Activation of CTP:Phosphocholine Cytidylyltransferase α Expression during the S Phase of the Cell Cycle Is Mediated by the Transcription Factor Sp1

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An essential step during cell division is induction of phosphatidylcholine biosynthesis. In this pathway, CTP:phosphocholine cytidylyltransferase α (CTα) plays an important regulatory role. Previous studies (Golfman, L. S., Bakovic, M., and Vance, D. E. (2001) J. Biol. Chem. 276, 43688–43692) demonstrated that CTα mRNA accumulates during S phase in preparation for cell division. We now demonstrate that increased binding of the transcription factor Sp1 to the proximal promoter of CTα is responsible for increased transcription during the S phase. The Sp1 binding element present in position −67/−62 is essential for activation, and the Sp1 site in position −31/−9 is required to enhance transcription. Inhibition of Sp1 expression by RNA interference abolished the enhanced expression of CTα. Immunoprecipitation studies demonstrated that Sp1 interacts with cyclin E, cyclin A, and cyclin-dependent kinase 2 during the S phase. We conclude that Sp1 binding to the CTα proximal promoter is necessary to enhance transcription during the S phase. This is the first elucidation of a mechanism by which expression of a key enzyme in phospholipid biosynthesis is regulated during the cell cycle.

Phosphatidylcholine (PC) biosynthesis is an important component of the cell cycle due to a doubling of PC mass prior to mitosis. PC biosynthesis occurs in all nucleated mammalian cells via the Kennedy (CDP-choline) pathway in which CTP:phosphocholine cytidylyltransferase (CT) catalyzes the regulated and rate-limiting step (1–4). Two genes encode CT activities, CTα and CTβ (5–10). CTα is ubiquitously expressed in nucleated cells (11), and its expression is tightly regulated. At the level of gene expression, CTα mRNA has been shown to increase after growth factor stimulation (12), during liver development (13), in proliferating liver tissue following partial hepatectomy (14), and during the S phase of the cell cycle (15).

Because the CTβ gene was only recently identified (8, 9), little is known about control of its expression. The CTα gene has been cloned and characterized (7). The gene is transcribed from two transcriptional start sites and lacks a TATA box, but contains a GC-rich region that is characteristic of many TATA-less promoters (16). The 200-bp proximal promoter contains sites for binding of Sp1, Sp3, transcriptional enhancer factor-4, Ets, and sterol response element-binding protein (16–23). There are additional candidate binding sites for other transcription factors that include Ap1, Ap2, signal transducer and activator of transcription, and NFκB (16). In the proximal promoter of the CTα gene three Sp1 binding elements have been identified that are involved in basal, activator, and suppressor activities (16). Sp1, Sp2, and Sp3 can competitively bind to these elements, and the relative abundance of these factors can regulate the expression of the CTα gene (17). Sp3 has recently been identified as being of central importance for the enhanced expression of CTα in C3H10T1/2 fibroblasts that have been stably transfected with a cDNA encoding Ras (20). Roles for transcriptional enhancer factor-4 and Ets in regulating the expression of CTα have also been identified (18, 19). A 2-fold stimulation of CTα expression by sterol response element-binding protein appears to occur upon binding to the proximal promoter (21). However, increased PC synthesis that occurs after the induction by sterol response element-binding protein appears to be largely a result of increased fatty acid biosynthesis (22, 23).

Unlike sterol or fatty acid biosynthesis, large changes in the rate of PC biosynthesis do not occur, probably because of the critical role of PC in cell survival (24–26). Moreover, genes that have TATA-less promoters are often referred to as “housekeeping” genes. However, to describe the CTα gene as “housekeeping” is inappropriate because the gene is critical for cell survival. Because the changes in CT activity are not dramatic under various perturbations, it is often assumed that the expression of the CTα gene is not differentially regulated. This is not the case, but instead the tight control of the expression of CTα gene presents a more difficult challenge for elucidation of its regulation.

We have used two cell model systems to begin to unravel cellular mechanisms that regulate the expression of the CTα gene. In cells stably transfected with Ras we have discovered that the enhanced expression of CTα is due to a higher affinity of Sp3 for the proximal promoter (20). In another model, in which expression of CTα was investigated during the cell cycle, the expression of the CTα gene was enhanced during the S phase (15). In the present study we have extended the cell cycle studies and provide evidence that Sp1 is the major transcription factor that stimulates the expression of CTα during the S phase of the cell cycle.
**EXPERIMENTAL PROCEDURES**

**Materials**—The luciferase vector, pGL3-basic, that contains the cDNA encoding *Photinus pyralis* luciferase, the control pRL-CMV vector that contains the cDNA encoding *Renilla reniformis* luciferase, pSV-β-galactosidase vector as a transfection control, and the dual-luciferase Reporter Assay System were obtained from Promega (Madison, WI). LipofectAMINE Plus reagent, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were from Invitrogen. Anti-Sp1, anti-Sp3, anti-CDK1, anti-CDK2, and anti-CDK2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. ECL® immunoblotting reagents were purchased from Amersham Biosciences.

**Cell Culture**—C3H10T1/2 mouse embryo fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) in a 5% CO2 humidified incubator at 37 °C. The cell cycle was arrested in G2, by incubation of cells in culture medium containing 0.5% FBS for 24 h and released by addition of fresh medium containing 10% FBS.

**[3H]Thymidine Incorporation into DNA**—For the analysis of DNA synthesis rates, [3H]thymidine (0.5 μCi, 1 μM) was added to triplicate 35-mm dishes 1 h before cells were harvested. At each time point, the medium was aspirated and the cells were washed three times with ice-cold phosphate-buffered saline and treated with 10% ice-cold trichloroacetic acid. The precipitated material was washed twice with 10% trichloroacetic acid, and the precipitated material was aspirated and the cells were washed three times with ice-cold phosphate-buffered saline and treated with 10% ice-cold trichloroacetic acid for 10 min on ice to precipitate DNA. The dishes were washed twice with 10% trichloroacetic acid, and the precipitated material was scraped into 0.2% SDS in 0.1N NaOH. Radioactivity associated with the trichloroacetic acid precipitate was measured by liquid scintillation spectrometry.

**Preparation of Deleted and Mutated CTA Promoter-Luciferase Reporters**—CTA promoter-luciferase constructs, LUC.C7 (−1268/+38), LUC.C8 (−201/+38), LUC.D1 (−90/+38), and LUC.D2 (−130/+38), were inserted into the promoter-less luciferase vector pGL3-basic (Promega) as described previously (18). To prepare mutated promoter constructs, GCCC (−139/−136) was mutated to AGCT and named LUC.msp1(C)/C7, CGGG (−67/−62) was changed to AATTCA and named LUC.msp1(B)/CT, and CGGG was changed for GGTAC (−211/−17) and named LUC.msp1(A)/CT. Double mutants were constructed by the same procedure but for template we used single mutants. Vectors enabling expression of recombinant Sp1 and Sp3 proteins were obtained from Dr. R. Tijan (pPacSp1 and pPac0) (27) and Dr. J. Noti (pPacSp3) (28).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)**—Total nuclear extracts of C3H10T1/2 cells grown to different stages of the cell cycle were prepared as described (29). Promoter fragments (−210/+38), (−130/+38), and (−90/+38) were released from pLUC.C8, D2 or D1 by restriction digestion with Kpn1 and HindIII. Promoter fragments were purified from 2% agarose gels using the Qiagen II gel extraction kits (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer’s instructions and end-labeled using Klenow polymerase fragments in the presence of [α-32P]dCTP. An oligonucleotide with the Sp1-consensus sequence (5′-ATTCGATCGCGCGGGCGAGC-3′) was synthesized by the University of Alberta Core Facility. Complementary oligonucleotides (100 μM of each) were heated at 90 °C for 5 min, then slowly cooled to room temperature, and 5 pmol of double-stranded oligonucleotide was 5′ end-labeled using T4 kinase (Invitrogen) and γ-[32P]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 MgCl2, 250 mM NaCl, 5 mM EDTA, 50% glycerol, 0.5% Nonidet P-40, 5 mM dithiothreitol), 1 μg nuclear extract, and labeled probe (20,000 cpm) were incubated for 30 min at room temperature.

For gel mobility shift experiments, 5 μl of antibody specific for either Sp1 or Sp3 (Santa Cruz Biotechnology) was added for 15 min prior to incubation of the probe with nuclear protein. For gel shift inhibition assays, protein and antibody were incubated for 30 min before addition of the probe. Binding reactions were terminated by the addition of 4 μl of gel loading buffer (30% [v/v] glycerol, 0.1% [v/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 Analyses**—Nuclear proteins (10 μg) from C3H10T1/2 fibroblasts were harvested 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 protein samples were electrophoresed on a 10% SDS-polyacrylamide gel in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 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, 0.1% Tween 20 and incubated for 1 h with antibody against the protein as 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 siRNA*s under the Control of the U6 Promoter**—DNA oligonucleotide templates for the in vitro synthesis of siRNAs were chemically synthesized by University of Alberta Core Facility. The oligonucleotides were designed to contain a nucleotide sequence specific for the mRNA of Sp1 (ggttgtacaccacuatc). After annealing, the DNA was cloned into the pSilencer and EcorI sites of pCBEd1 (Ambion). To confirm the insert, named pCBEd1, was confirmed by sequencing and transfected into cells with LipofectAMINE using concentrations indicated in each case.

**Immunoprecipitation**—Nuclear extracts were prepared as described above from cells collected after 20 h of cell cycle induction. Nuclear extract proteins (200 μg) 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, and 0.5% Nonidet P-40 [v/v]). The reaction was incubated for 1 h at 4 °C then incubated for 30 min with 50 μl 10% protein A-Sepharose (Staphylococcus 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, boiled, and the supernatant was electrophoresed on a polyacrylamide gel containing 0.1% SDS. Proteins were transferrable to polyvinylidene difluoride membranes and probed with the indicated antibodies.

**RESULTS**

**Sp1 Interacts with the CTA Promoter during the S Phase of the Cell Cycle**—Previous studies (15) showed that expression of CTA mRNA increased during cell cycle progression from G1 and reached a maximum during S phase. To identify the minimal promoter fragment that would stimulate CTA transcription during S phase, we analyzed luciferase activity in 3 different 5′ deletion constructs, LUC.C7 (−1268/+38), LUC.D1 (−90/+38), and LUC.D3 (−52/+38). Fig. 1 shows that partial transcriptional activation during S phase was maximal with LUC.C7 and was maintained with the LUC.D1 construct, but not with the LUC.D3 construct, indicating that the 40 bp between −52 and −90 are essential for activity during S phase. LUC.D3 showed basal activity as previously described (16). To investigate the transcription factor(s) involved in regulation of CTA promoter activity during S phase, we utilized electromobility shift assays to determine whether protein(s) present in C3H10T1/2 fibroblasts bind to the CTA proximal promoter region (−90/+38) during S phase. One DNA-protein complex was clearly observed at all points of the cell cycle (Fig. 2A). However, the abundance of the complex increased in nuclear extracts obtained 20 h after cell cycle induction (Fig. 2A). We confirmed that 20 h following cell cycle induction corresponded to S phase of the cell cycle by measuring the rate of [3H]thymidine incorporation into DNA (Fig. 2B) and by immunoblot analysis of cyclin D1 (Fig. 2C). Cyclin D1 expression was used as an indicator of cell cycle re-entry because cyclin D1 is not expressed in cells arrested in G0, but its expression is induced after serum stimulation, starting in middle G1 (30). In cycling cells, cyclin D1 is expressed throughout the cell-cycle, although its levels continue to peak at late G1.

We utilized super-shift EMSAs to identify which transcription factor(s) are involved in the formation of the DNA-protein complex during the S phase of the cell cycle. Previous studies (16) demonstrated that Sp1 and Sp3 bind the CTA promoter region and regulate its basal expression. Hence, for the EMSAs we used antibodies raised against Sp1 or Sp3. Fig. 2A shows that the addition of anti-Sp1 (2 μg) markedly reduced the
DNA-protein complex formation. The presence of anti-Sp3 antibody did not reduce formation of the DNA-protein complex. These results strongly suggest that Sp1 binds to the CTα proximal promoter during the S phase in C3H10T1/2 fibroblasts.

To confirm these results we used EMSAs to investigate the capacity of the same nuclear extract to bind a 20-bp DNA that
constructed containing the Sp1 binding consensus sequence (31). Consistent with the data shown in Fig. 2A, Fig. 3 shows the profile obtained with the Sp1 consensus element in which the protein-DNA complex formation increased in nuclear extracts obtained during S phase. As in the experiment depicted in Fig. 2A, interaction between DNA and protein decreased when we added anti-Sp1 antibody, but no decrease in the intensity of the retarded band was observed with anti-Sp3 antibody. The reason for the slower migrating smudge in lanes 6 and 7 is not clear and was not observed in other analyses. These results confirm that Sp1 is the major nuclear transcription factor that binds the CTA promoter during the S phase in C3H10T1/2 fibroblasts.

The Induction of CTA Promoter Activity During S Phase Is Abolished in a CTA Promoter Construct Lacking the Sp1 Binding Site B—In the CTA promoter region there are three Sp1 binding elements: A (−22/−15), B (−70/−58), and C (−144/−135) (Fig. 1A) that are involved in the regulation of CTA expression (16). Sp1, Sp2, and Sp3 can competitively bind to these regions, and the relative abundance of these factors regulates promoter activity of the CTA gene (17). We have investigated the role of these three binding sites in the regulation of CTA expression during different stages of the cell cycle. We analyzed luciferase activity using the Pcyt1a reporter plasmid LUC.C7 (−1268/+38) and different mutated constructs of one or more of the Sp1 binding sites. These plasmids were introduced into C3H10T1/2 murine fibroblasts by transient transfection. After synchronization and cell cycle induction, luciferase activity was measured. As demonstrated in Fig. 4A, the luciferase activity of the site A mutant (LUC.mSp1(A)/C7) increased with time with an activity profile similar to that of the wild-type reporter construct (LUC.C7). However, the level of expression was markedly (40%) lower than for the wild-type LUC.C7 after 18 h. The LUC.mSp1(C)/C7 mutant showed higher luciferase activity at all time points analyzed. Most strikingly, the mutant LUC.mSp1(B)/C7 showed low luciferase activity and no induction during S phase. Studies with constructs in which two Sp1 binding sites were mutated showed low activity when only site C was active (LUC.mSp1AB). In contrast, the luciferase activity increased during S phase in the LUC.mSp1AC mutant where only site B was active (Fig. 4B). Together these results indicate that the Sp1 binding site B is the minimal necessary element required for cell cycle induction of CTA promoter activity.

Because Ets has been previously implicated in the expression of the CTA gene (19), we examined the expression of LUC.C7 in which the Ets element of the CTA promoter was mutated. The expression of the mutated construct was decreased by 70% compared with LUC.C7, but there was still an increase of expression during the S phase of the cell cycle. Hence, Ets appears to be required for optimal reporter activity but does not seem to be involved in the enhanced expression of the CTA promoter observed in the S phase.

Interference with Sp1 Expression Decreases CTA Promoter Activity during S Phase—RNA interference is the process of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The mediators of sequence-specific messenger RNA degradation are 21 or 22 nucleotide small interfering RNAs (siRNA) generated by ribonuclease III cleavage from longer dsRNAs (32). We have used this technique to suppress expression of endogenous Sp1 for studying its function in CTA regulation during the cell cycle. Murine embryo fibroblasts were co-transfected with a pCBEd1 plasmid (0.1 µg), harboring the Sp1 RNA sequence specific for the in vitro synthesis of siRNAs that would attenuate reporter plasmid LUC.C7 (1 µg), and pSV-β-galactosidase (1 µg). Luciferase activity was analyzed at various time points after cell cycle induction. Fig. 5A shows that the CTA promoter activity clearly increased during cell cycle progression reaching a maximum in S-phase (20 h) in cells transfected with LUC.C7 and the empty plasmid pSilencer. However, in cells where Sp1 expression was attenuated with RNAi directed against Sp1, up to 50% reduction in S phase of the CTA promoter activity was observed (Fig.
Fig. 4. Sp1 binding site B (−67/−62) is essential for CTα promoter activity during S phase. A, truncated CTα reporter plasmids (1 μg) LUC.C7 (−1268/+38) (filled diamonds), or mutated constructs LUC.mSp1(A)/C7 (triangles), LUC.mSp1(B)/C7 (open squares), or LUC.mSp1(C)/C7 (dark squares), and pSV-β-galactosidase plasmids (0.1 μg) were transfected into C3H10T1/2 fibroblasts. Reporter activity is given relative to β-galactosidase activity and was measured at the indicated times after induction of the cell cycle. B, truncated CTα reporter plasmids (1 μg) LUC.C7 (−1268/+38) (filled diamonds), doubly mutated constructs LUC.mSp1(A-B)/C7 (filled squares), LUC.mSp1(B-C)/C7 (triangles), or LUC.mSp1(A-C)/C7 (open squares), and pCMV-β-galactosidase plasmids (0.1 μg) were transfected into C3H10T1/2 fibroblasts. Reporter activity was measured at different time points after induction of the cell cycle and is given relative to β-galactosidase activity. Values are the means ± S.D. from three dishes and two measurements for each dish.

5A). Because transfection efficiency was only about 10%, we were not able to detect a difference in the level of Sp1 expression or endogenous CT activity (data not shown). To evaluate the ability of pCBEd1 to inhibit Sp1 expression, cells were co-transfected with PacSp1 (0.05 μg) (an expression vector encoding Sp1) and pCBEd1 (1 μg) or pSilencer (1 μg). Immunoblot analysis showed a clear suppression of Sp1 expression in cells transfected with pCBEd1 (Fig. 5B). As indicated, the expression of the transcription factor TFII D was unaffected by pCBEd1 expression. These results demonstrate that inhibition of Sp1 expression by siRNA attenuated the induction of CTα expression. These results demonstrate that inhibition of Sp1 expression has enhanced CTα expression.

The Presence of Sp1 Is Necessary, but Not Sufficient, to Enhance CTα Promoter Activity during the S Phase—Previous studies (17) have established that the CTα promoter can be activated by both Sp1 and Sp3. We, therefore, next examined the ability of Sp1 and Sp3 to individually enhance CTα promoter activity during S phase of the cell cycle. We co-transfected Sp1 or Sp3 expression vectors (pPacSp1 or pPacSp3), or the empty plasmid pPac0, with a Pcyt1a reporter plasmid LUC.C7 (−1268/+38) or, as a control with pRL-cytomegalovirus vector that has the luciferase reporter under the control of the constitutive cytomegalovirus promoter. After transfection, cells were arrested for 24 h, after which cell cycle progression was induced by addition of 10% FBS. Luciferase activity was measured in samples obtained at different time points. As Fig. 6 shows, cells that were transfected with Sp1 have ~20 times higher luciferase activity than cells harboring the empty vector at each time point analyzed. Nevertheless, the luciferase activity was 2-fold higher at 19 h than at 0 or 15 h after cell cycle induction. Thus, although there was a marked increase in luciferase activity as a result of the over-expression of Sp1, luciferase expression was still induced in S-phase, indicating that S-phase-specific modifications of Sp1 are required to maximally activate the CTα promoter or that Sp1 interacts with other cell cycle regulated proteins, or both.

The expression of Sp3 enhanced luciferase activity 6-fold (Fig. 6), consistent with previous studies that showed that Sp3 activates CTα basal transcription (17). However, data presented in Figs. 2 and 3 demonstrate that Sp3 is not a major protein present in DNA-protein complexes isolated during S phase. A parallel experiment using the pRL-cytomegalovirus promoter-luciferase reporter as a control showed no change in luciferase activity at any time point analyzed (data not shown). Because the level of Sp1 and Sp3 expression determined by immunoblotting did not reveal substantial differences during the cell cycle (data not shown), we postulated that a modification of Sp1 had enhanced CTα expression.

Sp1 Interacts with a Complex Containing Cyclin A, Cyclin E, and CDK2 during S Phase—Activation of the CTα promoter was maximum at late G1/S phase of the cell cycle, when cyclin
E/CDK2 and cyclin A/CDK2 are activated. Because cyclin A/CDK2 and cyclin E/CDK2 form complexes with transcription factors and other regulatory proteins (such as retinoblastoma protein) and these complexes activate S-phase-specific promoters, we examined possible physical interactions between cyclin A, cyclin E, or CDK2 with Sp1. Cyclin E is expressed only from late G1 into early S phase, cyclin A is expressed from early S through M, and CDK2 is expressed throughout the cell-cycle. Both cyclins E and A activate CDK2. C3H10T1/2 cells were growth arrested by serum withdrawal for 48 h. After stimulation of cell division with 10% bovine serum albumin for 20 h, nuclear extracts were immunoprecipitated with anti-Sp1 antibody. The precipitated proteins were separated by electrophoresis and analyzed by immunostaining with anti-cyclin A or anti-cyclin E antibodies. Cyclin A and cyclin E were clearly co-immunoprecipitated with Sp1 from cells harvested 20 h after induction of the cell cycle (Fig. 7). We also performed immunoblotting with anti-CDK2 antibody and identified this kinase as a component of the complex (Fig. 7).

**DISCUSSION**

**Identification of Sp1 as the Transcription Factor that Enhances CTα Expression during the S Phase of the Cell Cycle**—During cell division each step is coordinately regulated to permit growth and development. One essential process is PC biosynthesis, which provides the principal phospholipid for the synthesis of new membrane and as a precursor for signaling molecules. Thus, CTα plays a critical role due to its regulatory function in the Kennedy pathway (1–4). Previous studies (15) showed that CTα mRNA increases during the S phase in preparation for mitosis. The focus of the current study was to elucidate the mechanism by which the expression of CTα mRNA increases during S phase in fibroblasts. The results demonstrate that a complex signaling pathway involving Sp1 interaction with cell cycle-specific cyclin A/cyclin E and CDK2 is essential for activation through the Sp binding element in the CTα proximal promoter, thereby enhancing transcription during the S phase. Moreover, we demonstrated that a Sp1 binding element (~671–62) is essential for activation of CT transcription during S phase.

Sp1 is present in nuclear extracts and binds the CTα promoter during all stages of the cell cycle, but maximally after 20 h of cell cycle induction corresponding with S phase. Previous studies (17) established that the CTα basal promoter is
inactive when transcription is not supported by the nuclear factors Sp1 and Sp3. We have now established that Sp1, but not Sp3, is responsible for the protein-DNA complex formed during S phase. Sp3 can act as a positive or negative regulator depending on the promoter and the cellular context (33, 34). Our data show that Sp3 does not bind to the CTα proximal promoter or the Sp-consensus element in appreciable quantities during the S phase, suggesting that this transcription factor is either not present in nuclear extracts of C3H10T1/2 fibroblasts in this phase, or is not specifically activated during the S phase. In contrast, Sp3 appears to be the factor responsible for enhanced CTα expression in fibroblasts stably transfected with Ras (20).

The CTα proximal promoter contains three Sp1 binding elements. Using specific mutants in each binding element we determined that the binding site B is essential for enhancement of transcription during the S phase. However, mutation of site A also diminished transcription, suggesting that both sites are important for maximal transcription during S phase. Interestingly we found that Sp1 binding site C has a negative effect on transcription indicated by the increased level of activity exhibited by the Sp1-mC mutant construct compared with the un-mutated promoter. The function of site C is unclear, but one possibility is that site C might govern CTα transcription during other phases of the cell cycle, such as G2, where PC is not required in such abundant quantities. This result is consistent with earlier findings that the Sp1 C site attenuates CTα expression (16).

An important role for Sp1 in CTα transcription was further indicated when we used siRNA. With this technology we were able to study the functional consequences of reducing the expression of Sp1 on CTα expression. The results demonstrated that inhibition of Sp1 by siRNA blocked enhanced CTα-promoter expression in S phase of the cell cycle.

Cyclins E/A and CDK2 Interact with Sp1 for Activation of CTα-Luciferase Expression—The next question we addressed was whether or not Sp1 in the nuclear extract was specifically required during the S phase to stimulate CTα transcription. If this were the case, we hypothesized that cells that over-expressed Sp1 would show transcriptional activity independent of the stage of the cell cycle. When we analyzed the luciferase activity in cells that over-expressed Sp1, we observed a large increase in the expression of CTα-luciferase relative to that in control cells. Nevertheless, CTα-luciferase activity clearly increased (2-fold) during the time corresponding to the S phase. This result suggests that the presence of Sp1 per se is not sufficient for the specific induction of CTα during S phase. Sp3 also stimulated CTα-luciferase expression but to a lesser extent than Sp1.

Certain post-translational modifications affect the level, capacity, and binding ability of transcription factors, and therefore transcriptional activity. Cell cycle-dependent phosphorylation of proteins is often catalyzed by cyclin/kinase complexes with cyclin A/E being the regulatory, and CDK2 the catalytic, subunit (35). Growth-dependent phosphorylation of Sp1 has been shown to occur during G1 (36). Several cellular kinases such as casein kinase II (37) and double stranded DNA-dependent protein kinase (37) have been reported to phosphorylate Sp1 and regulate its activity. Moreover, several proteins interact with Sp1 to alter its capacity as a transcriptional activator or repressor (36). Future experiments will be directed toward elucidating how Sp1 is modified to increase transcriptional activation of CTα during S phase of the cell cycle.

By co-immunoprecipitation assays we demonstrated that Sp1 interacts with cyclin E, cyclin A, and CDK2. This result is in agreement with previous results that cyclin E, in combination with CDK2, is a positive G1 cell cycle regulator controlling progression through G1 and initiation of DNA replication (38–41). Cyclin A binds both CDK2 and another kinase, CDK1, giving rise to two distinct cyclin A kinase activities, one appearing in S phase, the other in G2 (42). An interaction between cyclin A and Sp1 has been described in the regulation of thymidine kinase expression, where this interaction, and the phosphorylation of Sp1 by an associated kinase, enhance the transcriptional activity of the promoter (43). An interaction between cyclin E and Sp1 has not been previously reported. Considering that Sp1 regulates the expression of CTα during the S phase and that cyclin E/CDK2 are involved in the regulation of the transition to S phase it is not surprising that this interaction occurs. At the beginning of the S phase both cyclin E/CDK2 and cyclin A/CDK2 complexes co-exist.

CONCLUSION

We provide the first characterization of a mechanism by which the expression of a phospholipid biosynthetic gene is regulated during the cell cycle. During each division, cells coordinate gene expression to provide the required building blocks at the correct moment. Cyclin E/CDK2 complexes regulate the transition to S phase and cyclin A/CDK2 regulates events during S phase, and both of these complexes regulate the activity of Sp1 by phosphorylation. It is not surprising that genes that encode enzymes necessary for the synthesis of essential molecules like nucleic acids and phospholipids would be regulated by a common mechanism.

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Regulation of CTα by Sp1 during S Phase

32464

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