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Phosphorylation of Sp1 by Cyclin-dependent Kinase 2 Modulates the Role of Sp1 in CTP:Phosphocholine Cytidylyltransferase α Regulation during the S Phase of the Cell Cycle*

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Phosphatidylcholine is the major lipid component in mammalian membranes. Phosphatidylcholine synthesis increases in C3H10T1/2 fibroblasts during the G_1 and S phases of the cell cycle. Previous studies demonstrated that the mRNA encoding CTP:phosphocholine cytidylyltransferase α (CTα) increases during S phase (Golfman, L. S., Bakovic, M., and Vance, D. E. (2001) *J. Biol. Chem.* 276, 43688–43692) and that this activation is driven by increased binding of Sp1 to the CTα promoter (Banchio, C., Schang, L. M., and Vance, D. E. (2003) *J. Biol. Chem.* 278, 32457–32464). We now demonstrate that cyclin-dependent kinase 2 (CDK2) phosphorylation of Sp1 activates CTα transcription during S phase. Sp1 binds in a phosphorylated state to the CTα promoter. Sp1 binding is enhanced by association with cyclin A/E and CDK2, both *in vivo* and *in vitro*. In cells that overexpress Sp1, co-expression of cyclin A and CDK2 induces a high and constant level of CTα expression, whereas reduction in the expression of cyclin A, cyclin E, and CDK2 eliminates the induction of CTα expression in S phase. Furthermore, CTα expression is decreased in cells overexpressing a dominant-negative form of CDK2 and in cells treated with the CDK2 kinase inhibitors roscovitine and olomoucine. These results enhance our understanding of the regulatory mechanisms involved in the expression of CTα in preparation for cell division.

Sequential activation and inactivation of cyclin-dependent kinases (CDKs)[1] ensure an orderly progression through the cell cycle (1). CDK activities are regulated by a variety of mechanisms, such as a periodic accumulation of cyclins, degradation, nuclear localization, phosphorylation of CDKs, and association with different CDK inhibitors (1–3). Cyclin E, in combination with its associated cyclin-dependent kinase 2 (CDK2), is a positive cell cycle regulator controlling progression through G_1 and initiation of DNA replication (4–7). Cyclin A binds both CDK2 and CDK1 (Cdc2), giving rise to two distinct cyclin A kinase activities, one appearing in S phase (cyclin A-CDK2), and the other in G_2 phase (cyclin A-CDK1) (8). The cyclin-CDK complexes are thought to control the cell cycle by phosphorylating key regulatory proteins at specific points in the cell cycle.

Sp1 is a ubiquitous transcription factor that recognizes GC-rich sequences present in many promoters (9, 10). Regulation of Sp1-dependent transcription can be affected by changes in Sp1 abundance, DNA binding activity, and/or transactivation activity. Phosphorylation, as well as its interaction with other factors, has also been implicated in changes in Sp1 binding and transcriptional activation (11, 12). A large group of genes is activated in mid- or late G_1; these include several genes whose expression is required for DNA synthesis (*e.g.* those that encode adenosine deaminase, thymidine kinase, dihydrofolate reductase, and DNA polymerase), as well as genes whose products control cell cycle progression (cyclin A and cyclin E genes). Many of these late G_1 and S phase-expressed genes lack a TATAA box and have binding sites for the transcription factor Sp1 in their promoters.

Phosphatidylcholine (PC) biosynthesis is an important component of the cell cycle because PC mass/cell doubles prior to mitosis. PC is typically the major phospholipid of animal cells and is a precursor for the synthesis of sphingomyelin and phosphatidylserine. Cell cycle progression is sensitive to membrane PC content because choline deprivation of WI-38 fibroblasts, L6 myoblasts, or C3H10T1/2 fibroblasts results in decreased PC synthesis and mass with arrest in G_1 (13, 14). The addition of choline (which is converted to PC) restores PC content and progression into S phase. Chinese hamster ovary cells harboring a temperature-sensitive CTP:phosphocholine cytidylyltransferase (CT) do not synthesize PC at 40 °C and accumulate in G_1. These cells undergo apoptosis unless rescued by the addition of PC or lyso-PC (15).

PC biosynthesis occurs in all nucleated mammalian cells via the Kennedy (CDP-choline) pathway in which CTP:phosphocholine cytidylyltransferase catalyzes the regulated and rate-limiting step (16–18). Two genes encode CT activity, *Pcyt1a* and *Pcyt1b* (19–23). CTα is ubiquitously expressed in nucleated cells (24), and its expression is tightly regulated. CTα is regulated post-translationally by reversible association with membrane lipids, which are required for its activity (25–27). It was reported that the wave of PC synthesis that accompanies the G_0-G_1 transition is regulated by changes in the activity, membrane affinity, and intracellular distribution of CT (28). At the level of gene expression, CTα mRNA has been shown to increase after growth factor stimulation (29), during liver development (30), in proliferating liver tissue following partial hepatectomy (31), and during the S phase of the cell cycle (32). We recently reported that the expression of CTα is activated in late G_1-S phase by the action of Sp1 (33).

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; PC, phosphatidylcholine; CT, CTP:phosphocholine cytidylyltransferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LUC, luciferase; siRNA, small interfering RNA; CMV, cytomegalovirus.

In the present report we elucidate the mechanism(s) underlying growth/cell cycle-regulated induction of Sp1-dependent transcription of CTα. We demonstrate that complexes that regulate cell cycle progression, such as cyclin E-CDK2 or cyclin A-CDK2, phosphorylate and activate Sp1, thereby increasing CTα transcription during S phase.

EXPERIMENTAL PROCEDURES

Materials—The luciferase vector pGL3-Basic, which contains the cDNA for *Photinus pyralis* luciferase, and the Dual-Luciferase reporter assay system were obtained from Promega (Madison, WI). LipofectAMINE Plus reagent, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were from Invitrogen. Anti-Sp1, anticyclin A, anticyclin E, anticyclin B, anti-CDK2, and anti-CDK1 were purchased from Santa Cruz Biotechnology. ECL® immunoblotting reagents were purchased from Amersham Biosciences, and olomoucine and roscovitine were from Sigma.

Cell Culture—C3H10T1/2 mouse embryo fibroblasts were cultured in DMEM supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37 °C. Cells were arrested in G_0 by incubation in culture medium containing 0.5% serum for 36–48 h, and the growth arrest was released by addition of fresh medium containing 10% FBS. Transient transfections with CTα promoter-luciferase reporter plasmids containing deletions at the 5′-end of the murine promoter, LUC.C7 (−1268/+38) and LUC.C8 (−201/+38) (1 μg), were performed using a cationic liposome method. LUC.C7 (−1268/+38) and LUC.C8 (−201/+38), inserted into the promoterless luciferase vector pGL3-Basic (Promega), were prepared as described previously (34). All dishes received 0.1 μg of pSV-β-galactosidase (Promega) as a control for transfection efficiency. Luciferase assays were performed using a Promega assays system as recommended by the manufacturer, and luminometric measurements were made using a Fluskan Ascent FL Type 374 fluorometer (Thermo Electron Corporation). Luciferase activity was normalized to the protein content or β-galactosidase activity. Vectors enabling expression of recombinant cyclin A, CDK2, and dominant-negative CDK2 were obtained from Dr. E. Harlow, Harvard Medical School (35), and vectors enabling expression of recombinant Sp1 protein (pPacSp1 and pPac0) were obtained from Dr. R. Tjian (36).

Nuclear Extract Preparations and Electrophoretic Mobility Shift Assays—Total nuclear extracts of C3H10T1/2 cells grown to different stages of the cell cycle were prepared as described previously (37, 38). A dephosphorylation reaction was executed by suspending nuclear extracts (20 μg) in a buffer consisting of 25 mM HEPES, pH 7.5, 34 mM KCl, and 50 mM $MgCl_2$ containing protease inhibitors and treated with calf alkaline phosphatase (1.0 unit/50 μg of nuclear extract) at 30 °C for 5 min followed by 15 min on ice. The reactions were terminated by the addition of a mixture of inhibitors to final concentrations of 10 mM sodium fluoride, 10 mM sodium vanadate, 10 mM potassium pyrophosphate, and 5 mM sodium phosphate. Control nuclear extracts were prepared by addition of the inhibitor mix to nuclear extracts in the absence of phosphatase treatment or by addition of the inhibitor mix immediately after addition of the enzyme. An oligonucleotide carrying the Sp1 consensus sequence (5′-ATTCGATCGGGCGGGGCGAGC-3′) was synthesized by the University of Alberta Core Facility. Complementary oligonucleotides (100 μM each) were heated at 90 °C for 5 min and then were slowly cooled to room temperature, and 5 pmol of double-stranded oligonucleotide was 5′-end-labeled using T4 kinase (Invitrogen) and [γ-^{32}P]ATP (PerkinElmer Life Sciences). For each binding reaction (40 μl), 1 μg of poly(dI-dC)-poly(dI-dC), 20 μl of 2× binding buffer (100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 250 mM NaCl, 5 mM EDTA, 50% glycerol, 0.5% Nonidet P-40, 5 mM dithiothreitol), 1 μg of nuclear extract, and labeled probe (20,000 cpm) were incubated for 30 min at room temperature.

For supershift analysis, 1 μg of antibody specific for cyclin A, cyclin E, or CDK2 was added for 15 min after incubation of the probe with nuclear protein. Binding reactions were terminated by the addition of 4 μl of gel loading buffer (30% (v/v) glycerol, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol). The complex was separated on a non-denaturing 6% (w/v) polyacrylamide gel and visualized by autoradiography of the dried gel.

Immunoblot Analysis—Nuclear protein (10 μg) from C3H10T1/2 fibroblasts was heated for 3 min at 90 °C in 62.5 mM Tris-HCl (pH 8.3), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% SDS, and 0.004% bromphenol blue. The samples were electrophoresed on a 10% SDS-polyacrylamide gel in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS buffer. The proteins then were transferred to nitrocellulose by electroblotting in transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol). Following transfer, the membrane was incubated for 1 h at room temperature or overnight at 4 °C with 5% skim milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (T-TBS) and incubated for 1 h with antibody raised against the protein indicated. Immunoreactive proteins were detected using the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

Construction of Plasmids That Contain DNA Templates for the Synthesis of siRNAs under the Control of the U6 Promoter—DNA oligonucleotide templates for the *in vitro* synthesis of siRNAs were chemically synthesized by the University of Alberta Core Facility. The oligonucleotides were designed to contain nucleotides specific for cyclin A (TGTAATATCTATTTGGGTC), cyclin E (ATTGCCAAGATTGACAAGA), and CDK2 (GAGTGAACAATTATATTTA). After annealing, the DNA was cloned into p*Silencer*™ 2.1-U6 hygro (Ambion) double digested with HindIII and EcoRI. The identity of the plasmids harboring the insert, named siCyE, siCyA, and siCDK2, respectively, was confirmed by sequencing, and the plasmids were transfected using the concentrations indicated. As a negative control we used the same plasmid harboring a sequence that does not have homology with the expressed genes (ACTACCGTTGTTATAGGTGTT).

Immunoprecipitation—Nuclear extracts were prepared as described above from cells collected after 20 h of cell cycle induction. Nuclear extracts (200 μg of protein) were incubated with 5 μg of polyclonal anti-Sp1 (Santa Cruz Biotechnology) in 1-ml final volume containing immunoprecipitation buffer (1% Triton X-100 (v/v), 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) Nonidet P-40). The reaction was incubated for 1 h at 4 °C and then incubated for 30 min with 50 μl 10% protein A-Sepharose (*Staphilococcus aureus*, Cowan strain), and the complex was washed three times with immunoprecipitation buffer. The pellet was resuspended in 30 μl of concentrated electrophoresis sample buffer and boiled, and the supernatant was loaded onto an SDS-polyacrylamide gel and electrophoresed. Proteins were transferred to polyvinylidene difluoride membranes and probed with appropriate antibodies.

In Vivo Labeling and Immunoblot Analysis—For $^{32}PO_4$ labeling, cells that had been synchronized by serum deprivation for 36 h were rinsed with phosphate-buffered saline (135 mM NaCl, 4 mM KCl, 10 mM $NaPO_4$ (pH 7.4)) and placed in phosphate-free DMEM containing 10% dialyzed fetal bovine serum. After 30 min, cells were labeled for 2 h in the same medium containing 7.5 μCi/ml $^{32}PO_4$. At the indicated times following serum stimulation, cells were rinsed with phosphate-buffered saline, lysed directly in boiling 10 mM Tris-HCl (pH 7.2) containing 1% SDS, reboiled, and DNA was sheared (9). Following the addition of 2.2 volumes of ice-cold buffer containing 15 mM Tris-HCl (pH 7.2), 7.5 mM EDTA, 150 mM sodium fluoride, 230 mM NaCl, 1.5% Triton X-100, 0.75% Nonidet P-40, 100 mM β-glycerophosphate, 15 mM sodium pyrophosphate, 400 μM Na_2VO_3, 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 10 μg/ml aprotinin, particulate material was removed by centrifugation (13,000 × *g*, 10 min). Supernatants were precleared with normal rabbit serum and protein A-Sepharose, and Sp1 was immunoprecipitated with anti-Sp1 antibody and protein A-Sepharose. Immunoprecipitates were washed four times with radioimmune precipitation assay buffer (phosphate-buffered saline containing 1% (w/v) Ipegal CA-630, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS), separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to autoradiography. Membranes then were blocked in 5% nonfat dried milk, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20, and subjected to immunoblotting with anti-Sp1 antibody and anti-rabbit IgG linked to horseradish peroxidase as secondary antibody as prescribed by the manufacturer (Santa Cruz Biotechnology).

In Vitro Kinase Assays—Nuclear extracts (200 μg) obtained during S phase were incubated with Sp1 antibody. After addition of protein A-Sepharose, the precipitate was washed three times with radioimmune precipitation assay buffer, resuspended in 35 μl of kinase buffer (40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 10 mM $MgCl_2$) with 1 mCi of [γ-^{32}P]ATP, and incubated for 20 min at 30 °C. The reaction was terminated by addition of 10 ml of 6× SDS-PAGE loading buffer. For phosphorylation of His-tagged protein, equal amounts of protein (assayed by immunoblot analysis) were used in the assay.

Treatment with Roscovitine and Olomoucine—C3H10T1/2 fibroblasts were stably transfected with LUC.C8 (−201/+38) and grown under normal conditions for 24 h, after which they were synchronized in G_0 phase with DMEM containing 0.5% FBS for 24 h. The cell cycle was induced by adding DMEM containing 10% FBS, and 2 h before collecting the samples, roscovitine, olomoucine, or dimethyl sulfoxide was

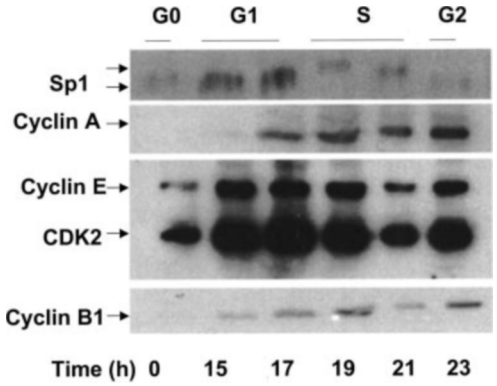

FIG. 1. **Expression of cell cycle-related proteins during G_0, G_1, S, and G_2 phases of the cell cycle in C3H10T1/2 fibroblasts.** Shown are immunoblots of nuclear extracts obtained from cells at various times after synchronization. From *top* to *bottom* are the protein levels of Sp1, cyclin A, cyclin E, CDK2, and cyclin B1.

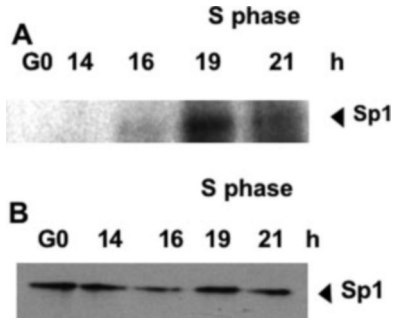

FIG. 2. **Cell cycle-dependent phosphorylation of Sp1.** Equal numbers of C3H10T1/2 fibroblasts were serum-starved for 48 h prior to readdition of 10% serum. Cells were then labeled with $^{32}PO_4$ for 2 h prior to harvest at the indicated times. Sp1 was immunoprecipitated from the cells, and then proteins were electrophoresed by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Incorporation of ^{32}P into Sp1 was detected by autoradiography (*A*). The position of phosphorylated Sp1 is indicated by the *arrowhead*. Sp1 levels were compared by immunoblot analysis (*B*). The blots represent two independent experiments.

added at the concentrations indicated. Samples were collected 20 h after induction of the cell cycle, and luciferase activity was measured.

RESULTS

Sp1 Interacts with Cyclin A, Cyclin E, and CDK2—The expression of CTα during the S phase of the cell cycle in C3H10T1/2 embryo fibroblasts is mainly regulated by the binding of Sp1 at positions −67/−62 and −31/−39 in the CTα promoter (33). We have demonstrated previously that overexpression of Sp1 increases expression of CTα in all phases of the cell cycle (33). However, the increase in CTα expression during the S phase was maintained, suggesting that overexpression of Sp1 did not explain the increase in CTα expression during the S phase. In the same study we demonstrated that Sp1 interacts with cyclin A, cyclin E, and CDK2 during the S phase.

To evaluate the expression pattern of Sp1 and Sp1 binding partners during the cell cycle, we performed immunoblot analyses. C3H10T1/2 fibroblasts were synchronized at G_0 phase by serum depletion. After serum addition, nuclear extracts were isolated at various time points. We monitored the expression of Sp1, cyclin A, cyclin E, CDK2, and cyclin B, which together with CDK1 has been reported to regulate mitosis (39–41). The result shown in Fig. 1 is consistent with the expression patterns of cyclins and kinases during the cell cycle defined previously (42). Interestingly, we detected a retardation in mobility for the Sp1 signal in the S phase that may represent post-translational modification. We also confirmed that all proteins defined previously as part of the Sp1 complex (cyclin A, cyclin E, and CDK2) are expressed coincidentally. CDK1 was not detected in the phases analyzed, and cyclin B was present at lower levels at the time point that corresponds to the G_1-S phase.

Time Course for Serum Induction of Sp1 and Phosphorylation—Serum induction of Sp1-dependent transcription from the CTα promoter occurred in S phase (33). Therefore, Sp1 levels and phosphorylation were assessed in C3H10T1/2 fibroblasts at various times following serum stimulation. As seen in Fig. 2*A*, $^{32}PO_4$ incorporation into Sp1 increases with time, becoming apparent at 16 h and increasing to the maximum at 19 h after serum stimulation. Because S phase occurs 18–20 h following serum stimulation of these cells (data not shown and Refs. 32 and 33), Sp1 phosphorylation is induced in late G_1-S phase and therefore occurs concurrent with, or slightly before, the induction of Sp1-dependent CTα transcription. The level of Sp1 expression analyzed by immunoblot analysis showed that Sp1 is present in all phases of the cell cycle analyzed (Figs. 1 and 2). From this experiment, the shift in Sp1 mobility during the S phase (shown in Fig. 1) is likely because of phosphorylation, suggesting that this mechanism might regulate Sp1 activity in this phase of the cell cycle.

The Overexpression of Cyclin A and CDK2 Enhances CTα Expression—To determine whether or not phosphorylation affects Sp1 activity and the subsequent activation of CTα expression during the S phase, we determined whether the overexpression of cyclin A and CDK2 affected CTα expression. To address this question, we used luciferase reporter assays. Cells were transfected with the reporter construct LUC.C7 (−1268/+38), Sp1 expression vector pPacSp1, pSV-β-galactosidase, and CMV-cyclin A, CMV-CDK2, or the empty plasmid as a control. After synchronization, the cell cycle was induced, and samples were collected at various times. The luciferase/β-galactosidase ratios are summarized in Fig. 3. Enhanced expression of cyclin A or CDK2 with Sp1 increased the transcriptional activity of the CTα promoter by ~1.5–2-fold at early points (0 and 13 h) in the cell cycle compared with cells co-transfected with empty plasmids. In cells transfected with either cyclin A or CDK2, the expression profile showed an increase in all phases of the cell cycle prior to S phase (18 and 21 h), indicating that S phase transactivation is dependent on both proteins.

CDK2 Phosphorylates and Physically Associates with Sp1—It has been proposed that association between kinases and their substrate proteins can be an important factor in their specificity and activity (43). Therefore we investigated by *in vitro* assays whether or not the increase in Sp1 phosphorylation was due to its association with CDK2. Nuclear extracts obtained 20 h after induction (S phase) were immunoprecipitated with anti-Sp1 antibody. The precipitate was then incubated with histone H1 as a substrate for CDK2 and [γ-^{32}P]ATP for 15 min. The proteins were subsequently electrophoresed by SDS-PAGE. As Fig. 4*A* shows, several phosphorylated proteins were detected. From the apparent molecular weights, one of the phosphorylated proteins was assumed to be histone H1 (Fig. 4*A*). By immunoblot analysis we detected Sp1 (Fig. 4*B*). However, other phosphorylated proteins that we did not identify also co-precipitated with Sp1. When the reaction was performed in the presence of roscovitine (10 μM) (Fig. 4*A*), the labeling of Sp1 and histone H1 was greatly attenuated, indicating that the kinase activity was inhibited. Roscovitine competes specifically for the ATP-binding domains of CDK1, CDK2, CDK5, CDK7, and possibly CDK9 (44–47).

Phosphorylated Sp1 Binds to the CT Promoter—Of the many types of post-translational modifications that might regulate transcription, most attention has focused on phosphorylation. Several studies have indicated that there are a multitude of

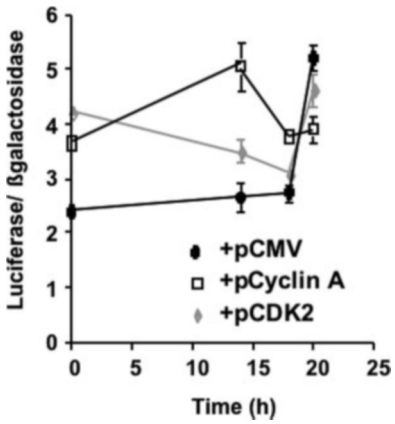

FIG. 3. **Overexpression of cyclin A and CDK2 alters CTα promoter-luciferase expression during the cell cycle.** Truncated CTα reporter constructs and pSV-β-galactosidase as a control (1 μg) were transfected into C3H10T1/2 fibroblasts with the constructs LUC.C7 (−1268/+38) (1 μg) + pPacSp1 (1 μg) and CMV (empty plasmid) (1 μg), CMV-cyclin A (1 μg), or CMV-CDK2 (1 μg). Reporter activity is relative to β-galactosidase activity and was measured at the indicated times after induction of the cell cycle. The data represent two independent experiments, and each point was measured in triplicate. These six numbers were used to calculate the error bars.

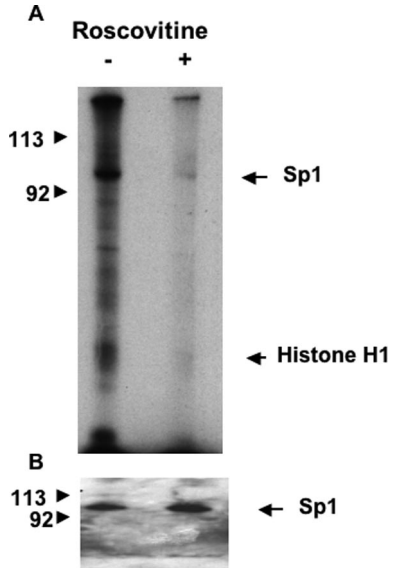

FIG. 4. **CDK2 is responsible for Sp1 phosphorylation.** *A*, *in vitro* kinase assay. Histone H1 protein was used as a substrate for phosphorylation with a kinase complex prepared by immunoprecipitation of nuclear extracts with anti-Sp1 antibody obtained from fibroblasts that were grown to S phase ± 10 μM roscovitine. *Arrows* indicate Sp1 that was detected by immunoblot and histone H1 that was indicated by molecular weight. *B*, immunoblotting to detect Sp1 on the same membrane. The blots represent three independent experiments.

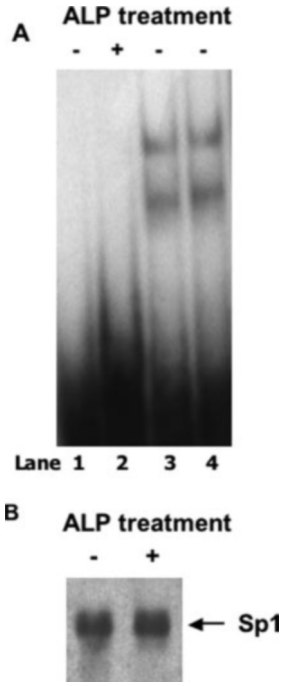

FIG. 5. **Dephosphorylation decreases the binding of Sp1 to DNA.** *A*, electromobility shift assays were performed using nuclear extracts obtained from fibroblasts in S phase and a ^{32}P-labeled oligonucleotide containing a Sp1 binding element. *Lane 1*, free probe; *lane 2*, nuclear extract treated with alkaline phosphatase (*ALP*); *lane 3*, nuclear extract treated with alkaline phosphatase buffer as a control; *lane 4*, nuclear extract treated with alkaline phosphatase followed immediately by a stop buffer. The shifted bands may be Sp1-cyclin A/E-CDK2 complexes or different stages of Sp1 phosphorylation. *B*, immunoblot of Sp1 from cells treated with or without alkaline phosphatase. The blots represent two independent experiments.

ways in which phosphorylation can influence transcription factor activity (11, 12). Phosphorylation can influence the DNA binding activity of transcription factors by inducing the translocation of a transcription factor from the cytoplasm to the nucleus. Alternatively, phosphorylation might modulate transcription by altering the transcriptional activation potential of the factor.

Because an increase in the level of Sp1 was not sufficient to induce CTα promoter activity (33), we investigated whether or not the phosphorylation state of Sp1 influenced CTα promoter activity. To test whether or not Sp1 was phosphorylated when it bound to the CTα promoter, we determined whether treatment with alkaline phosphatase abrogated the ability of total nuclear extracts (obtained at 19 h after cell cycle induction) to bind to the Sp1 DNA binding consensus element. The gel shift (Fig. 5*A*) shows that hypophosphorylated Sp1 failed to bind to the CTα promoter. Because the phosphatase treatment did not affect the quantity of Sp1 (Fig. 5*B*), these results indicate that phosphorylated Sp1 is the active form that binds to DNA.

To determine whether Sp1 was released from the cyclin A-CDK2 complex after phosphorylation of Sp1 or whether the complex is also present when Sp1 binds the CTα promoter, we performed supershift assays. Nuclear extract obtained during the S phase was incubated with labeled DNA (Sp1 binding consensus element) in the presence or absence of antibody raised against Sp1, cyclin A, cyclin E, or CDK2. Fig. 6 shows a supershift signal when any of these antibodies was added, suggesting that Sp1 drives the complex to the binding site. We observed three different bands when we added anti-CDK2 or anticyclin A. However, one additional band (*arrowhead* in Fig. 6) was also observed in the presence of anti-Sp1 antibodies. The multiplicity of the bands might represent different populations of complexes, for example between Sp1 and cyclin E or Sp1, cyclin A, and CDK2.

Inhibition of CDK Activity Reduces Sp1-dependent Expression of a Reporter Gene—If cyclins/CDK2-mediated phosphorylation resulted in increased DNA binding of Sp1 and, thereafter, CTα promoter activity, inhibition of the kinase activity would be expected to reduce the promoter activity. To test this hypothesis, we determined the concentration of roscovitine and olomoucine necessary to inhibit CDK activity in cultured cells by measuring cell cycle arrest. C3H10T1/2 fibroblasts were incubated with 60, 100, or 180 μM olomoucine or 10, 30, or 100 μM roscovitine and used for fluorescence-activated cell sorting analysis that discriminates among various phases of the cell cycle (data not shown). The optimal inhibitory concentration

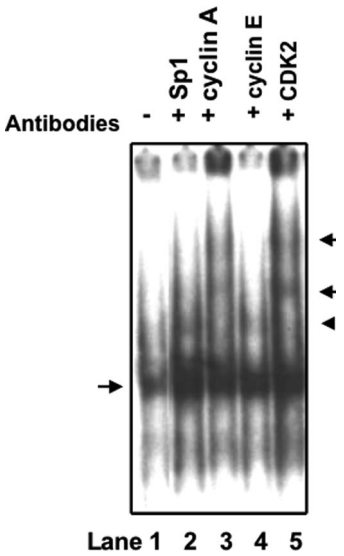

FIG. 6. **Complexes formed between Sp1 and cyclin A-CDK2 or cyclin E-CDK2 are stable and bind DNA.** Electromobility shift assays were performed using nuclear extracts obtained from fibroblasts in S phase with a ^{32}P-labeled oligonucleotide containing a Sp1 binding element in the absence (*lane 1*) or presence of antibodies raised against Sp1 (*lane 2*), cyclin A (*lane 3*), cyclin E (*lane 4*), or CDK2 (*lane 5*). *Arrows* indicate complexes formed. The blots represent two independent experiments.

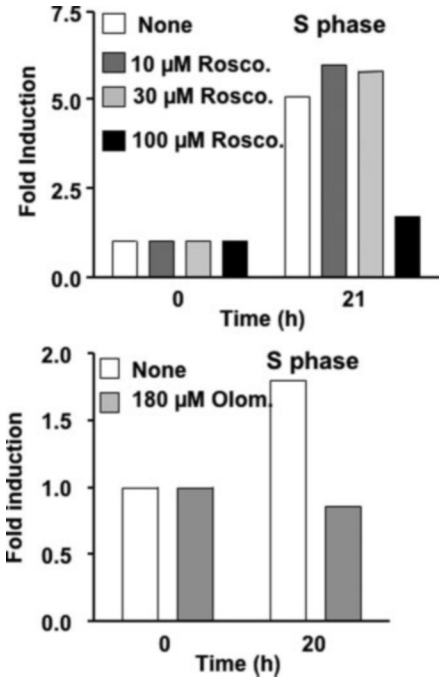

FIG. 7. **Incubation of C3H10T1/2 cells with roscovitine or olomoucine inhibits CTα-luciferase expression.** Stable cell lines that express the CTα reporter construct LUC.C8 were synchronized, and the progression of the cell cycle was induced. At different time points roscovitine (*Rosco.*), olomoucine (*Olom.*), or dimethyl sulfoxide (*None*; as a control) was added at the concentration indicated. Samples were taken 2 h after addition of the inhibitor. Luciferase activity was measured and normalized to cellular protein. The results are reported as –fold induction at 20 or 21 h compared with the luciferase activity at 0 h. Results represent the average of two independent experiments.

was 180 μM for olomoucine and 100 μM for roscovitine. Cells expressing the LUC.C8 reporter construct were synchronized, and after induction of the cell cycle, cultures were treated with the CDK inhibitors roscovitine (100 μM), olomoucine (180 μM), or dimethyl sulfoxide for 2 h before harvest. Addition of the inhibitors 2 h before harvest ensures that the effect is on the kinase and not on progression of the cell cycle. Samples were taken 0 and 20 h after cell cycle induction. Under these conditions, cell cycle progression was not affected, but CTα promoter activity was clearly reduced during S phase in cells that were treated with either of the CDK inhibitors (Fig. 7). However, cells that received only dimethyl sulfoxide showed the normal profile.

Inhibition of Cyclin A, Cyclin E, and CDK2 Using siRNA Decreases the Expression of CTα-Reporter Activity—CDKs have been referred to as the "traffic light" of the cell cycle (48). They promote and coordinate DNA replication during S phase and chromosome segregation during mitosis. The type E and A cyclins associate with CDK2 to regulate initiation of DNA replication and progression through S phase. To confirm the role of these complexes in CTα expression during the cell cycle, we used siRNA to "knock down" their expression. We constructed plasmids designed to generate *in vivo* double-stranded RNA. Each plasmid was co-transfected into C3H10T1/2 fibroblasts with the reporter construct LUC.C7 and pSV-β-galactosidase as a control for transfection efficiency. After synchronization and cell cycle induction, samples were taken at different time points, and the luciferase and β-galactosidase activities were analyzed. As shown in Fig. 8*A*, the presence of cyclin E and cyclin A interfering RNAs reduced CTα induction in the S phase by 30%. When siRNAs that blocked expression of both cyclins and CDK2 were present, CTα expression was decreased at all time points analyzed.

We also expressed a dominant-negative CDK2 that contained a mutation in an arginine residue essential for its kinase activity (35). Luciferase activity was not induced when cells reached the S phase, reflecting an essential role of CDK2 in CTα promoter expression (Fig. 8*B*).

Many cases (2, 49–51) have been described in which the interactions and subsequent phosphorylation of a protein by a cyclin-CDK complex result in diverse effects depending on the protein phosphorylated. Our results clearly indicate that Sp1 phosphorylation is necessary for CTα promoter expression during S phase.

DISCUSSION

Progression through the mammalian cell cycle is driven by the orderly activation of CDKs. Each cyclin binds to a preferred subset of CDKs, and the resulting cyclin-CDK complexes typically display peaks of kinase activity during a defined period in the cell cycle. Different cyclin-CDK complexes regulate distinct downstream effector proteins via phosphorylation, thus giving rise to the different biochemical characteristics of each stage of the cell cycle.

A number of protein kinases are known to phosphorylate Sp1. Growth-dependent phosphorylation of Sp1 has been shown to occur during G_1 phase (52). Kinases such as casein kinase II (11), protein kinase A (12), double-stranded DNA-dependent protein kinase (52), and cyclin A-CDK2 (53) have been reported to phosphorylate Sp1 and regulate its activity. Moreover, several proteins interact with Sp1 to modulate its activity as an activator or repressor (52). DNA-dependent protein kinase increases Sp1 activity, whereas phosphorylation of the C terminus of Sp1 by casein kinase II decreases its DNA binding properties (11). Haidweger *et al.* (54) and Fojas de Borja *et al.* (53) reported that cyclin A-CDK2 interacts with and phosphorylates Sp1 on Ser-61, enhancing its activity.

Phosphorylated Sp1 Activates the CTα Promoter—In the present study we show that in mouse embryo fibroblasts, Sp1 is phosphorylated in late G_1-S phase (Fig. 2). However, the level of Sp1 protein does not change during the cell cycle, indicating that Sp1 is specifically phosphorylated in S phase to regulate

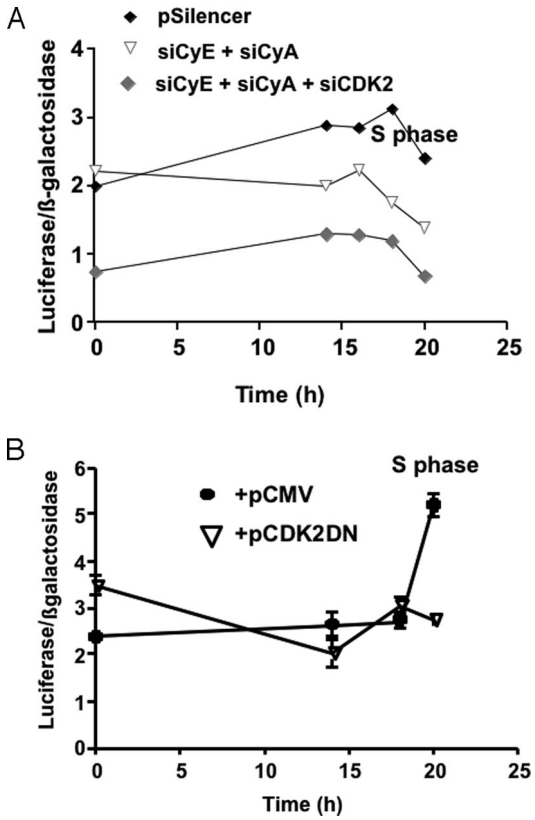

FIG. 8. **siRNAs block cyclin A, cyclin E, CDK2, and CTα expression during S phase.** *A*, the truncated CTα reporter construct LUC.C7 (−1268/+38) (1 μg) was co-transfected with pSV-β-galactosidase (1 μg) into C3H10T1/2 fibroblasts with siCyE (a plasmid designed to generate cyclin E-siRNA) and siCyA (a plasmid designed to generate cyclin A-siRNA), with siCyE, siCyA, and siCDK2 (a plasmid designed to generate CDK2-siRNA), or with p*Silencer*™ as a negative control (1 μg of each). Luciferase activity was measured relative to β-galactosidase activity at the indicated times after cell cycle induction. The values are averages of two independent experiments, the results of which did not differ by more than 10%. *B*, truncated CTα reporter constructs and pSV-β-galactosidase as a control (1 μg) were transfected into C3H10T1/2 fibroblasts with the plasmids LUC.C7 (−1268/+38) (1 μg) + pPacSp1 (1 μg) and either CMV (empty plasmid) (1 μg) or a dominant-negative form of CDK2 (*CMV-CDK2DN*) (1 μg). Luciferase activity is given relative to β-galactosidase activity and was measured at the indicated times after induction of the cell cycle. The data represent two independent experiments, and each point was measured in triplicate. These six numbers were used to calculate the error bars.

its activity. These results are in agreement with our previous report, which showed that increasing the level of Sp1 protein does not completely activate CTα transcription (33).

We also provide evidence that Sp1 is a substrate for a kinase that we identified as CDK2. In the *in vitro* phosphorylation assay, Sp1-phosphorylating activity was specifically precipitated from nuclear extracts obtained during S phase using an anti-Sp1 antibody. Addition of roscovitine, a CDK2 inhibitor, decreased Sp1 phosphorylation, indicating that phosphorylation is dependent on CDK2. We detected proteins other than Sp1 and histone H1 that were phosphorylated during S phase both *in vitro* and *in vivo*. We also demonstrate that Sp1 binds to the CTα promoter only when phosphorylated because nuclear extracts containing phosphatase-treated Sp1 were unable to bind the DNA probe. It seems likely that both the promoter and the context of the Sp1 binding site determine whether or not expression of a gene is influenced by Sp1 phosphorylation. For example, the DNA binding activity of the E2F family of transcription factors can be decreased when their heterodimerization partner, DP1, is phosphorylated by the E2F-cyclin A complex (55). Our data demonstrate that phosphorylation of Sp1 by a CDK2-cyclin E or CDK2-cyclin A complex increases the DNA binding of Sp1. This observation is consistent with our finding that in cells that overexpress cyclin A and CDK2, Sp1-dependent CTα-luciferase transcription is increased.

In agreement with our *in vitro* experiments, we have demonstrated that cells which overexpress Sp1 and either cyclin A or CDK2 have increased CTα promoter activity. Overexpression of cyclin A and Sp1 increases CTα promoter activity by ~2-fold. This observation confirms that Sp1 is activated by cyclin A. Expression of cyclin A in other phases of the cell cycle, when this protein is not normally expressed, induces a change in the CTα expression profile. When CDK2 was overexpressed, the increase was less dramatic than for cyclin A, probably reflecting residual cyclin levels in cells that had been arrested and synchronized. Overexpression of the dominant-negative CDK2 mutant reduced CTα expression. Thus we conclude that CDK activity is essential for normal induction of CTα expression during S phase. These findings are consistent with the idea that cyclin-CDK2 interacts with and phosphorylates Sp1, thereby activating Sp1-mediated transcription. This conclusion is supported by two additional results. First, an increase in CTα expression during the S phase is abolished after treatment with the CDK2 inhibitors roscovitine or olomoucine. Because the experimental design did not affect cell cycle progression, the possibility that cell cycle arrest was responsible for reduced CTα expression can be eliminated. Second, knock-down of the expression of cyclin A, cyclin E, and CDK2 using specifically designed siRNAs reduced CTα-luciferase expression. Simultaneous down-regulation of cyclin A and cyclin E abolished the induction of CTα during S phase. Cyclin E controls G_1 progression and transition to S phase (56), whereas cyclin A controls S phase events (8). With attenuated expression of cyclin A and cyclin E, the loss of CTα induction in the S phase of the cell cycle might be explained by the absence of cyclin-dependent activation of Sp1 or might be because the cells were arrested in G_1. In other studies, when cyclin E activity was inhibited by antibody microinjection during G_1, the cells failed to progress to the S phase, possibly preventing assembly and activation of cyclin E-CDK2 (56). Both possibilities might affect Sp1 activation and consequently also CTα induction during S phase.

We were unable to examine cell cycle progression ([^{3}H]thymidine incorporation into DNA) in the cells transfected with siRNAs because C3H10T1/2 fibroblasts have a low transfection efficiency (10%). However, this did not prevent us from examining siRNA effects because the cells transfected with the luciferase reporters are assumed to be the same as those transfected with siRNA. Moreover, we considered that generation of stable cell lines harboring multiple siRNAs would not be viable. The transiently transfected cells are viable as we measured β-galactosidase activities in these cells and noted that their appearance was normal. The knock-down of expression of CDK2, cyclin A, and cyclin E dramatically decreases the CTα promoter activity at all time points analyzed.

It is interesting to note that Sp1 can interact with two different protein complexes, cyclin E-CDK2 and cyclin A-CDK2. The interaction between cyclin A and Sp1 has been defined previously (53, 54). However, our finding that Sp1 not only interacts with cyclin E but also affects Sp1 transcriptional activity has not been reported. Both cyclin E-CDK2-Sp1 and cyclin A-CDK2-Sp1 are stable complexes that bind to DNA. We detected both cyclins A and E and CDK2 as part of the Sp1-DNA complex. In contrast, Fojas de Borja *et al.* (53) reported that the interaction between Sp1 and cyclin A is transient. However, the interaction between E2F and cyclin A-CDK2 involved in dihydrofolate reductase regulation is stable, and the complex binds to DNA (55).

Considerable evidence supports a close relationship between cell cycle progression and the requirement for PC biosynthesis. Most of this evidence is based on how the activity of CTα is increased to provide PC in appropriate amounts during different phases of the cell cycle (25, 27, 28). In the present study we demonstrate that CTα expression is regulated at the level of transcription by the state of Sp1 phosphorylation during the cell cycle.

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REFERENCES

1. Morgan, D. O. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 261–291
2. Sherr, C. J. (1996) *Science* **274,** 1672–1677
3. Nasmyth, K. (1996) *Science* **274,** 1643–1645
4. Hunter, T., and Pines, J. (1991) *Cell* **66,** 1071–1074
5. Hunt, T. (1991) *Semin. Cell Biol.* **2,** 213–222
6. Sherr, C. J. (1993) *Cell* **73,** 1059–1065
7. Sherr, C. J. (1994) *Cell* **79,** 551–555
8. Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992) *EMBO J.* **11,** 961–971
9. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Wade, M. (1993) *Crit. Rev. Eukaryotic Gene Expression* **3,** 229–254
10. Black, A. R., Jensen, D., Lin, S. Y., and Azizkhan, J. C. (1999) *J. Biol. Chem.* **274,** 1207–1215
11. Armstrong, S. A., Barry, D. A., Leggett, R. W., and Mueller, C. R. (1997) *J. Biol. Chem.* **272,** 13489–13495
12. Rohlff, C., Ahmad, S., Borellini, F., Lei, J., and Glazer, R. I. (1997) *J. Biol. Chem.* **272,** 21137–21141
13. Cornell, R., Grove, G. L., Rothblat, G. H., and Horwitz, A. F. (1977) *Exp. Cell Res.* **109,** 299–307
14. Terce, F., Brun, H., and Vance, D. E. (1994) *J. Lipid Res.* **35,** 2130–2142
15. Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E., and Terce, F. (1996) *J. Biol. Chem.* **271,** 14668–14671
16. Vance, D. E. (1990) *Biochem. Cell Biol.* **68,** 1151–1165
17. Johnson, J. E., and Cornell, R. B. (1999) *Mol. Membr. Biol.* **16,** 217–235
18. Lykidis, A., Jackson, P., and Jackowski, S. (2001) *Biochemistry* **40,** 494–503
19. Lykidis, A., Murti, K. G., and Jackowski, S. (1998) *J. Biol. Chem.* **273,** 14022–14029
20. Lykidis, A., Baburina, I., and Jackowski, S. (1999) *J. Biol. Chem.* **274,** 26992–27001
21. Tang, W., Keesler, G. A., and Tabas, I. (1997) *J. Biol. Chem.* **272,** 13146–13151
22. Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., and Cornell, R. B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6029–6033
23. Feldman, D. A., and Weinhold, P. A. (1987) *J. Biol. Chem.* **262,** 9075–9081
24. Wang, Y., and Kent, C. (1995) *J. Biol. Chem.* **270,** 18948–18952
25. Tronchere, H., Record, M., Terce, F., and Chap, H. (1994) *Biochim. Biophys. Acta* **1212,** 137–151
26. Cornell, R. B. (1998) *Biochem. Soc. Trans.* **26,** 539–544
27. Kent, C. (1997) *Biochim. Biophys. Acta* **1348,** 79–90
28. Northwood, I. C., Tong, A. H., Crawford, B., Drobnies, A. E., and Cornell, R. B. (1999) *J. Biol. Chem.* **274,** 26240–26248
29. Tessner, T. G., Rock, C. O., Kalmar, G. B., Cornell, R. B., and Jackowski, S. (1991) *J. Biol. Chem.* **266,** 16261–16264
30. Sesca, E., Perletti, G. P., Binasco, V., Chiara, M., and Tessitore, L. (1996) *Biochem. Biophys. Res. Commun.* **229,** 158–162
31. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochim. Biophys. Acta* **1346,** 1–9
32. Golfman, L. S., Bakovic, M., and Vance, D. E. (2001) *J. Biol. Chem.* **276,** 43688–43692
33. Banchio, C., Schang, L. M., and Vance, D. E. (2003) *J. Biol. Chem.* **278,** 32457–32464
34. Bakovic, M., Waite, K., Tang, W., Tabas, I., and Vance, D. E. (1999) *Biochim. Biophys. Acta* **1438,** 147–165
35. van den Heuvel, S., and Harlow, E. (1993) *Science* **262,** 2050–2054
36. Courey, A. J., and Tjian, R. (1988) *Cell* **55,** 887–898
37. Labbe, J. C., Capony, J. P., Caput, D., Cavadore, J. C., Derancourt, J., Kaghad, M., Lelias, J. M., Picard, A., and Doree, M. (1989) *EMBO J.* **8,** 3053–3058
38. Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989) *Cell* **56,** 829–838
39. Pines, J., and Hunter, T. (1990) *Nature* **346,** 760–763
40. Minshull, J., Golsteyn, R., Hill, C. S., and Hunt, T. (1990) *EMBO J.* **9,** 2865–2875
41. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. (1992) *Science* **257,** 1689–1694
42. Obaya, A. J., and Sedivy, J. M. (2002) *Cell. Mol. Life Sci.* **59,** 126–142
43. Karin, M., and Hunter, T. (1995) *Curr. Biol.* **5,** 747–757
44. Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G., and Moulinoux, J. P. (1997) *Eur. J. Biochem.* **243,** 527–536
45. Wang, D., de la Fuente, C., Deng, L., Wang, L., Zilberman, I., Eadie, C., Healey, M., Stein, D., Denny, T., Harrison, L. E., Meijer, L., and Kashanchi, F. (2001) *J. Virol.* **75,** 7266–7279
46. Schang, L. M., Bantly, A., Knockaert, M., Shaheen, F., Meijer, L., Malim, M. H., Gray, N. S., and Schaffer, P. A. (2002) *J. Virol.* **76,** 7874–7882
47. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) *Biochem. J.* **371,** 199–204
48. Nasmyth, K. (2001) *Annu. Rev. Genet.* **35,** 673–745
49. Hatakeyama, M., and Weinberg, R. A. (1995) *Prog. Cell Cycle Res.* **1,** 9–19
50. Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J., and Nevins, J. R. (1991) *Cell* **65,** 1243–1253
51. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992) *Cell* **70,** 993–1006
52. Jackson, S. P., MacDonald, J. J., Lees-Miller, S., and Tjian, R. (1990) *Cell* **63,** 155–165
53. Fojas de Borja, P., Collins, N. K., Du, P., Azizkhan-Clifford, J., and Mudryj, M. (2001) *EMBO J.* **20,** 5737–5747
54. Haidweger, E., Novy, M., and Rotheneder, H. (2001) *J. Mol. Biol.* **306,** 201–212
55. Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. (1994) *Cell* **78,** 161–172
56. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995) *Mol. Cell. Biol.* **15,** 2612–2624