Cell Cycle-dependent Regulation of the Cyclin B1 Promoter*

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Cyclin B1 mRNA expression varies through the cell cycle with its peak in G2/M. In cycling mammalian cells, its lowest level is in G1, with a steady increase in S until a level 50-fold greater than that in G1 is reached. In order to characterize the transcriptional component to this variation in expression, we cloned the upstream region 872 base pairs upstream from the start site of the cyclin B1 gene and have demonstrated that it confers cell cycle-dependent regulation onto two reporter genes, both chloramphenicol acetyltransferase and luciferase. Its activity was 25-fold greater in G2/M than in G1 in HeLa cells with intermediate activity in S. This cyclical activity could be seen with sequences encompassing only 90 base pairs upstream from the start site. Protein binding to this region was demonstrated using electrophoretic mobility shift assays, and the binding profiles appeared to vary depending upon the phase of the cell in which the extracts are made. Thus, transcriptional control plays an important role in determining cyclin B1 mRNA levels, and cell cycle-dependent activity is regulated through interactions with the region 90 bases upstream from the start site.

The importance of the fluctuations of cyclin B1 mRNA in controlling the cell cycle raises the question of how cyclin B1 mRNA level is regulated. Understanding cyclin B1 mRNA regulation could be important in understanding the mechanisms leading to cell cycle control in G2/M. Both transcriptional and post-transcriptional controls are likely to be involved. Pines and Hunter (7), using nuclear run-on assays, suggested that the transcription of cyclin B1 was increased in cells synchronized by thymidine-aphidicolin treatment in G2/M as compared to cells in G1. Maity et al. (9) showed that the stability of cyclin B1 mRNA also fluctuated through the cell cycle with a half-life of 1–2 h in G1, 8 h in S, and 13 h in G2/M. While changes in the cyclin B1 mRNA stability are clearly significant, they alone are not of sufficient magnitude to account for the variation in level of 50-fold that is seen in many cycling cells. To further investigate the role of transcription in the cell cycle regulation of the human cyclin B1 gene, we have cloned its 5′ upstream regulatory region and in this report describe its cell cycle-regulated promoter activity. We have also defined a short region of the promoter that can confer cell cycle-regulated transcription and that shows variation in protein binding through the cell cycle as evaluated by electrophoretic mobility shift assays.

MATERIALS AND METHODS

Library Screening, Polymerase Chain Reaction Amplification, and DNA Sequence Analysis—A human placental genomic library cloned in the Lambda FIX II vector (Stratagene) was screened using a full-length (1.4-kilobase) human cyclin B1 cDNA as a radiolabeled probe. We obtained a positive insert of approximately 20 kilobases. From this clone, we polymerase chain reaction-amplified a 949-bp clone using 5′ upstream and coding sequences of the human cyclin B1 gene as primers. This 949-bp clone was sequenced using the dideoxynucleotide chain termination method (10). It includes 872 bp upstream from the transcriptional start site identified by Pines and Hunter (7). Piaggio et al. (11) and Cogswell et al. (12) have also identified start sites at slightly different positions from that of Pines and Hunter (7). We have used the designation of Pines and Hunter (7) to position our clones numerically.

The 949-bp fragment was subcloned into pCRII (Invitrogen) using T4 DNA ligase. The 949-bp clone was also generated with polymerase chain reaction using sequences at PstI and 3′ KpnI sites. The cyclin D1-CAT and DNA polym- erase α-CAT plasmids were obtained from Bruno Calabretta (Thomas Jefferson University) (14). The hTK-CAT and histone H3.2-CAT plasmids were obtained from Amy S. Lee (University of Southern California) (15).

Cell Culture, DNA Transfection, and Cell Synchronization—HeLa cells were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37°C in 5% carbon dioxide. The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U36898.

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Cells were grown to 50–70% confluence and then transfected
using the calcium phosphate method (10). Briefly, 30 μg (for 100-mm
dishes) or 18 μg (for 60-mm dishes) of DNA in TE (10 mM Tris-HCl, pH
8.0, 1 mM EDTA) and 250 μL CaCl₂ was added dropwise to 2 ×
HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄,
12 mM dextrose, 50 mM HEPES). The calcium phosphate-DNA comple-
cix was allowed to form for 30 min before being added dropwise
to cells. The following day, the calcium phosphate-DNA coprecipitate
was removed, and the cells were washed twice with serum-free media.

For the experimental assay, we tested several other promoters
specific for the cyclin B1 upstream sequences and not an artifi-
cial promoter, pCAT-CONTROL was similarly trans-
ferred into HeLa cells which were then blocked in different
phases of the cell cycle. The activity of pCAT-BASIC was much
stronger in cells arrested in S phase by thymidine, 2 mM for 16 h; or
aphidicolin, 1 μg/ml for 16 h. Cells were then harvested using Reporter
Lysis Buffer (Promega), and light intensity was measured for 10 s on a Berthold
L9590 luminometer.

Flow Cytometry and Cell Cycle Analysis—Cells were collected and
resuspended in 40 mM citrate buffer and 5% dimethyl sulfoxide and
ixed in TGE buffer (25 mM Tris, 200 mM glycine, 1 mM EDTA). The gels were
screened as described by Schreiber et al. (18) from HeLa cells that were
treated with thymidine-aphidicolin, mimosine, or nocodazole. Oligonu-
ucleotides were prepared as described by Schreiber et al. (18) from HeLa cells that were
treated with thymidine-aphidicolin, mimosine, or nocodazole. Oligo-
ucleotides corresponding to the minimal 90-bp cyclin B1 promoter were
amplified and 32P-end-labeled with T4 kinase. DNA-protein complexes
were formed in a 20 μL reaction mixture containing 20 mM HEPES pH
7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% (v/v) glycerol, 1 mM
dithiothreitol, 1 μg/ml phenylmethylsulfonyl fluoride, 10 μM leupeptin,
1 μg/ml pepstatin A, 1 μg/ml aproitin, 2 ng (40 fmol) of the labeled oligonucleotide, 3 μg of extract, and 300 ng of salmon sperm DNA
(nonspecific competitor) for 20 min at room temperature. The DNA-
protein complexes were run on 5% nondenaturing polyacrylamide gels
in TGE buffer (25 mM Tris, 200 mM glycine, 1 mM EDTA). The gels were
then dried, and autoradiography was performed with an intensifying
screen.

RESULTS

Cloning of the 5' Upstream Region of the Cyclin B1 Gene—To isolate
the 5' upstream sequences of the human cyclin B1 gene, we
screened a human placental genomic library with the hu-
man cyclin B1 cDNA as a probe. From this clone we subcloned
a 949-bp fragment that comprised 872 bases upstream of the 5'
start site and extended into the transcribed portion of the
human cyclin B1 gene. Fig. 1 shows the sequence of this clone. Several
consensus sequences characteristic of eukaryotic promoter
 elements are present. At —25 upstream from the transcriptional
start site published by Pines and Hunter (7), there is a "TATA-
like" element and at —60 there is a CAAT box. Further up-
stream at —135 there is an Sp1 consensus element.

Promoter Activity of the 5' Upstream Region of Cyclin B1—In order to test the upstream sequences for promoter activity, we
placed the 5' upstream region of the cyclin B1 gene into a
chloramphenicol acetyltransferase (CAT) reporter vector,
pCAT-BASIC, a plasmid that has no promoter or enhancer
sequences. The plasmid containing the 5' upstream sequences,
pCAT-upB, was then transfected into HeLa cells using the
calcium phosphate method. The transfected cells were treated
with various drugs to arrest them in different phases of the cell
cycle: nocodazole to block in G2/M phase, mimosine to block in
late G1 phase, or thymidine to block in S phase. Fig. 2 shows
representative flow cytometry profiles of cell cycle position as
determined by DNA content after treatment with these drugs.

After a 24-h treatment with nocodazole, 85–90% of the cells were
arrested at the G2/M boundary. Mimosine, a plant amino
acid that has been shown to arrest cells in late G1 (19, 20),
blocked 65–75% of the cells in G1 in the presence of thymidine.
Thymidine blocked 55–65% of the cells in S phase as deter-
mined by flow cytometry after a 16-h treatment. Those cells
blocked in G2 at the G2/S interface will appear as G1 cells by
this type of analysis. Following cell cycle arrest for 16 or 24 h,
the cells were harvested and assayed for CAT activity. A repre-
sentative experiment is shown in Fig. 3. The transfected cells
arrested in G2 by nocodazole had 11–26-fold greater CAT
activity than cells arrested in late G1 by mimosine. Cells ar-
rested in S phase by thymidine had an intermediate level.
pCAT-BASIC had essentially no activity in the transfected cells
in any phase. A CAT vector with the SV40 early promoter
and enhancer sequences (pCAT-CONTROL) was similarly trans-
ferred into HeLa cells which were then blocked in different
phases of the cell cycle and showed no variation in activity with
the cell type. The activity of pCAT-upB in nocodazole-arrested
was essentially equivalent to that of pCAT-CONTROL
indicating that cyclin B1 promoter activity at that phase of the
cycle was strong.

To be sure that the cell cycle differences we observed were
specific for the cyclin B1 upstream sequences and not an arti-
factual of the experimental assay, we tested several other prom-
ters in the same system. We transfected plasmids containing
the human DNA polymerase α, the human cyclin D1, the
human thymidine kinase, or the hamster histone H3.2 promoters
(14, 15) upstream of the CAT reporter gene into HeLa cells and
then treated the cells with either nocodazole, mimosine,
or thymidine. Each of these promoters has been characterized as
being specific for S phase (14, 15). The human DNA polymerase
α and the human cyclin D1 promoters have both been charac-
terized as being dependent upon E2F activation (14, 21–24).
Since HeLa cells are effectively deficient for the retinoblastoma
protein (pRb) due to the binding of pRb to the E7 protein of
human papilloma virus (25), and the regulation of E2F trans-
activation is affected by its interaction with pRb, these cells
can not regulate E2F-activated genes in the same way as cells
having normal pRb activity (26). Cells deficient in pRb,
for example, bypass the requirement for cyclin D1 in G1 (26). In
Evaluating these experiments, it should also be noted that the CAT protein has a half-life of about 50 h (27). The long half-life can affect the interpretation of experiments because once a cell has synthesized the CAT protein, CAT activity would be detectable for the duration of the experiment. Since the majority of the cells in an exponentially growing population of HeLa cells are in either G₁ or S phase, the cells in S would lead to considerable synthesis of the CAT protein by an S phase-specific promoter. The CAT protein because of its long half-life would remain detectable as cells progressed into G₂/M. In contrast, few cells would be in G₂/M and spend little time at that point since it is the shortest component of the HeLa cell cycle. Thus, this method should clearly distinguish transcription in G₂/M from that in G₁ or S, but would not be expected to clearly separate transcription in G₁ from S phase.

After transient transfection and cell cycle blockade, the S phase-specific promoters showed 1.3–3.4 times more CAT activity in S phase than in G₁ and slightly more CAT activity in S than in G₂/M, as shown in Fig. 4. In contrast, however, the cyclin B1 upstream region vector had markedly higher CAT activity in G₂/M compared to other phases of the cell cycle. Thus, it is unlikely that the cell cycle-regulated activity of the cyclin B1 promoter is an artifact of our experimental assay or of the drugs used to induce blockage in the cell cycle. These experiments indicate that the upstream region of the human cyclin B1 gene has promoter activity that is low in G₁, high in S, and maximal at G₂/M.

To further confirm the cell cycle-regulated activity of the cyclin B1 promoter, we subcloned the cyclin B1 promoter upstream of the luciferase reporter gene in the pGL2-BASIC vector (pGL2-upcB). While the CAT mRNA has a short half-life, its protein has a long half-life of 50 h. In contrast, both the luciferase mRNA and protein have short half-lives. The half-life of the luciferase protein is approximately 3 h (27). Thus, we could transfect a luciferase expression vector driven by the cyclin B1 promoter, follow the cells as they cycled, and assess the activity of the cyclin B1 promoter throughout the cell cycle by monitoring luciferase activity. These experiments did not depend upon pharmacological blockade of the cell cycle. HeLa
cells were cotransfected with pGL2-upcB and pSVβ-gal (as an internal control for transfection efficiency) and treated with aphidicolin for 16 h. The cells were then released from the aphidicolin block, and cell lysates were assayed for luciferase and β-galactosidase activity and by flow cytometry for DNA content. As shown in Fig. 5A, the cells were predominantly in G1 at the start of the experiment. At this point, the level of luciferase activity was low. As the cells entered S phase, luciferase activity rose. Luciferase activity peaked (at 9 times greater than the level at the time of release) 10 h after release when the majority of cells were in G2/M. Once the cells exited mitosis and re-entered G1, luciferase activity began to drop. At 21 h after release, only 11% of the cells remained in G2/M while 62% were in G1. At this point, the luciferase activity had fallen to a level only twice that of the level at the time of release from the aphidicolin block. This decline in luciferase activity was not a result simply of activity declining with time after transfection, but was a result of cell cycle progression. This can be seen from the experiment shown in Fig. 5B. Some of the transfected synchronized cells from the experiment shown in Fig. 5A were also treated with nocodazole. By blocking the cells in G2/M and following them with time, we could assess the effect that increasing time after transfection had on the luciferase activity. As shown in Fig. 5B, nocodazole blocked the cells in G2/M, yet luciferase activity remained high throughout the remainder of the experiment. Thus, the cyclin B1 promoter conferred cell cycle variation to the expression of the luciferase reporter gene. It resulted in the lowest level of luciferase activity in G1, an increasing level in S phase, and the maximal level in G2/M corresponding to a pattern paralleling the level of cyclin B1 mRNA in cycling cells.

Identification of a Minimal Promoter Sequence—To map the sequences responsible for conferring the cell cycle-regulated transcription of cyclin B1, we generated a series of plasmids containing smaller fragments of the cyclin B1 promoter. The full-length upcB construct was further truncated to generate fragments that are 207, 140, and 90 bp upstream of the transcriptional start site. All of these constructs included the putative TATA element and the CAAT box. The −207 and −140 constructs include the Sp1 consensus site, whereas the −90 construct does not. These constructs were then subcloned into pCAT-BASIC and used in transient transfection assays as described in Fig. 3. As shown in Fig. 6, the −207 construct was found to have 7-fold greater CAT activity in transfected cells blocked in G2/M compared to transfected cells blocked in late G1. The −140 and −90 constructs were found to have 16-fold greater CAT activity in transfected cells blocked in G2/M compared to transfected cells blocked in late G1. These results indicate that a fragment as small as 90 bp upstream of the transcriptional start site of the human cyclin B1 gene is suffi-
Promoter activity of truncations of the cyclin B1 promoter. The full-length cyclin B1 promoter (~872) was truncated to generate fragments of 207, 140, and 90 bp upstream of the transcriptional start site and subcloned into a promoterless CAT vector (pCAT-Basic). HeLa cells were transfected with these truncated cyclin B1 promoter-CAT plasmids and treated with nocodazole to block in G2/M or mimosine to block in G1. Cells were then harvested and assayed for CAT activity indicated as counts/min.

Electrophoretic Mobility Shift Assays Using the Minimal Cyclin B1 Promoter—In order to search for proteins that may be important for the cell cycle-regulated transcription of the human cyclin B1 gene, we performed electrophoretic mobility shift assays using the cyclin B1 promoter as the DNA probe. The 90-bp region of the cyclin B1 promoter that was found to be sufficient for conferring cell cycle-regulated activity was 32P-end-labeled and incubated with nuclear extracts prepared from HeLa cells that were synchronized and harvested when the majority of cells had exited from mitosis into G1 (early G1), cells that were blocked in G1 with mimosine (late G1), or blocked in G2/M with nocodazole. As shown in Fig. 7, four bands were seen when the 90-bp fragment of the cyclin B1 promoter was incubated with G1 extracts. The arrow indicates the position of a fainter, but reproducible band that was seen when extracts were made from late G1 cells, but not from extracts of early G1 cells. When the assay was performed using extracts from nocodazole-blocked cells, only three faint bands were seen. This suggests that there may be inhibitory protein(s) present during G1 that down-regulate transcription of cyclin B1.

**DISCUSSION**

In this study, we have described the isolation of the 5’ upstream sequences of the human cyclin B1 gene, and, using two different experimental assay systems, we have shown that these sequences show promoter activity that is strongly cell cycle-regulated. Its activity was lowest in G1, rose to an intermediate level in S phase, and reached a peak in G2/M. This activity parallels the pattern of expression of the cyclin B1 mRNA. We found that the cyclin B1 promoter was 11-26 times more active in G2/M than in G1. Previous studies have shown that the level of cyclin B1 mRNA present during G2/M is up to 50-fold greater than the amount of mRNA present in G1. Data from our laboratory have shown that variations in cyclin B1 mRNA stability during the cell cycle contribute to the vast differences in the cyclin B1 mRNA level. The half-life of the cyclin B1 message is 13 h in G2, and 2 h in G1. The cyclical activity of the cyclin B1 promoter, along with the differences in the cyclin B1 half-life, suggest that both changes in the rate of transcription and changes in mRNA stability are responsible for the cell cycle-regulated expression of this gene.

Piaggio et al. (11) have recently cloned the upstream region of the human cyclin B1 gene. They found that the upstream region had promoter activity that was minimal in quiescent or G0 cells, but increased in activity gradually after serum stimulation. However, they were unable to show any cell cycle-specific regulation of activity. In their experiments, the activity of the cyclin B1 promoter did not fall following mitosis and entry into G1. This may have been due to their use of chloramphenicol acetyltransferase (CAT) as their reporter gene, since CAT has a half-life that is considerably longer than the cell cycle time of about 24 h in the cells they used. High levels of CAT protein present during mitosis would still be present once cells cycled back into G1.

Cogswell et al. (12) have also reported the isolation of a cyclin B1 promoter element using the upstream sequences from the cyclin B1 gene. They found that the activity of the cyclin B1 promoter was enhanced in vivo during G2/M as compared to S. Their experiments also use CAT as the reporter gene and hence do not show cyclical activity of the cyclin B1 promoter. In this study, Cogswell et al. (12) identified a region as being important for cell cycle regulation that is distal to the -90 region that we studied. This region contained a binding site for the upstream stimulatory factor and is required for in vitro transcription of the cyclin B1 gene. Using an electrophoretic mobility shift assay, they were able to identify upstream stimulatory factor binding to this region. This binding was greater in extracts from G2/M phase cells than in S phase cells, suggesting that upstream stimulatory factor stimulates transcription of cyclin B1 during G2/M. This is in contrast to our finding that suggests the presence of inhibitory protein(s) that repress cyclin B1 transcription during G1. Together these experiments suggest that regulation of the cyclical activity of the cyclin B1 promoter may be controlled by several regions and that their action may be redundant.

The isolation of the cyclin B1 promoter and its further characterization will provide us with a better understanding of how the cyclin B1 gene is regulated and thus how transcription may be triggered in a cell cycle-specific fashion. The Cdc25C promoter has been identified as being specific for G2/M transcription (28). Cdc25C dephosphorylates the inhibitory phospho-
rylations on p34\textsuperscript{cdc2} (cdk-1) and is required for activation of maturation promoting factor and for transition into M and G1 (3, 5). Its mRNA cycles with the highest levels in G2/M and the lowest in G1, yet in spite of the cyclical expression of the mRNA, the protein itself appears to be present in constant amounts through the cell cycle making the physiological significance of the cell cycle regulation ofcdc25C mRNA difficult to understand (29, 30). Thecdc25C promoter lacks a canonical TATA element (28). An element termed the cycle-dependent element (29, 30). The cycle-dependent element contains the sequence GC\textsuperscript{CGG} that was identified as important in the cycle-dependent element is located downstream of the transcriptional start site of cyclin B1 making it less likely to play a role in the regulation of cyclin B1, but not excluding that possibility.

The finding of promoters that are actively transcribed during G2/M raises another interesting question with respect to cell cycle-dependent transcription. Generally, transcription is turned off during mitosis at which point the chromosomes are condensed (31, 32). Since the cyclin B1 gene is maximally active during this phase of the cell cycle, there must be novel mechanisms that allow transcription of this gene to occur under conditions in which transcription generally is repressed. Further study of this promoter should reveal important insights into transcriptional control during chromosome condensation and perhaps will reveal regions protected from chromosome condensation.

Currently, the B-type cyclins are a well-characterized class of proteins, but the mechanisms underlying their transcriptional regulation are poorly understood. Although genes have been identified in yeast that regulate the transcription of cyclin B1, their mammalian homologs are unknown (33, 34). A trans-acting factor that acts positively during the G2/M transition or negatively during G1 has yet to be identified in mammalian cells. The existence of such proteins is likely given that the cyclin B1 promoter is maximally active during G2/M and given the differential patterns of gel retardation found through the cell cycle using portions of the cyclin B1 promoter.

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