiological conditions.

DISCUSSION

These studies suggest that cyclin E and cyclin A can both modulate transcription when bound to DNA. Cyclin E can activate transcription and this activity requires the ability to bind to, and activate, cdk2. Furthermore, we found that physiological levels of cyclin E could activate transcription in a cell-cycle–dependent manner from a chromosomally integrated reporter gene. In contrast, cyclin A represses transcription when bound to DNA and this activity does not require cdk2 activation. We hypothesize that cyclin A and cyclin E might be concentrated at certain regions of the genome in a temporally controlled manner by virtue of their ability to form stable complexes with specific proteins that directly or indirectly bind to DNA (Bandara *et al.*, 1991; Mudryj *et al.*, 1991; Devoto *et al.*, 1992; Ewen *et al.*, 1992; Faha *et al.*, 1992; Lees *et al.*, 1992; Hannon *et al.*, 1993; Dynlacht *et al.*, 1994, 1997; Krek *et al.*, 1994; Xu *et al.*, 1994; Zhu *et al.*, 1995b; Adams *et al.*, 1996; Perkins *et al.*, 1997; Smith *et al.*, 1998; Zhao *et al.*, 1998, 2000; Felzen *et al.*, 1999; Ma *et al.*, 2000). An example of the latter would be the pRB homologue p107. p107 binds to DNA via its association with members of the E2F transcription factor family. In late G1-phase, cyclin E/cdk2/p107/E2F complexes are formed as pRB/E2F complexes begin to dissociate. In S-phase, cyclin A replaces cyclin E in the p107 complex (Lees *et al.*, 1992). The highly choreographed appearance and disappearance of these complexes was previously difficult to understand in light of earlier findings that suggested that p107 and pRB were functionally equivalent with respect to their ability to inhibit E2F activity (Schwarz *et al.*, 1993; Zamanian and La Thangue, 1993; Beijersbergen *et al.*, 1995; Zhu *et al.*, 1995a; Lee *et al.*, 1996; Starostik *et al.*, 1996). Recent studies suggest

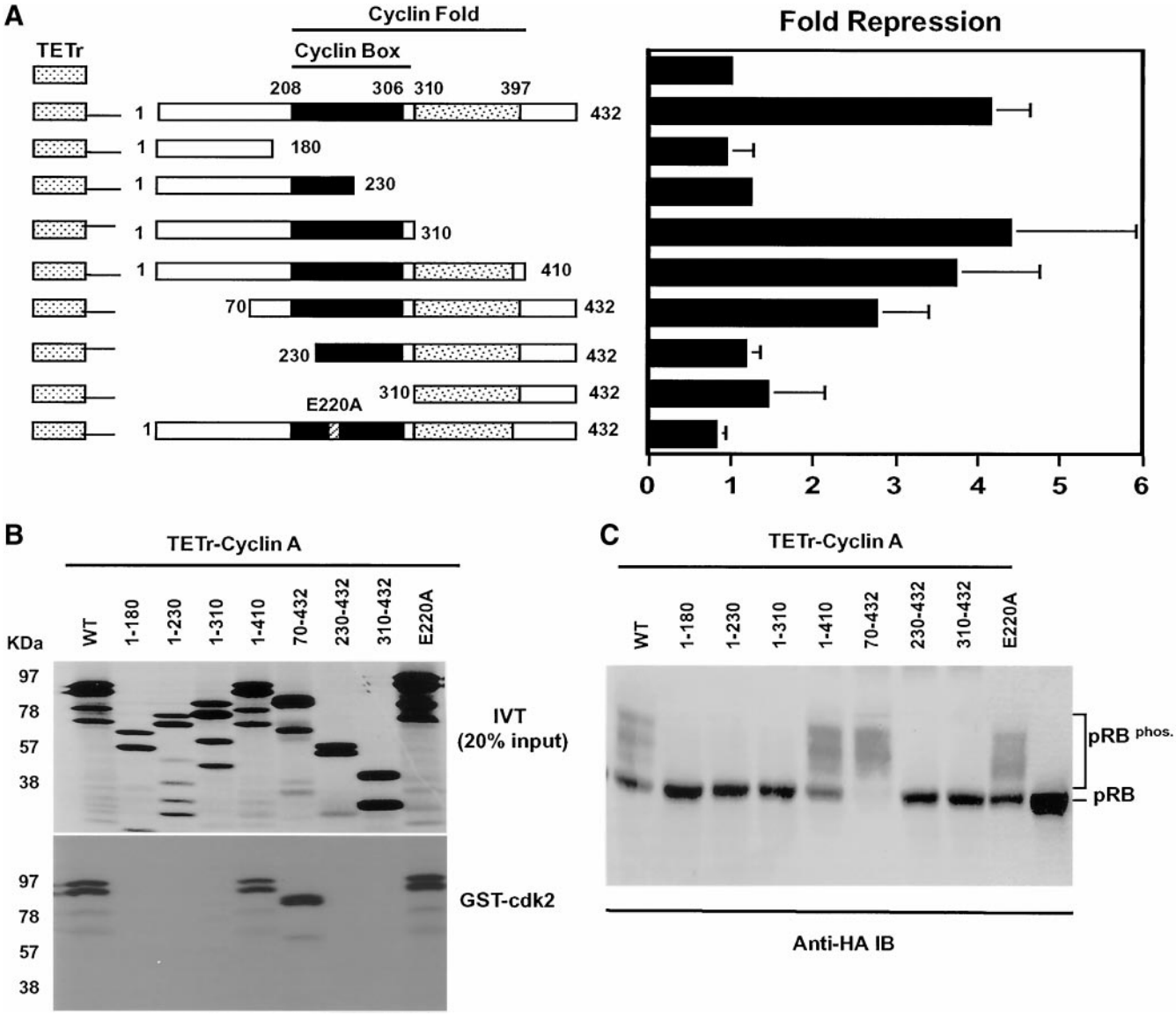

Figure 4. Cyclin box is required for transcriptional repression by DNA bound cyclin A. (A) U2OS cells were cotransfected with plasmids encoding the indicated TETr-cyclin A variants along with the pUHC 13-3 reporter plasmid and a plasmid encoding β-galactosidase. Cell extracts were prepared and luciferase activity, corrected for β–galactosidase activity, was expressed as fold repression relative to cells producing TETr alone. (B) The indicated TETr-cyclin A variants were translated in vitro in the presence of [^{35}S]methionine and incubated with GST-cdk2 and glutathione Sepharose. Specifically bound proteins were resolved by SDS-PAGE and detected by autoradiography. In parallel, 20% of the input proteins were resolved by SDS-PAGE and detected by autoradiography. (C) pRB defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated TETr-cyclin A variants were lysed and immunoblotted with anti-HA antibody. WT, wild type.

that cyclin E/cdk2, bound to NPAT, is linked to the regulation of histone gene expression in S-phase (Ma *et al.*, 2000; Zhao *et al.*, 2000). Both NPAT and cyclin E/cdk2 physically associate with histone gene loci as determined by chromatin immunoprecipitation and high-resolution fluorescence microscopy (Zhao *et al.*, 2000; Brian Kennedy, personal communication).

There is precedence for other cyclins playing relatively direct roles in transcriptional regulation (Dynlacht, 1997; Yankulov and Bentley, 1997; Hengartner *et al.*, 1998; Kimmelman *et al.*, 1999; Lania *et al.*, 1999; Majello *et al.*, 1999; Rickert *et al.*, 1999). Two cdks have been identified in yeast and mammalian transcription initiation complexes. The TFIIH complex, which contains MO15, cyclin H, and cdk7, phosphorylates the C-terminal domain (CTD) of RNA polymerase II after the assembly of the transcriptional initiation complex and positively regulates transcription (Dahmus, 1996; Yankulov and Bentley, 1997; Kimmelman *et al.*, 1999).

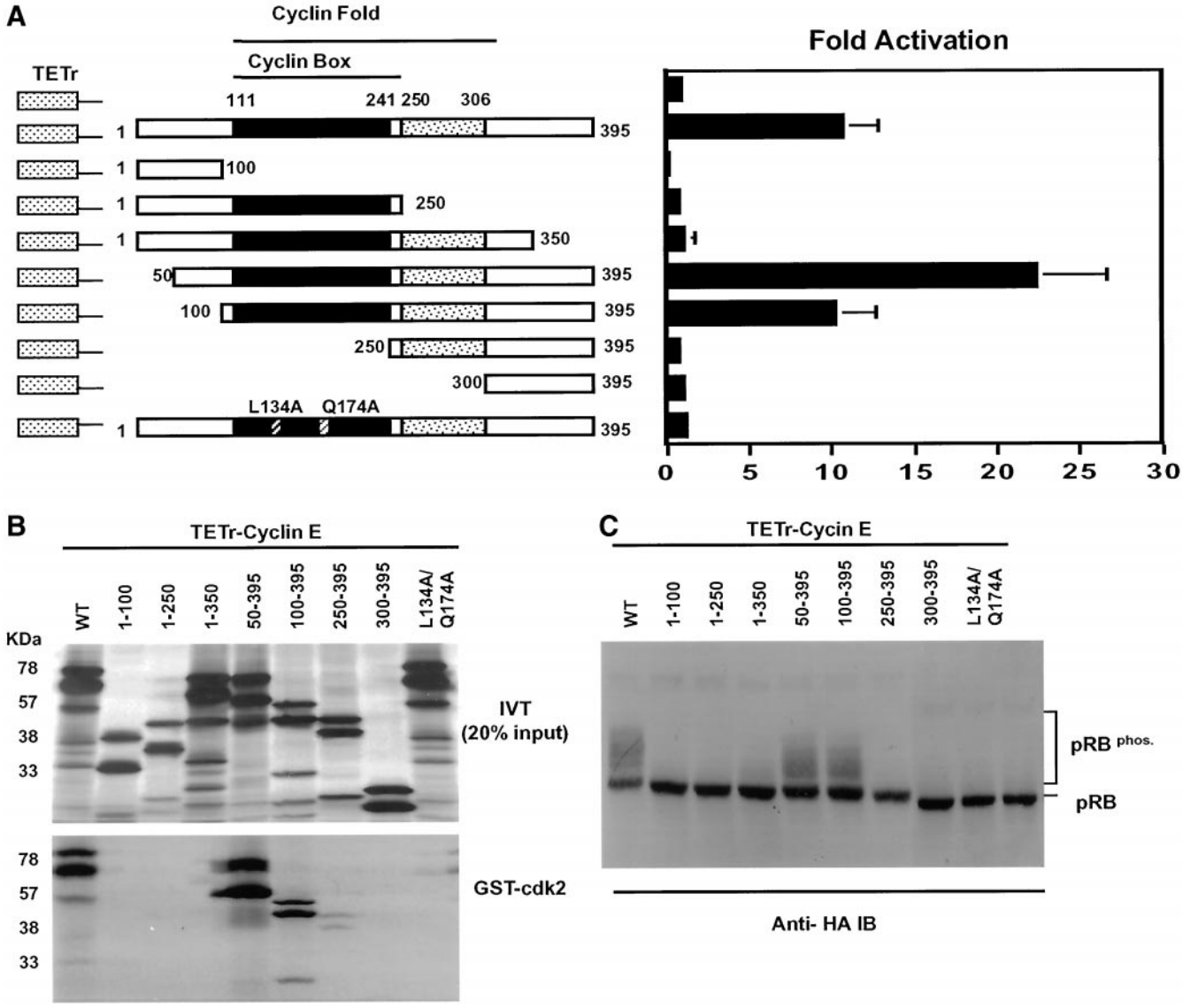

Figure 5. Transcriptional activation by cyclin E linked to its ability to bind to cdk2 and interact with substrates. (A) U2OS cells were cotransfected with plasmids encoding the indicated TETr-cyclin E variants along with the pUHC 13-3 reporter plasmid and a plasmid encoding β-galactosidase. Cell extracts were prepared and luciferase activity, corrected for β-galactosidase activity, was expressed as fold activation relative to cells producing TETr alone. (B) The indicated TETr-cyclin E variants were translated in vitro in the presence of [^{35}S]methionine and incubated with GST-cdk2 and glutathione Sepharose. Specifically bound proteins were resolved by SDS-PAGE and detected by autoradiography. In parallel, 20% of the input proteins were resolved by SDS-PAGE and detected by autoradiography. (C) pRB defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated TETr-cyclin E variants were lysed and immunoblotted with anti-HA antibody. WT, wild type.

Interestingly, transactivation by the HIV Tat protein has been linked to its ability to promote the phosphorylation of CTD by cdk7 (Zhu *et al.*, 1997). In contrast, a complex containing SRB10, the yeast homologue of human cdk8, phosphorylates CTD before assembly of the initiation complex and inhibits transcription (Hengartner *et al.*, 1998). The positive transcription elongation factor (P-TEFb) also phosphorylates CTD and contains cdk9 in addition to cyclin T and cyclin K (Jones, 1997; Bieniasz *et al.*, 1999; Fu *et al.*, 1999). Cyclin C/cdk8 copurifies with CTD and has been implicated in transcriptional regulation as well (Rickert *et al.*, 1999; Akoulitchev *et al.*, 2000). Recent studies suggest that mammalian cyclin D1 can bind to the estrogen receptor and enhance estrogen receptor-dependent transcriptional activation (Neuman *et al.*, 1997, Zwijsen *et al.*, 1997, 1998). This activity of cyclin D1 is distinct from its ability to activate cdk4.

It is intriguing that a structural motif, called the cyclin box fold, has been identified in bona fide transcriptional regulators, such as TFIIB and pRB, as well as in the cyclins (Bagby

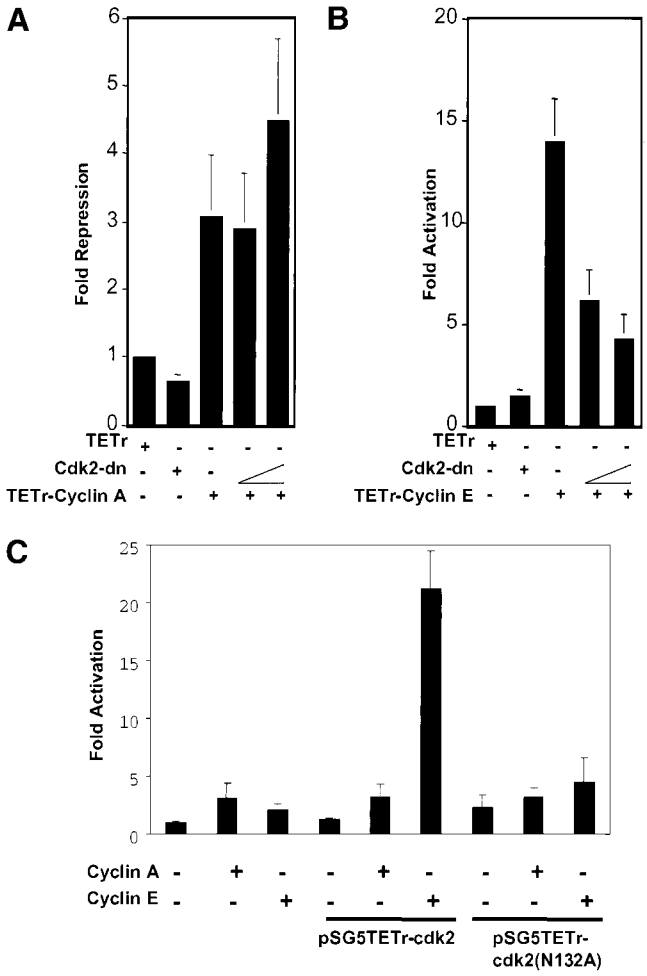

Figure 6. Transcriptional activation by DNA-bound cyclin E depends on cdk2 catalytic activity. (A and B) U2OS cells were transiently cotransfected with plasmids encoding TETr-cyclin A or E and, where indicated, increasing amounts of a plasmid encoding a dominant-negative (dn) form of cdk2. Cell extracts were prepared and luciferase activity, corrected for β-galactosidase activity, was determined. Corrected luciferase values were expressed as fold repression (A) or activation (B) relative to TETr alone. (C) U2OS cells were transiently transfected with plasmids encoding TETr-cdk2 or TETr-cdk2 (N132A) and a plasmid encoding either cyclin A or E. Cell extracts were prepared and luciferase activity, corrected for β-galactosidase activity, was determined. Corrected luciferase values were expressed as fold activation relative to TETr alone.

et al., 1995; Jeffrey *et al.*, 1995; Noble *et al.*, 1997). Thus, cyclin A and cyclin E would appear to be both structurally and functionally related to proteins that are known to control transcription. Furthermore, our mutagenesis strongly implicates the cyclin box fold as important for transcriptional regulation by cyclin A and E.

How, mechanistically, do cyclin A and cyclin E affect transcription? We found that both cyclin A and cyclin E can phosphorylate TBP and CTD in vitro (Kim and Kaelin, unpublished results). It is possible that cyclin A and cyclin E phosphorylate these proteins on different sites, leading to different functional consequences. Experiments can now be performed to address this possibility. A potential role for TBP phosphorylation is suggested by our finding that TETr-E2F1, but not TETr-cyclin E, activated transcription from a naturally occurring TATA-less promoter in which TETo sites had been introduced (Kim and Kaelin, unpublished results). Based on the behavior of TFIIH and SRB10, it is also possible that cyclin A and cyclin E phosphorylate substrates such as TBP and CTD at different times with respect to the formation of a competent transcriptional initiation complex.

Lees and coworkers (Shanahan *et al.*, 1999) have reported that cyclin E, but not cyclin A, can efficiently phosphorylate components of the mammalian SWI-SNF complex implicated in chromatin remodeling. This finding suggests as additional mechanism for cyclin E-dependent transcriptional activation. In addition, it raises the interesting possibility that cyclins A and E might affect processes such as DNA replication and repair by inducing changes in chromatin structure.

Cyclin A-dependent transcriptional repression, in contrast to cyclin E-dependent activation, does not clearly depend on cdk2 activity and hence would not appear to depend on its ability to target proteins for phosphorylation. Many transcriptional repression domains directly or indirectly recruit histone deacetylase complexes to DNA and are therefore inhibited by drugs such as trichostatin. In pilot experiments, however, we found that transcriptional repression by cyclin A is unaffected by trichostatin (Kim and Kaelin, unpublished results). One model, which remains to be tested, is that the cyclin A cyclin box binds to a corepressor molecule. Of note, cdk7 stimulates transcription in association with TFIIH but represses transcription as part of a trimeric CAK complex. In this latter context, repression is independent of cdk7 kinase activity (Bochar *et al.*, 1999).

As cells pass through G1- and into S-phase, cyclin E/cdk2 complexes form and dissolve before the formation of cyclin A/cdk2 complexes. This precise temporal regulation clearly suggests that cyclin E/cdk2 and cyclin A/cdk2 complexes are fundamentally different with respect to certain key functions. The simplest explanation would be that the substrates for these two complexes differ. Nonetheless, cyclin E and cyclin A are fairly similar and, with a few exceptions, it has been difficult to identify substrates that are differentially phosphorylated by these two cyclins (Roberts, 1999). Based on our study, we propose that cyclin E and cyclin A are fundamentally different with respect to their effects on transcription when recruited to DNA. According to this model, cyclin E would facilitate transcription in late G1-phase, whereas cyclin A would inhibit transcription as cells enter and traverse S-phase. Of note, an earlier study suggested that cdc2, which is another kinase partner for cyclin A, inhibited transcription during mitosis when bound to cyclin B (Leresche *et al.*, 1996; Gebara *et al.*, 1997). Thus, the coordinated appearance and disappearance of specific cyclins throughout the cell cycle may differentially influence transcription.

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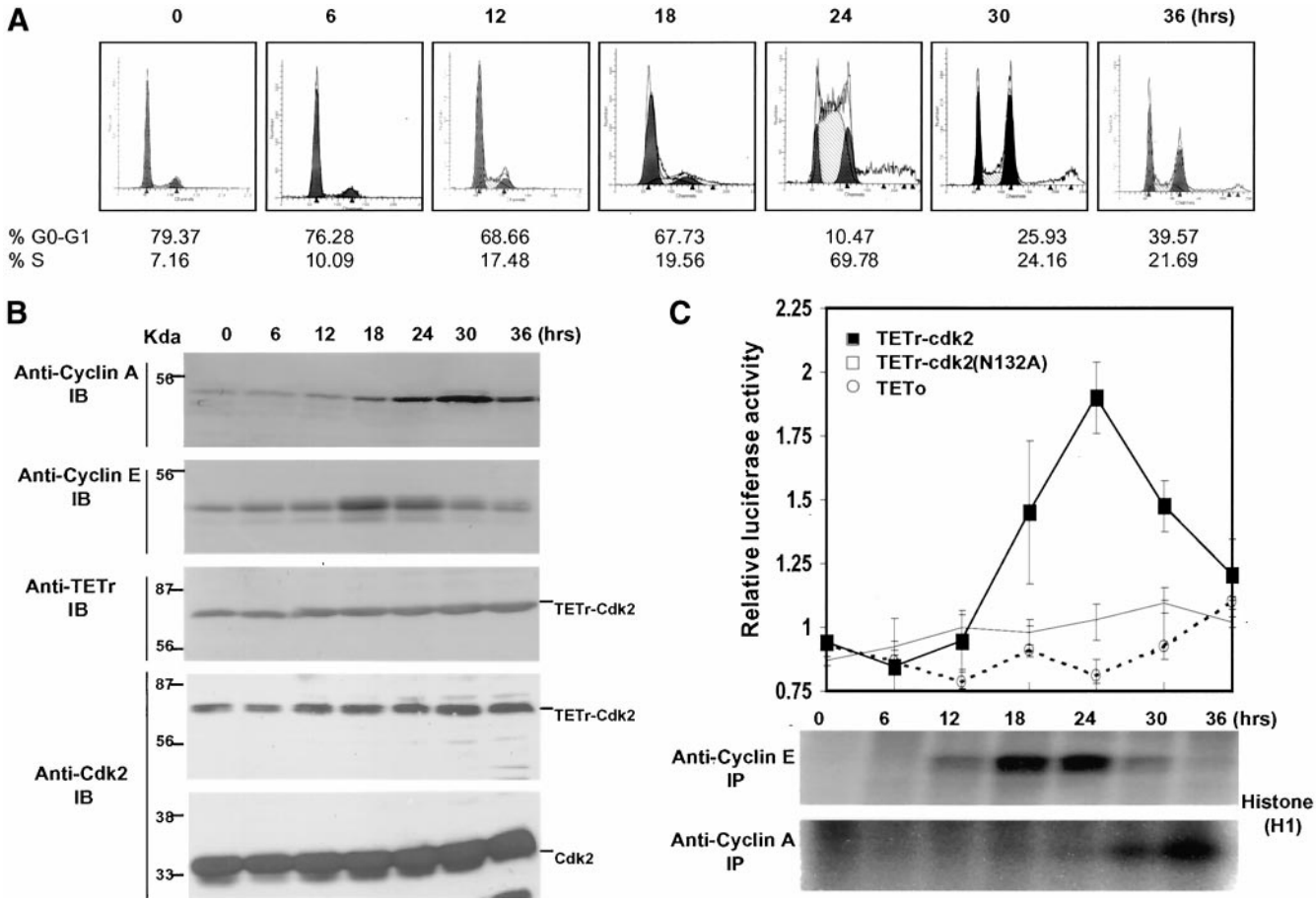

Figure 7. Transcriptional effects mediated by cell-cycle–dependent changes in endogenous cyclins E and A. (A and B) 3T3 cells stably transfected with a luciferase reporter plasmid containing seven TETo sites (pUHC13-3) and a plasmid encoding TETr-cdk2 were serum starved for 72 h and subsequently refed with serum. At various times thereafter aliquots of cells were removed and either lysed for immunoblot (IB) analysis with the indicated antibodies or analyzed for DNA content by propidium iodide staining followed by FACS. (C) 3T3 cells stably transfected with a luciferase reporter plasmid containing seven TETo sites (pUHC13-3) in the absence (open circles) or presence of a plasmid encoding TETr-cdk2 (closed squares) or TETr-cdk2 (N132A) (open squares) were serum starved for 72 h and then refed with serum in the presence or absence of doxycycline. At various times thereafter luciferase assays were performed. To correct for general effects due to serum, the luciferase values at each time in the absence of doxycycline were corrected by subtracting the luciferase assay obtained in the presence of doxycycline. After correction the luciferase values for the two cell populations were expressed relative to the corresponding luciferase values obtained at time 0. In parallel, the cells producing TETr-cdk2 were lysed and immunoprecipiated with anti-cyclin A or anti-cyclin E antibodies. The immunoprecipitates were then used to phosphorylate histone H1 in vitro.

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