Effects of Depletion of CREB-binding Protein on c-Myc Regulation and Cell Cycle G1-S Transition*

We recently reported that the transcriptional coactivator and histone acetyltransferase p300 plays an important role in the G1 phase of the cell cycle by negatively regulating c-myc and thereby preventing premature G1 exit (Kolli, et al. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4646–4651; Baluchamy, et al. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9524–9529). Because p300 does not substitute for all CREB-binding protein (CBP) functions, we investigated whether CBP also negatively regulates c-myc and prevents premature DNA synthesis. Here, we show that antisense-mediated depletion of CBP in serum-deprived human cells leads to induction of c-myc and that such cells emerge from quiescence without growth factors at a rate comparable with that of p300-depleted cells. The CBP-depleted cells contained significantly reduced levels of the cyclin-dependent kinase inhibitor p21 and low levels of p107 and p130 (but not pRb) phosphorylation, suggesting that these factors, along with elevated levels of c-Myc, contribute to induction of DNA synthesis. Antisense c-Myc reversed the phosphorylation of p107 and p130 and the induction of S phase in CBP-depleted cells, indicating that up-regulation of c-myc is directly responsible for the induction of S phase. Furthermore, the serum-stimulated p300/CBP-depleted cells did not traverse beyond S phase, and a significant number of these cells died of apoptosis, which was not related to p53 levels. These cells also contained significantly higher levels of c-Myc compared with normal cells. When c-myc expression was blocked by antisense c-Myc, the apoptosis of the serum-stimulated CBP-depleted cells was reversed, indicating that high levels of c-Myc contribute to apoptosis. Thus, despite their high degree of structural and functional similarities, normal levels of both p300 and CBP are essential for keeping c-myc in a repressed state in G1, and thereby preventing inappropriate entry of cells into S phase. In addition, both these proteins also provide important functions in coordinated cell cycle progression.

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p300 and the CBP1 are two large highly homologous and conserved transcriptional adapter proteins containing histone acetyltransferase activity that link transcription with chromatin remodeling (1). CBP and p300 also acetylate a number of sequence-specific transcription factors, including p53, E2F, and T-cell factor-4, affecting their transcriptional activation and DNA binding activities (2–4). Mice in which both alleles coding for either CBP or p300 are disrupted die at the embryonic stage due to multiple developmental abnormalities, suggesting that both the p300 and CBP genes are required for the normal cell proliferation and development (5, 6). Patients with haploinsufficiency of the CBP gene display severe abnormalities characteristic of Rubinstein-Taybi syndrome, comprising mental retardation and physical deformities (7). These patients also have an increased incidence of cancer. Mutations in the p300 and CBP genes have been reported in several cancers, including gastric and colon cancers, and certain forms of leukemia, indicating that they may have tumor suppressor properties (reviewed in Refs. 1 and 8). The intrinsic ubiquitin ligase activity of p300 plays a role in the proteasomal degradation of p53 (9, 10). p300 and CBP also function as coactivators for tumor suppressor proteins such as p53, suggesting an indirect role for these proteins in tumor suppression (2, 11, 12).

Previous adenovirus (Ad) E1A studies have indicated that p300 may play a role in maintaining cells in the G1 phase of the cell cycle. For example, E1A can stimulate DNA synthesis in quiescent baby rat kidney cells, which is dependent on its binding to p300 (13–15). E1A can also re-stimulate DNA replication in terminally differentiated cardiac myocytes through its p300/CBP-binding domain (16). There is evidence that p300 is involved in terminal differentiation in several cell types, including muscles, neurons, and enteroendocrine cells (reviewed in Ref. 1). During terminal differentiation, p300 trans-activates p21 by cooperating with Sp1, Sp3, or tissue-specific transcription factors, suggesting that p300 may play a role in keeping cells in G0/G1 (1, 17, 18). However, fibroblasts from p300 knockout mice are unable to replicate and appear to undergo cell cycle arrest, indicating that some p300 function may be necessary for cell cycle progression (5). Furthermore, CBP has been shown to be associated with Cdk2 in vivo and may be a substrate for cyclin E/Cdk2 (19). p300 provides a coactivator function for both growth stimulatory transcription factors such as E2F (3) and growth inhibitory proteins such as p53 (2, 11, 12). p300 is underphosphorylated during G0/G1 and hyperphosphorylated during S and G2/M phases (20). Therefore, the overall roles played by p300/CBP at different stages of

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1 The abbreviations used are: CBP, cAMP-responsive element-binding protein-binding protein; Ad, adenovirus; Cdk, cyclin-dependent kinase; CREB, cAMP-responsive element-binding protein; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.
the cell cycle remain to be determined.

Although, in most studies, p300 was able to substitute for CBP functions, not all p300 functions can be substituted by CBP (reviewed in Ref. 1). For example, in F9 cells, both differentiation and cell cycle arrest induced by retinoic acid are dependent on normal levels of p300, but not of CBP (21). Mice lacking one copy of the CBP gene display some of the characteristics found in Rubinstein-Taybi syndrome patients, including the increased development of hematological neoplasm (6). In striking contrast, p300 heterozygous mice do not display this phenotype (5). These proteins also appear to play distinct roles in maintaining normal hematopoiesis (22). Mouse p300 fi- 

broblasts are also defective in retinoic acid-induced transcription despite the presence of normal level of CBP in these cells. In contrast, these cells maintain normal levels of CREB-acti-
tated transcription (5). In transient assays, transcription of the cyclin D1 gene can be induced by p300, but not by CBP (23). Other studies related to functional differences between p300 and CBP include the involvement of p300 (but not CBP) acetyl-transferase activity during myogenesis (24) and complexes containing p300 (but not CBP) and the nuclear proto-oncoprotein SYT in the proper activation of β1 integrin and control of cell adhesion (25).

Recently, we showed that quiescent human cells depleted of p300 enter S phase without serum and that this premature DNA synthesis is due to up-regulation of c-myc (26). Similarly, we also showed that conditional overexpression of p300 re-
presses c-myc and S phase entry (27). In view of the above-noted functional differences between p300 and CBP, we inves-
tigated the effects of depletion of CBP on c-myc regulation and G1/S transition. We now show that reduced synthesis of CBP in quiescent cells also results in the induction of c-myc and S phase entry. However, p300- or CBP-depleted cells with or without serum stimulation did not undergo normal cell cycle progression. Such cells expressed c-myc to much higher levels compared with control cells, and a significant portion of them died of apoptosis after serum stimulation, which did not appear to be related to p53 levels. Furthermore, we also show that in growth-arrested p300/CBP-depleted cells, the Cdk inhibitor p21 is dramatically down-regulated, and p107 and p130 (but not pRb) are hyperphosphorylated at low levels, suggesting that in addition to the induction of c-myc, the alterations in these negative regulators of the cell cycle may also contribute to the induction of S phase. Finally, we show that the inappropri-
ate up-regulation of c-myc in CBP-depleted cells is primarily responsible for the induction of S phase and apoptosis, as inhibition of c-myc expression reverses both these effects. Thus, our results suggest that despite their structural and functional similarities, normal levels of both p300 and CBP are required for keeping c-myc in a repressed state and maintaining cells in the G1 phase of the cell cycle. These two proteins may have a universal role as checkpoint proteins, preventing the untimely onset of DNA synthesis in senescent or differentiated cells. Our results also indicate that normal levels of both these proteins are essential for coordinated cell cycle progression.

EXPERIMENTAL PROCEDURES

Cells and Viruses—MCF10A cells are a nontransformed human breast epithelial cell line that arose as a spontaneous immortalization of normal breast epithelial cells derived from a reduction mammaplasty specimen (28). The growth conditions for these cells were described previously (29). AS-p300 is a replication-defective Ad vector that ex-
presses 1784 bp of p300 cDNA S-sequence in an antisense orientation from the cytomegalovirus promoter in the E1A region (26). AS-CBP is identical to AS-p300 except that it expresses 2.0 kb of human CBP cDNA sequence (30) and was constructed as described (31). Adβ-gal expresses β-galactosidase from the cytomegalovirus promoter (26). AdM4 is an Ad vector that contains a promoter-luciferase reporter cassette with four copies of the c-Myc-binding site (E-box element) cloned upstream of the herpes simplex virus thymidine kinase minimal promoter and linked to the luciferase reporter gene (27). AS-Myc is an Ad vector that expresses antisense c-myc sequences (26).

Cell Cycle Analysis—Cells were seeded overnight at a density of 1 × 10^6/100-mm dish and serum-starved. Thirty-six hours after serum star-
vation, they were infected with appropriate Ad vectors at a multiplicity of infection of 200 and maintained under serum-starved conditions for the duration of the experiments shown in the figure legends. For the serum stimulation experiments, cells were stimulated with serum 18 h after vector infection and harvested at the indicated time points. For experiments in which cell cycle analysis was carried out under continued serum starvation, cells were maintained in serum-free medium for the duration of the experiments and then harvested and processed for FACS analysis as described (29).

RNA Analysis—MCF10A cells were seeded, starved, and infected with Ad vectors as described above; starvation was continued for 18 h after virus infection; and the cells were stimulated with pentol medium containing 5% horse serum. Total RNA was isolated from cells using an RNA isolation kit (Promega catalog no. Z3100). Poly(A)-containing RNA was isolated from total RNA using a poly(A)+ RNA isolation kit (QIA-
GEN catalog no. 72012). Equal quantities of RNA were analyzed by Northern blotting using a human c-myc probe corresponding to 1 kb of N-terminal coding sequence.

Apoptosis Assays—Apoptosis assays for p300- or CBP-depleted serum-starved cells were carried out using the annexin V binding assay to assess the loss of phospholipid asymmetry of the plasma membrane (32) and by staining the mitochondria with chloromethyl-X-rosamine to assess the changes in the mitochondrial membrane potential (33). Cells were serum-starved, infected with Ad vectors, and serum-stimulated as described above for the cell cycle experiment. At the indicated times, the adherent cultures were harvested after trypan blue and pooled after the previously collected floating cells, which were washed once with calcium/magnesium-free phosphate-buffered saline. Annexin V binding ass-
ay was then carried out using a commercially available kit (Immuno-
tech catalog no. PN IM3546). Briefly, the phosphate-buffered saline-
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CBP Depletion, c-Myc Up-regulation, and G1-S Transition

Quiessential MCF10A cells were infected with antisense vectors, lysed, and immunoblotted using anti-p300 or anti-CBP antibodies as described (28). c-Myc, p53, p21, and the three Rb family proteins were analyzed by Western immunoblotting using appropriate antibodies (see figure legends for antibodies and other details).

**RESULTS**

**Quiessential Cells Depleted of CBP Exit G1, without Serum at Levels Similar to Those Depleted of p300**—Our previous study showed that an antisense Ad vector expressing 1.7 kb of p300 cDNA 5'-sequence (AS-I; referred to here as AS-p300) effectively reduces the levels of p300 in quiescent cells (26). To block the synthesis of CBP in quiescent cells, we used a replication-defective Ad vector expressing 2 kb of human CBP cDNA sequence from the cytomegalovirus promoter (AS-CBP). To determine whether AS-CBP would reduce CBP synthesis without affecting p300 levels, serum-starved MCF10A cells (see “Experimental Procedures” for a description of these cells) were infected with antisense vectors; and 20 h after infection, the levels of p300 and CBP in the cells were quantified by Western immunoblotting using appropriate antibodies. An Ad vector expressing β-galactosidase from the cytomegalovirus promoter (Adβ-gal) was used as a control in these and all other experiments in this work. Fig. 1A shows that AS-CBP infection resulted in a significant reduction in CBP levels without affecting p300 levels. Consistent with our previous report, infection of cells with AS-p300 resulted in a considerable reduction in p300 levels. This result suggests that our antisense vectors are effective in reducing the synthesis of respective gene products with a high degree of specificity despite the fact that these two proteins are highly homologous (35–37).

To determine whether serum-starved cells infected with AS-CBP would exit G0/G1 in a manner similar to AS-p300-infected cells, MCF10A cells were serum-starved and infected with the antisense vectors or Adβ-gal, and serum starvation was continued (see Fig. 1B for the time course of infection and harvesting). After a total of 52 h of serum starvation, cells were harvested at 4-h time intervals up to 36 h post-infection. Fig. 1C shows that both CBP- and p300-depleted cells exited G1 at similar rates, although AS-CBP was slightly more effective in this regard. For example, at 36 h after infection, ~35% of the AS-CBP-infected cells were in S phase fractions, whereas at this time point, ~28% of the AS-p300-infected cells were found in S phase. In contrast, the number of Adβ-gal-infected cells in S phase at this time point was <5%, ruling out the effects of genes expressed by the vector backbone contributing to the induction of S phase. Both AS-CBP- and AS-p300-infected cells steadily accumulated in S phase, but did not traverse into G2/M, suggesting that these cells lack the ability to complete the cell cycle. This aspect is addressed below. Thus, our results show that both p300 and CBP provide an activity that allows the cells to remain in G0/G1 phase.

Surprisingly, we did not observe a significant increase in the number of S phase-specific cells when cells expressed both types of antisense sequences simultaneously as opposed to cells expressing only antisense p300 or CBP sequences (data not shown). At present, we do not know the reasons for the lack of an additive or synergistic effect.

**c-myc Is Up-regulated at Similar Levels in Antisense CBP and p300-expressing Cells**—Our previous study showed that premature DNA synthesis that occurs in cells expressing antisense p300-specific sequences is due to up-regulation of c-myc

(26). To determine whether the S phase entry of cells infected with AS-CBP was also due to up-regulation of c-myc, we assayed the levels of c-Myc protein in serum-starved cells expressing antisense CBP (see Fig. 2A for the time course of infection and harvesting). As a comparison, the levels of Myc in cells expressing antisense p300 were also determined in parallel. Myc levels increased significantly in both antisense CBP and p300-expressing cells compared with control cells infected with Adβ-gal (Fig. 2, B and C, respectively). To determine the endogenous Myc transcriptional activation activity, we used an Ad vector (AdM4) that expresses a promoter-luciferase reporter cassette containing four copies of the c-Myc protein-binding site (E-box element) (27).

The luciferase activity was assayed using histone H1 as a substrate (27).

**FIG. 1.** p300 and CBP levels and S phase induction in quiescent MCF10A cells infected with AS-p300, AS-CBP, and Adβ-gal. A, Western blot showing reduced levels of CBP (but not p300) in AS-CBP-infected cells and reduced levels of p300 (but not CBP) in AS-p300-infected cells. Serum-starved MCF10A cells were infected with AS-p300, AS-CBP, or Adβ-gal (B-gal) at 20 plaque-forming units/cell. Protein (100 μg) from each lysate was separated on an SDS-7.5% polyacrylamide gel and immunoblotted using polyclonal antibodies specific for p300 and CBP (N-15 and A-22, respectively; Santa Cruz Biotechnology). Only the relevant portion of the autoradiogram is shown. B, schematic representation of the time course used for vector infections and harvesting of cells for cell cycle analysis. C, induction of DNA synthesis in quiescent MCF10A cells infected with antisense vectors or Adβ-gal. MCF10A cells were infected with Ad vectors as shown in B, and the cells were harvested at the indicated times. The percentage of cells in G1, S, and G2/M was quantified by FACS analysis as described under “Experimental Procedures.” Assays were done in triplicate, and the means ± S.D. are shown.
with an increase in c-Myc protein levels (27). To assay the induction of c-myc in p300-depleted quiescent cells, serum-starved cells were first infected with AS-CBP, AS-p300, or Adβ-gal and again infected 1 h later with AdM4. Seventeen hours following AdM4 infection and every 2 h thereafter, they were harvested, and the luciferase activities of the infected cells were determined. As shown in Fig. 2 (D and E), the reporter activity in the antisense-expressing cells began to rise at 19 h post-infection and continued to increase up to 25 h (later time points were not tested). The luciferase activity of Adβ-gal-infected cells increased only at moderate levels, indicating that the 3–4-fold increase in luciferase activity observed in antisense-infected cells was due to an increase in Myc protein levels. Thus, we conclude that depletion of either p300 or CBP leads to up-regulation of c-myc. Furthermore, the above results indicate that both proteins are essential for the negative regulation of c-myc and the prevention of cells from exiting G1.

Using antisense c-Myc, we have shown previously that the induction of DNA synthesis in p300-depleted cells is due to c-myc up-regulation (26). Using this strategy, we show below that S phase induction in CBP-depleted cells is also due to c-myc up-regulation. Consistent with the lack of additive effect...
of the two antisense viruses on DNA synthesis discussed above, we did not observe an increase in Myc activity when cells were infected simultaneously with two antisense vectors in our several independent experiments (data not shown).

p21 Levels Decrease Significantly in Antisense p300/CBP-expressing Cells—We showed previously that p300 negatively regulates c-myc expression in G1, which is primarily responsible for preventing early entry of cells into S phase (26, 27). However, we were curious to know whether there are any changes in the status of the well known negative regulators of the cell cycle in p300/CBP-depleted cells that would contribute to DNA synthesis, including the cyclin/Cdk activity inhibitor p21 and pRb, p107, and p130, which sequester E2F. Because p300/CBP is a coactivator of transcription factors that control transcription of the p21 gene, we determined whether reduced synthesis of CBP affects the levels of p21 in antisense-expressing cells. The levels of p21 in cell extracts prepared from growth-arrested cells expressing antisense CBP sequences were determined by Western blotting (see Fig. 3A for the time course of infection and harvesting of cells). The data presented in Fig. 3B show that a significant reduction of p21 levels was observed as early as 18 h after AS-CBP infection compared with control samples, and the levels dropped further by 28 h post-infection. Interestingly, using the same cell lysates, we observed that at the 18-h time point, there was only a marginal induction of c-myc (Fig. 3H; note that same cell lysates were used for the data shown Figs. 3 (B-H) and 8A). This indicates that the down-regulation of the p21 genes occurs before a significant rise in Myc levels. We have shown previously that p21 RNA levels decline in antisense p300-expressing cells (26). Thus, the p21 gene is down-regulated in both CBP- and p300-depleted cells.

p107 and p130 (but Not pRb) Are Hyperphosphorylated at Low Levels in Antisense p300/CBP-expressing Cells—A normal growth-stimulated G1-S transition involves phosphorylation of pRb and the related proteins p107 and p130, resulting in the release of E2F from the chromatin repressor complexes, which is essential for S phase progression (reviewed in Refs. 38–40). Thus, we were interested to know whether phosphorylation of pRb and the related proteins would occur in p300/CBP-depleted cells, which might contribute to the induction of DNA synthesis. The Western blot data presented in Fig. 3C show that there were negligible amounts of slower migrating forms of pRb in lanes corresponding to cell extracts prepared from quiescent cells infected with AS-CBP for 24 or 28 h (the B-gal + serum lane is a positive control and represents phosphorylation of pRb in serum-stimulated cells undergoing a normal S phase progression, and the phosphorylated form of pRb is shown by the arrow; see legend to Fig. 3C). Studies have shown that in G0 and early G1, p130 appears as a hypophosphorylated doublet representing two phospho forms in Western blots, and a new hyperphosphorylated form appears in late G1 and S phases (41–43). Consistent with these reports, p130 migrated as a doublet in control Adβ-gal lanes, and a new band migrating slower than the two phosphorylated forms was evident in serum-stimulated cells, which served as a positive control (Fig. 3D, phosphorylated form shown by the arrow). A faint slower migrating band was evident in AS-CBP-infected cells at 24 h after infection, suggesting a low level phosphorylation of p130 (Fig. 3D; the 28-h sample could not be completed in this experiment). Similarly, we observed a faint band migrating slower than the p107 band in AS-CBP-infected cells at the 24- and 28-h time points, indicating that p107 was also phosphorylated at low levels. Note that in serum-stimulated cells serving as a positive control, the band corresponding to phosphorylated p107 was merged with the p107 band (Fig. 3E). Because pRb was not hyperphosphorylated in antisense-expressing cells, we determined whether the cyclin D/Cdk activity was elevated in these cells. As shown in Fig. 3F, there was no change in the levels of cyclin D/Cdk activity at 20 and 24 h post-infection. In contrast, AS-CBP-infected cells showed elevated levels of cyclin E/Cdk activity beginning 20 h after virus infection (Fig. 3G). We believe that cyclin A/Cdk activity is also elevated in these cells, as we showed previously that both cyclin E and cyclin A kinase activities are induced in antisense p300-expressing cells (26). The lack of induction of cyclin D/Cdk activity in these cells could be attributed to the lack of growth factors in the medium because induction of cyclin D is dependent on growth factors (reviewed in Ref. 44).

Antisense c-Myc Reverses the Induction of S Phase and the Phosphorylation of p107 and p130—To determine whether the induction of S phase and phosphorylation of the Rb family proteins in CBP-depleted cells was the result of induction of c-myc, we inhibited c-myc expression by expressing antisense c-Myc sequences as described previously (26), followed by monitoring the capacity of the CBP-depleted cells to exit G1. Serum-starved cells were co-infected with AS-CBP and an Ad vector that expresses antisense c-myc sequences (AS-Myc) (26). To maintain a constant multiplicity of infection, the Adβ-gal vector was included in the protocol where appropriate. To confirm the effectiveness of AS-Myc in blocking the synthesis of c-Myc, c-myc transcriptional activity was monitored using the reporter assay described above. As shown in Fig. 4B (Fig. 4A shows the time course for infection and harvesting of cells), at 16 h post-infection, the luciferase activity in AS-CBP-infected cells was comparable with that detected in different control samples. In contrast, at 25 h post-infection, the AS-CBP-infected cells expressed ~3-fold more luciferase activity compared with cells infected with Adβ-gal or with Adβ-gal and AS-Myc. Importantly, in cells co-infected with AS-Myc and AS-CBP, Myc activity was reduced to ~50% of that observed in AS-CBP-infected cells, indicating that AS-Myc was effective in blocking c-myc expression.

Next, the S phase entry of cells co-infected with AS-CBP and AS-Myc was compared with that of AS-CBP-infected cells. As shown in Fig. 4C, at 16 h post-infection, <5% of the cells were detected in S phase in all samples. At 36 h post-infection, ~33% of the AS-CBP-infected cells accumulated in S phase, whereas <5% of the Adβ-gal- and Adβ-gal/AS-Myc-infected cells accumulated in S phase. Importantly, the number of S phase-specific cells co-infected with AS-CBP and AS-Myc was reduced to <10%, indicating that AS-Myc efficiently reversed the induction of S phase in CBP-depleted cells. Thus, we conclude that the induction of S phase in CBP-depleted cells is the result of induction of c-myc.

To determine whether AS-Myc would also reverse the phosphorylation of p107 and p130, cell extracts were prepared from cells infected with AS-CBP and with AS-CBP and AS-Myc for 28 h along with appropriate controls as shown in Fig. 4C. The phosphorylation status of p107 and p130 was then determined by Western immunoblotting as described in the legend to Fig. 3. Fig. 4D shows that p107 was not phosphorylated in cells infected with Adβ-gal or with Adβ-gal and AS-Myc, p107 was phosphorylated at moderate levels in AS-CBP-infected cells, which was reversed by AS-Myc (Fig. 4D, AS-CBP + AS-Myc lane). Similarly, low level phosphorylation of p130 in CBP-depleted cells observed at 28 h post-infection was reversed by AS-Myc. In summary, we conclude that the induction of S phase and the phosphorylation of p107 and p130 in CBP-depleted cells are the result of induction of c-myc.

Serum-starved p300- and CBP-depleted Cells Fail to Progress beyond S Phase after Serum Stimulation—Our FACS
analysis of the serum-starved antisense-expressing cells suggested to us that these cells accumulated in S phase steadily, but did not traverse beyond S phase (data not shown). As growth factor-stimulated activation of cyclin D is essential for the G₁-S transition of serum-starved cells, we considered it possible that serum stimulation of the cells accumulating in S

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**Fig. 3.** p21 levels; phosphorylation of pRb, p130, and p107; and cyclin D and cyclin E kinase activities in serum-starved AS-CBP-infected cells. The same cell lysates were used for all assays shown. A, time course used for starvation, infection with Ad vectors, and harvesting cells for the assays described here. B, Western blot showing decreased levels of p21 in AS-CBP-infected cells. Protein (100 µg) from each cell lysate was analyzed on an SDS-12% polyacrylamide gel. The blot was probed with anti-p21 monoclonal antibody (Labvision catalog no. MS-891). To detect tubulin, the blot was reprobed with anti-tubulin antibody (Labvision catalog no. MS-581). C, Western blot showing the phosphorylation status of pRb. Protein (60 µg) from each cell lysate was analyzed on an SDS-7.5% polyacrylamide gel. The blot was probed with anti-Rb monoclonal antibody (Santa Cruz Biotechnology catalog no. sc-50). The B-gal-serum, 16h lanes in C–F represent positive controls in which Adβ-gal (B-gal)-infected cells were serum-stimulated, and cell lysates were prepared 16 h after serum stimulation. D, Western blot showing the phosphorylation status of p130. The cell lysates were analyzed as described for C using anti-p130 monoclonal antibody (Santa Cruz Biotechnology catalog no. sc-317). E, Western blot showing the phosphorylation status of p107. Cell lysates were analyzed as described for C using anti-p107 monoclonal antibody (Santa Cruz Biotechnology catalog no. sc-318). The phosphorylated forms of pRb, p130, and p107 are shown by arrows in C–E. F, cyclin D kinase activity in serum-starved AS-CBP-infected cells. The cyclin D-Cdk complex was immunoprecipitated using anti-cyclin D antibody (Santa Cruz Biotechnology catalog no. sc-753), and the kinase activity in the immunoprecipitated complex was assayed using a recombinant truncated Rb protein as a substrate (see “Experimental Procedures”). G, cyclin E kinase activity in serum-starved AS-CBP-infected cells. The cyclin E-Cdk complex was immunoprecipitated using anti-cyclin E antibody (Santa Cruz Biotechnology catalog no. sc-248), and the associated kinase activity was determined using histone H1 as a substrate as described under “Experimental Procedures.” H, Western blot showing the levels of c-Myc protein in cell lysates. Serum-starved cells were infected with antisense or Adβ-gal vectors as shown in A. At the indicated time points, 60 µg of protein from each cell lysate was Western-immunoblotted on an SDS-7.5% polyacrylamide gel using anti-c-Myc monoclonal antibody 9E10 (gift of J. DeCaprio). These data are taken from Fig. 8A after cropping, and tubulin levels are shown in Fig. 8A.
phase would allow them to progress into G2/M and complete the cell cycle. Accordingly, cells were serum-starved for 36 h and infected with antisense vectors or Ad\(\text{H9252}\)-gal, and starvation was continued for another 18 h as described above (Fig. 5A). Cells were then stimulated with serum, and the distribution of cells in G1, S, and G2/M was quantified by FACS analysis as described under “Experimental Procedures.” The results were highly reproducible in two independent experiments. D, reversal of phosphorylation of p107 and p130. The details of this experiment are described in the legend to Fig. 3 (D and E). The arrows indicate the phosphorylated forms.

Fig. 4. Reversal of S phase induction and p107 and p130 phosphorylation by antisense c-Myc. A, time course of starvation, infection, and harvesting of cells. B, reversal of luciferase activity in response to c-Myc activation in serum-starved AS-CBP-infected cells by AS-Myc. Cells serum-starved for 36 h were co-infected with AS-CBP and AS-Myc, superinfected 1 h later with AdM4, and harvested at the indicated times, and the luciferase (Luc) activity was determined. The Ad\(\beta\)-gal (B\(\beta\)-gal) control vector was included where appropriate to maintain a constant multiplicity of infection. Assays were done in triplicate, and the means \(\pm\) S.D. are shown. AU, activity units. C, reversal of CBP depletion-dependent S phase induction by AS-Myc. Cells were serum-starved, infected with the indicated vectors, and harvested as shown in A. The percentage of cells in G1, S, and G2/M was quantified by FACS analysis as described under “Experimental Procedures.” The results were highly reproducible in two independent experiments. D, reversal of phosphorylation of p107 and p130. The details of this experiment are described in the legend to Fig. 3 (D and E). The arrows indicate the phosphorylated forms.

Fig. 5. Effect of serum stimulation on cell cycle progression of serum-starved cells expressing antisense p300- or CBP-specific sequences. A, time course for starvation, infection, stimulation, and harvesting of AS-p300- and AS-CBP-infected cells. B, quantification of S phase-specific cells after serum stimulation. Cells infected with Ad vectors were harvested at the indicated times, stained with propidium iodide, and analyzed by flow cytometry as described under “Experimental Procedures.” C, quantification of cells in G2/M phase after serum stimulation. Data shown are from a representative experiment that was repeated twice. B-gal, Ad\(\beta\)-gal.
to 29 h (Fig. 5C). After ~29 h, the number of cells in the G2/M phase fraction began to decline, with a corresponding increase in the G1 phase fraction (data not shown). This pattern of accumulation was comparable with that observed for mock-infected cells, indicating that the Ad-p300-infected cells cycled normally. However, the distribution of antisense-expressing cells during these time points was dramatically different. For example, significantly more antisense-expressing cells appeared in S phase at 14 h after serum stimulation compared with control cells and continued to accumulate, reaching ~50% by 35 h (Fig. 5B). Surprisingly, unlike control cells, even after prolonged serum stimulation, neither antisense p300- nor CBP-expressing cells accumulated in the G2/M fraction (Fig. 5C), indicating that these cells were unable to traverse beyond S phase. These results suggested to us that a substantial number of the antisense-expressing cells did not survive beyond S phase and might be undergoing apoptosis.

**A Considerable Portion of p300- or CBP-depleted Cells Die of Apoptosis after Serum Stimulation**—To determine whether antisense-expressing cells die of apoptosis after serum stimula-
tion, annexin V binding assays were carried out to assess the loss of phospholipid asymmetry of the plasma membrane that occurs during the initial stages of apoptosis (32). Cells were also assayed for the loss of mitochondrial membrane potential that is characteristic of apoptosis by staining cells with the fluorescent stain trimethyl-X-rosamine (33). The percentage of antisense-expressing cells undergoing apoptosis after serum stimulation is further induced upon serum starvation in antisense-expressing cells (Fig. 6). The presence of high levels of c-Myc in cells deprived of growth factors and a further increase in its levels induced as a result of p300/CBP depletion would lead to non-physiological levels of c-Myc in cells, which may contribute to the apoptotic effects. Thus, we considered it possible that c-myc that is already induced in the serum-starved antisense-expressing cells is further induced upon serum stimulation. The presence of high levels of c-Myc in cells deprived of growth factors and a further increase in its levels after serum stimulation would lead to non-physiological levels of Myc in the cell, which may contribute to the apoptotic effects. We show below that c-myc induced as a result of p300/CBP depletion in antisense-expressing cells was further induced by serum stimulation.

First, increases in c-Myc RNA levels in antisense-expressing
cells were determined by Northern blot hybridizations. Cells were serum-starved for 36 h and infected with AS-CBP, AS-p300, or Adβ-gal, and serum starvation was continued. After a total of 54 h of starvation (18 h after antisense virus infection), one set of plates was stimulated with serum, and the other set was maintained under starvation conditions (schematically shown in Fig. 7A). Two and 4 h after stimulation, serum-stimulated and unstimulated cells were harvested, and poly(A)⁺ RNA was isolated and subjected to Northern blot hybridizations using a human c-myc probe. Fig. 7B shows that Myc RNA levels began to rise by 18 h post-infection in AS-CBP- and AS-p300-infected cells in the absence of serum (0-h time point) (Fig. 7B). At this time point, Myc RNA was barely detectable in control cells. Myc RNA levels began to rise by 2 h after serum stimulation in cells infected with Adβ-gal. Quantitation of RNA bands of the autoradiogram using a densitometer indicated that the RNA levels in unstimulated AS-p300- and AS-CBP-infected cells increased by 2–3- and 4–5-fold, respectively, compared with the serum-stimulated Adβ-gal-infected cells. After serum stimulation, the antisense-expressing cells showed a further increase of 2–3-fold in Myc RNA levels compared with those without serum stimulation (Fig. 7B, compare the 0, 2, and 4 lanes in the Unstimulated panels with those in the Stimulated panels). These RNA levels were much higher than those observed in Adβ-gal-infected cells at these time points and were also higher than those found in antisense-expressing cells without serum stimulation. Thus, we conclude that c-myc induced in serum-starved p300/CBP-depleted cells is further induced upon serum stimulation.

Next, by Western immunoblotting, we determined the Myc protein and activity levels in MCF10A cells infected with AS-p300, AS-CBP, and Adβ-gal viruses under serum-starved and serum-stimulated conditions. A, Western blots showing c-Myc protein levels. Serum-starved cells were infected with antisense and Adβ-gal (B-gal) vectors and stimulated with serum as shown in Fig. 7A. At the indicated time points, 60 µg of protein from each cell lysate was Western-immunoblotted on an SDS-7.5% polyacrylamide gel using anti-c-Myc monoclonal antibody 9E10. Tubulin levels were determined by reprobing the membrane used for Myc analysis with anti-tubulin antibody (Sigma catalog no. T5168). The numbers in parentheses refer to hours after vector infection. B, assay of Myc activity in cells expressing antisense CBP sequences. Quiescent cells infected with AS-CBP and AdM4 were serum-stimulated, and the luciferase (LUC) activity in starved and stimulated cells was assayed at the indicated time points using 5 µg of protein as described under "Experimental Procedures." AU, activity units. C, assay for Myc activity in cells infected with AS-p300 with and without serum stimulation. Assays in both B and C were carried out in triplicate, and the means ± S.D. are shown. This experiment was repeated twice with highly reproducible results.
protein levels in cell lysates prepared at the time points described above (see Fig. 7A), and the data are shown in Fig. 8A. The numbers in parentheses refer to hours after vector infection, whereas the numbers 0, 2, 4, and 6 refer to hours after serum stimulation, with 18 h after vector infection as 0 h (see Fig. 7A). These results show that in serum-starved AS-CBP-infected cells, c-myc was induced transiently beginning at 20 h post-infection (Fig. 8A, lanes 2, 4, 8, and 12), whereas Myc was barely detectable in control samples (lanes 1, 3, 7, and 11). However, when these cells were stimulated with serum, c-myc was induced in control cells as expected (2-, 4-, and 6-h samples; lanes 5, 9, and 13, respectively). Importantly, there was a further increase in Myc levels in samples corresponding to AS-CBP-expressing cells at 2, 4 and 6 h after serum stimulation compared with AS-CBP-expressing cells not treated with serum (compare lanes 6, 10, and 14 with lanes 4, 8, and 12). Thus, serum-stimulated AS-CBP-infected cells contained the highest levels of Myc compared with any other vector-infected cells, indicating that even though c-myc was already induced in antisense-infected cells before serum stimulation, they remained responsive to serum stimulation.

To determine whether the induction pattern of c-myc correlates with its transcriptional activation activity, an experiment identical to that described above was performed, but including AdM4 infection following AS-CBP infection as shown in Fig. 2A. Luciferase activities in the stimulated and unstimulated cells at 0 and 4 h (time points in relation to serum stimulation) were assayed. As shown in Fig. 8B, at 4 h after serum stimulation, the luciferase activity in control samples (Adβ-gal infection) increased by 2.5-fold compared with that in unstimulated control cells (compare bars 1 and 3 at the 4-h time point). At this time point, the luciferase activity in the unstimulated antisense-expressing cells had already increased by ~2.5-fold compared with that in the unstimulated control cells (compare bars 1 and 2 at the 4-h time point). Upon serum stimulation, the Myc activity of the AS-CBP-infected cells further increased by another 2-fold (compare bars 2 and 4 at the 4-h time point). This would translate into an overall increase of 2.5-fold in Myc activity in the serum-stimulated antisense CBP-expressing cells compared with the unstimulated antisense-expressing cells and an 8-fold increase compared with the unstimulated control cells. This pattern of increase in Myc activity was also observed 10 h after serum stimulation (data not shown). This experiment was repeated using the AS-p300 vector. The results shown in Fig. 8C indicate that cells infected with AS-p300 also showed a similar pattern of increase in Myc activity, with the highest level of Myc activity in serum-stimulated AS-p300-infected cells. These results were reproducible in at least three independent experiments. In summary, these results show that the c-myc induction pattern was similar in both antisense AS-CBP- and AS-p300-expressing cells and that both cells contained higher levels of Myc after serum stimulation compared with serum-stimulated control cells undergoing normal G1-S transition.

Antisense c-Myc Reverses Apoptosis of CBP-depleted Cells—If the superinduction of c-myc observed in serum-stimulated p300/CBP-depleted cells contributes to apoptosis, then inhibition of c-myc expression should reverse this effect. To test this prediction, serum-starved cells were infected with AS-CBP and with AS-CBP and AS-Myc along with appropriate control infections as shown in Fig. 9B (Fig. 9A shows the time course of infection and harvesting). Cells were harvested at 8 and 16 h after serum stimulation, and the percentage of apoptotic cells in the total population was determined by annexin V binding assays as described under “Experimental Procedures.” As shown in Fig. 9B, at 8 h after serum stimulation, ~7–8% of the

![Fig. 9](http://www.jbc.org/)
DISCUSSION

We previously showed that p300 plays an important role in the negative regulation of c-myc in the G1 phase of cell cycle. A decrease in p300 levels in G1 leads to induction of c-myc and DNA synthesis (26), whereas an increase in p300 levels leads to repression of c-myc and inhibition of DNA synthesis (27). Although p300 and CBP are highly homologous proteins and can substitute for each other in most assays (1), several previous studies indicated that there may be some unique functions that these two proteins provide in cell growth (summarized in the Introduction). These observations prompted us to ask whether CBP also negatively regulates c-myc and whether reduced synthesis of CBP in G1 would cause the cells to enter S phase prematurely. In this study, we showed that reduced synthesis of CBP also leads to induction of c-myc and that such cells escape G1 without growth factor stimulation. Thus, although both proteins are highly homologous and can substitute for each other in many functions, they do not appear to be redundant, and normal levels of both p300 and CBP are required to keep c-myc in a repressed state in G1.

To gain insight into the mechanism of induction of S phase in antisense-expressing cells, we determined the levels of p21, a general inhibitor of cyclin/Cdk activities (50), and the phosphorylation of three pocket proteins, pRb, p107, and p107, which sequester E2F (51). We showed that p21 levels are reduced in antisense-expressing cells before a rise in c-myc levels. The reduction of p21 levels might have contributed to the S phase induction, as cyclin-Cdk complexes are not under the influence of p21. Besides, p21 also has been shown to interact with proliferating cell nuclear antigen and to inhibit DNA replication (52). Therefore, decreased levels of p21 in antisense-expressing cells would provide a favorable environment for DNA synthesis. Down-regulation of the p21 genes most likely occurs at the transcriptional level, as we showed previously that p21 RNA levels are reduced in antisense p300-infected cells (26). Because p53 levels did not significantly change in serum-starved antisense-expressing cells (Fig. 10), coactivation of other transcription factors related to p21 gene activation might have been impaired (17). Furthermore, at later time points after antisense virus infection, high levels of Myc that accumulate in the cell also might contribute to repression of the p21 gene (53).

We have shown that in antisense-expressing cells, cyclin D/Cdk activity did not change, whereas cyclin E and A/Cdk activities were elevated to considerable levels. Furthermore, pRb was not phosphorylated, and p107 and p130 were phosphorylated in antisense-expressing cells at low levels. Because pRb is a major player in sequestering E2F, this suggests that only limited amounts of E2F might have been released from the repressor complexes, presumably by the phosphorylation of p107 and p130. Thus, E2F might play a limited role in the induction of S phase. For reasons that we cannot explain at present, even though there was a considerable induction of cyclin E and cyclin A kinases in antisense p300/CBP-expressing cells (Fig. 3G and data not shown) (26), there were negligible levels of pRb phosphorylation.

It is likely that induction of c-myc in p300/CBP-depleted cells is an initial event that leads to the phosphorylation of p107 and p130 and the induction of S phase. This is based on our observation that blocking the induction of c-myc in antisense CBP-expressing cells reversed S phase induction as well as phosphorylation of p107 and p130 (Fig. 4, C and D). These results are in agreement with our previous report in which we showed that inhibiting the induction of c-myc can reverse the induction of S phase in p300-depleted cells (26). Similarly, inhibition of G1 exit in p300-overexpressing cells as a result of repression of c-myc can be reversed by overexpression of c-myc (27). A number of recent reports suggest the existence of two pathways that
promote mitogen-stimulated G1-S transition. One mechanism involves induction of c-myc, which then transcriptionally targets several cell cycle-related genes, including cyclin E, cdc25A, and the genes that are responsible for DNA synthesis (reviewed in Refs. 54–57). In the second well understood mechanism, mitogenic signals inactivate Rb and release E2F, which then activate cyclin E, cdc25A, and other E2F target genes involved in DNA synthesis. Both these pathways operate independently and mutually cooperate in inducing DNA synthesis and coordinated cell cycle progression. The contribution of both these pathways is essential for efficient S phase induction (58–61).

Thus, we believe that in antisense-expressing cells, DNA synthesis is primarily due to the Myc pathway, and insufficient contribution by the Rb/E2F pathway likely results in inefficient S phase induction.

In this study, we have also shown that the antisense-expressing cells that accumulated in S phase in the absence of serum stimulation did not transit into G2/M phase. Serum stimulation of these cells also did not promote their transition from S to G2/M phase. In contrast, cell cycle progression was normal in cells infected with the Adβ-gal control. For many reasons, the antisense-expressing cells may have failed to transit from S to G2/M phase. For example, both p300 and CBP provide coactivator functions for a number of transcription factors, many of which are directly involved in the activation of genes that control DNA synthesis. These include activator protein-1 (cycR D1) (23), E2F (cycR E and A) (62), and activating transcription factor (cycR A) (63). These coactivators also acetylate nucleosomes of transcriptionally active promoters and stimulate transcription. The histone acetyltransferase activity of these proteins is also known to acetylate E2F and thereby increase its DNA binding and transcriptional activation activities (3). Other studies have suggested that the histone acetyltransferase activity of CBP increases during the G1-S transition (64). Therefore, lack of p300 or CBP, combined with lack of a contribution by the Rb pathway discussed above, would result in incomplete DNA synthesis, and such cells would not transit to G2/M phase.

We have shown that a significant number of serum-starved cells expressing antisense p300/CBP sequences underwent apoptosis upon serum stimulation. At 16 h after serum stimulation, the number of apoptotic cells increased by ~2-fold for antisense p300- or CBP-expressing cells compared with Adβ-gal-expressing cells (Fig. 6). We provided genetic evidence that apoptosis of the CBP-depleted cells could be reversed if expression of c-myc is blocked by antisense c-Myc sequences (Fig. 9). This shows that c-Myc plays an important role in the induction of apoptosis in CBP-depleted cells. A number of studies have shown that high levels of c-Myc can cause apoptosis (reviewed in Refs. 57, 65, and 66). In serum-starved antisense p300- and CBP-expressing cells, c-myc was induced at considerable levels. This induced c-myc was further induced upon serum stimulation (Fig. 7 and 8). For example, at 4 h after serum stimulation, the AS-p300- and AS-CBP-infected cells contained ~3-fold higher levels of c-myc activity compared with serum-stimulated control cells undergoing normal cell cycle progression (Fig. 8, B and C). It is conceivable that the inappropriate induction of c-myc and the unscheduled DNA synthesis that occur in serum-starved cells prime these cells for apoptosis. Further induction of c-myc by serum and perhaps other yet to be identified factors contribute to apoptosis. This interpretation is consistent with the view that induction of apoptosis by E1A, Myc, or E2F is the result of a conflict between the growth-promoting action of the oncprotein and simultaneous growth inhibitory signals such as low serum (67, 68). We believe that p53 does not play a role in this apoptosis, as the levels of p53 in antisense-expressing cells dropped beginning at 4–6 h after serum stimulation (Fig. 10B). This is consistent with published studies suggesting that c-myc can induce apoptosis in certain cell types by both p53-dependent and p53-independent mechanisms (69–71). Other studies have shown that during myc-induced apoptosis, Myc targets the pro-apoptotic gene bax, which mediates apoptosis (72, 73). Western blot experiments did not detect a significant increase in the levels of Bax protein in antisense-expressing cells after serum stimulation (data not shown). Thus, at the present time, we do not know the Myc target genes in antisense-expressing cells that may be responsible for apoptosis.

In summary, normal levels of both p300 and CBP are essential to keep c-myc in a repressed state in G1, and to prevent inappropriate DNA synthesis. Similarly, normal levels of both these proteins are also needed in S phase. Previously knockout mice studies showed that embryos lacking p300 are significantly smaller than control embryos (5). In vivo bromodeoxyuridine labeling studies showed a striking reduction of the number of cells in S phase in the embryos of these mice (5). Fibroblasts derived from p300−/− mice also grow slowly and cease to divide after a few generations (5). These data indicate a defect in cell cycle progression in these mice and are in agreement with the data presented here.

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Effects of Depletion of CREB-binding Protein on c-Myc Regulation and Cell Cycle G1-S Transition
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