MOLECULAR AND CELLULAR BIOLOGY, May 1995, p. 2782–2790 Vol. 15, No. 5
0270-7306/95/$04.00+0

Upstream Stimulatory Factor Regulates Expression of the Cell Cycle-Dependent Cyclin B1 Gene Promoter

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Received 21 October 1994/Returned for modification 21 December 1994/Accepted 17 February 1995

Progression through the somatic cell cycle requires the temporal regulation of cyclin gene expression and cyclin protein turnover. One of the best-characterized examples of this regulation is seen for the B-type cyclins. These cyclins and their catalytic component, cdc2, have been shown to mediate both the entry into and maintenance of mitosis. The cyclin B1 gene has been shown to be expressed between the late S and G_2 phases of the cell cycle, while the protein is degraded specifically at interphase via ubiquitination. To understand the molecular basis for transcriptional regulation of the cyclin B1 gene, we cloned the human cyclin B1 gene promoter region. Using a chloramphenicol acetyltransferase reporter system and both stable and transient assays, we have shown that the cyclin B1 gene promoter (extending to −3800 bp relative to the cap site) can confer G_2-enhanced promoter activity. Further analysis revealed that an upstream stimulatory factor (USF)-binding site and its cognate transcription factor(s) are critical for expression from the cyclin B1 promoter in cycling HeLa cells. Interestingly, USF DNA-binding activity appears to be regulated in a G_2-specific fashion, supporting the idea that USF may play some role in cyclin B1 gene activation. These studies suggest an important link between USF and the cyclin B1 gene, which in part explains how maturation promoting factor complex formation is regulated.

Cyclins are a family of related proteins which are present at specific stages of the somatic cell cycle (58). They function as regulatory subunits for cyclin-dependent kinases (cdks), which phosphorylate key substrates that mediate cell cycle transit (37, 43, 56, 58). Catalytic activation of the cdks requires sufficient accumulation of cyclin protein at particular stages of the cell cycle (41–43, 56). Dysregulation of cyclin gene expression through overexpression and/or unscheduled cdk activity results in inappropriate entry into the S or M phase and may be characteristic of some human cancer cells (7, 20, 26, 30, 31, 38, 45, 53, 60). The best-characterized cyclin-cdk complex is maturation-promoting factor, which consists of a B-type cyclin and cdc2 kinase (13, 17, 18, 33, 41, 56). The B-type cyclins (B1, B2, and B3), as well as cyclin A, have been implicated in control of the G_2/M transition (13, 16, 37, 38, 41, 42, 56, 58). The interaction between B-type cyclins and cdc2 during the G_2 cell cycle phase is necessary for cdc2 kinase activation, and the resultant phosphorylations mediate structural changes crucial for the G_2/M transition (12, 16, 37, 43). Activation of the cdc2 kinase does not occur until sufficient cyclin B protein has been synthesized (56), whereas proteolysis of cyclin B via ubiquitination at the end of mitosis is critical for entry into interphase (19). The accumulation of cyclin B protein, as with many other cyclins, is correlated with nascent-gene expression. Cyclin B mRNA can be detected in late S phase, peaks in late G_2 phase, and cannot be detected in M, G_1, or early S (41). Models that explain the regulation of cyclin B gene expression invoke both transcriptional and posttranscriptional control (41).

A key question is how cyclin gene transcriptional initiation is restricted to particular stages of the cell cycle. The recent cloning and initial promoter analyses of the cyclin A and D1 genes have provided some insights. These genes share features characteristic of housekeeping genes in that they lack a consensus TATA box and have multiple Sp1 factor DNA elements (2, 24, 28, 34, 40, 52, 62). However, their responses to particular cell cycle regulators such as E2F, Rb, c-Myc, and p53 may differ (24, 27, 28, 40, 62). For example, Rb has been shown to induce expression of cyclin D1 but not cyclin A (24, 36). The cyclin D1 and cyclin A genes each contain E2F sites, although the role of E2F in regulating these promoters has not been assessed (24, 34, 36, 62). A p53-like site may be a negative regulator of cyclin A transcription, although p53 repression of this gene has not been linked to its cognate binding site (62). c-Myc has received the most attention since its expression correlates with the proliferative status of the cell, and overexpression of c-Myc has been reported to repress cyclin D1 transcription and to activate cyclin E and A transcription (27, 28, 40). A recent analysis of the cyclin D1 promoter has shown that c-Myc antagonizes upstream stimulatory factor (USF)-mediated transactivation via a core promoter element called an initiator repeat (INR) (40). The way in which c-Myc transactivates the cyclin A promoter is currently being assessed.

In this paper we describe the genomic structure and promoter sequence of the cyclin B1 gene. We demonstrate that a minimal promoter specifies G_2-enhanced expression to a chloramphenicol acetyltransferase (CAT) reporter gene either stably or transiently expressed in HeLa cells. Further analysis revealed that a USF-binding site and its cognate transcription factor(s) are critical for cyclin B1 expression. Interestingly, USF-mediated transcription depends on active cell cycle progression. These studies suggest an important link between USF and the cyclin B1 gene, which in part explains how maturation-promoting factor complex formation is regulated.

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MATERIALS AND METHODS

Genomic clones and mapping. PCR primers against the 5′ end of the cyclin B cDNA were designed. Their sequences are as follows: PCR1, 5′-TGGTTTCTG CTGGGTGTAGGTCC-3′, PCR2, 5′-CCTGCCATGTTGATCTTCGC-3′. These primers were used to screen the DuPont Merck Pharmaceutical Co. Human Foreskin Fibroblast P1 library no. 1 P1 by Genome Systems Inc., St. Louis, Mo. Clones DMPC-HFF1-0657-B11 and DMPC-HFF1-1338-A12 were provided by Genome Systems, and both contained the cyclin B1 gene as determined by Southern blotting. Clone 1338-A12 was used for all further subcloning. Plasmid DNA was prepared by alkali lysis from 20 liters of cells and purified by fast protein liquid chromatography as described previously (8). Southern blotting was used to identify a 10-kb *Hin*dIII fragment (CB1), which contained the 5′ promoter sequence, and two additional overlapping fragments, a 3-kb *Kpn*I fragment (CB2) and an 8-kb *Bam*HI fragment (CB3), which contained the rest of the cyclin B gene. All three fragments were subcloned into pBluescript II SK+ (Stratagene, La Jolla, Calif.) by a shotgun approach involving colony hybridization with ^{32}P-labeled fragments of the cyclin B1 cDNA to identify positive clones. A restriction enzyme map was generated for each fragment. Nested primers spaced approximately every 100 to 200 bp within the cDNA were used to sequence across intron/exon boundaries in all three clones. A *Bam*HI-*Nco*I fragment containing 1,170 bp of 5′ sequence was generated by deleting the 5′ end (*Bam*HI-*Bam*HI) and 3′ end (blunt *Nco*I-*Hin*dIII) of the 10-kb *Hin*dIII fragment in pCB1-SK. The resultant construct, p(BamHI-NcoI)CB1-SK, was sequenced by primer walking along both strands (50). Consensus transcription factor-binding sites were identified by searching the cyclin B1 promoter sequence against the GenBank transcription factor database.

RNase protection assay. A riboprobe template was prepared by *Stu*I digestion of p(BamHI-NcoI)CB1-SK. A uniformly [^{32}P]UTP-labeled riboprobe was synthesized from the T7 promoter, and the full length product was purified after polyacrylamide gel electrophoresis on a 6% polyacrylamide gel. The probe was eluted in 0.5 M ammonium acetate–1 mM EDTA–0.2% sodium dodecyl sulfate, and 100,000 cpm bound to 50 μg of HeLa RNA (9) or to tRNA as a control. RNase digestion was performed with the RPA II kit (AMBION, Austin, Tex.) as described by the manufacturer.

Plasmid constructs. pCycB(−1050)-CAT, which contains the first 1,170 bp of the cyclin B1 sequence 5′ to the ATG in pCAT basic (Promega, Madison, Wis.), was generated in four steps. First, pCB1-SK was digested with *Bam*HI and religated to remove the 5′ promoter sequence to −1050. Second, this new clone was opened at the *Nco*I (translation start) site and digested with S1 nuclease to remove the ATG, the clone was then opened at the 3′ *Hin*cII polylinker site, and the blunt ends were religated. Third, a 3′ *Xho*I polylinker site was adapted to an *Xba*I site with a linker oligonucleotide. Finally, the clone was digested with *Xba*I to remove an 1,170-bp fragment (5′ polylinker site plus 3′ *Xba*I adapter) for cloning into the *Xba*I site of pCAT basic. Orientation was confirmed by restriction enzyme digestion and sequencing. pCycB(−290)-CAT was made from pCycB(−1050)-CAT by deleting a *Hin*dIII-*Pst*I fragment between the 5′ polylinker and −224 bp and then using oligonucleotides to reconstruct the sequence to −290. pCycB(−290 mut USF)-CAT was constructed in the same way, except that the CACGTG USF site was mutated to the *Bgl*II recognition sequence AGATCT. pCycB(PstI-BamHI)-SK was constructed by cloning a 2,750-bp *Pst*I-*Bam*HI fragment (−3800 to −1050 bp) from pCB1-SK into the identical sites of pBluescript II SK+. pCycB(−3800)-CAT was made by destroying the *Bam*HI site 3′ to the simian virus 40 poly(A) tail in pCAT basic, inserting the *Xba*I-*Xba*I fragment from pCycB(−1050)-CAT (5′ *Bam*HI site brought over from pBluescript II SK+), and inserting the 2,750-bp *Pst*I-*Bam*HI 5′ cyclin B1 promoter fragment as a *Hin*dIII-*Bam*HI fragment from pCycB(PstI-BamHI)-SK. For in vitro transcription assays, the G-less cassette plasmid pC2AT was used (44). Promoter deletion constructs were made by PCR amplification of the corresponding regions of pCycB(−1050)-CAT and subcloning the resultant products into pC2AT (52). G-less cassette vector was prepared by opening the 3′ *Sst*I site, blunt ending with T4 polymerase, and opening the 5′ *Eco*RI site. The 3′ amplimer sequence 5′-GAGAAGAGCCAG-3′ was designed so as not to encode a C residue on the noncoding strand and contained an *Eco*RV adapter for blunt-end ligation onto the *Sst*I site of the G-less vector. The five prime amplimers (listed with their corresponding plasmids) contained *Eco*RI adapter sequence to facilitate complementary end cloning onto the *Eco*RI site of the vector: pCycB (−414) GLC, 5′-GTGCAACACTAG-3′; pCycB (−258) GLC, 5′-CGCACTGG CTTC-3′; pCycB (−184) GLC, 5′-AGAGCCTGGCCA-3′; pCycB (−171) GLC, 5′-GCCTTCCGGC-3′; pCycB (−42) GLC, 5′-GGAGGGAGCAGT-3′; and pCycB (−17) GLC, 5′-CTGAGGCTAGGC-3′. The MLP plasmid contains the IVa2 promoter and the adenovirus major late promoter with a G-less cassette of 400 bases fused at nucleotide +11, relative to the adenovirus major late promoter (MLP) cap site. pAlb286 has been described previously (1). pRSVneo was obtained from the American Type Culture Collection (ATCC 37149), and p β-actin-CAT was a gift of Nevis Fregian, University of Miami, Miami, Fla. The human cyclin B1 cDNA was a generous gift of Tony Hunter, Salk Institute, San Diego, Calif.

Cells, transfections, and CAT assays. HeLa cells were transfected by electroporation with a Bio-Rad apparatus, using 3×10^6 cells per 0.4-cm cuvette and 5 μg of pCycB-CAT plasmid or 0.5 μg of pβ-actin-CAT. Stable cell lines were transfected with 10 μg of pCycB(−3800)-CAT and 1 μg of pSV2neo and rested for 24 h prior to selection for neomycin resistance with 500 μg of G418 per ml. Twenty-four colonies were picked for further expansion and testing. Six clones with low CAT expression were blocked in the G_1 or G_2/M phases of the cell cycle (see below), and their CAT expression was compared. Cells were assayed for CAT as described by Gorman et al. (21). Protein contents were made equivalent by the Bradford assay (Bio-Rad, Hercules, Calif.). Conversion of [^{14}C]chloramphenicol substrate was quantified with a radioanalytic scanner (AMBIS Systems, San Diego, Calif.). All transfections and CAT assays were confirmed in at least three independent experiments.

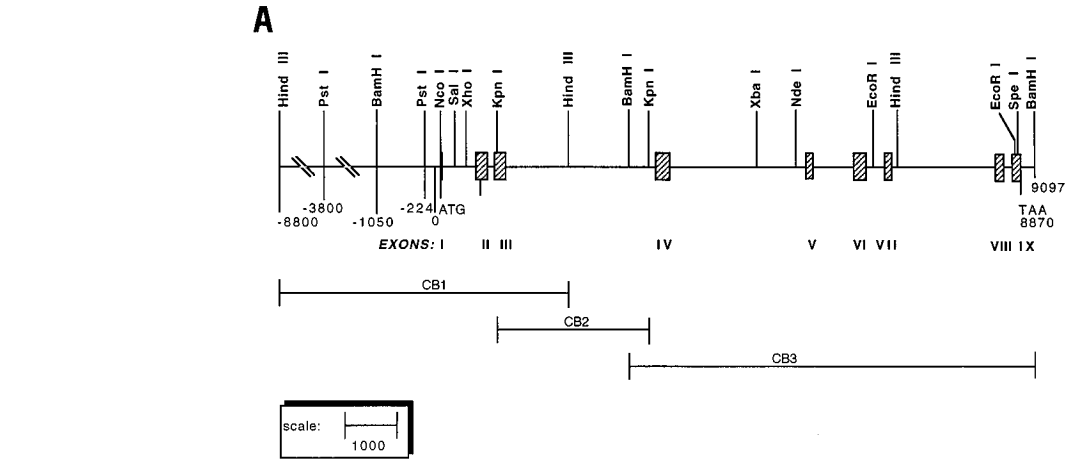
Cell cycle synchronization. Transiently transfected HeLa cells were synchronized in early S phase by a single thymidine block. For the single thymidine block, cells were transfected, rested for 6 h, and incubated in 4.0 mM thymidine for 40 h before harvest. Stable HeLa lines were synchronized in G_1 by a thymidine-aphidicolin block (23, 41). Briefly, cells were blocked for 16 h in 4.0 mM thymidine, released into fresh medium for 8 h, and reblocked with 5 μg of aphidicolin per ml for 16 h. Transiently transfected or stable HeLa lines were blocked in G_2/M by the thymidine-nocodazole method (41, 63). Briefly, transiently transfected HeLa cells were rested for 6 h (not necessary for stable cell lines), blocked with thymidine for 16 h as above, released for 4 h, and blocked overnight with 0.15 μg of nocodazole per ml for 20 h. Nonadherent cells blocked in prometaphase were isolated. The cells were harvested for CAT 48 h posttransfection. For flow cytometry analysis, HeLa cells were harvested for two-color cell cycle analysis by a modification of the protocol of White et al. (61). The modifications were as follows: (i) cells were incubated in 10 μM bromodeoxyuridine (Sigma, St. Louis, Mo.) for 1 h and afterwards fixed in 70% methanol; and (ii) IFA buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 150 mM NaCl, 4% fetal calf serum, 0.1% sodium azide) was substituted for PBTP, and Tween was added as indicated; the anti-bromodeoxyuridine-fluorescein isothiocyanate (Becton Dickinson, San Jose, Calif.) was diluted 1:5 with IFA buffer. Stained nuclei were analyzed on a Becton Dickinson FACSort machine.

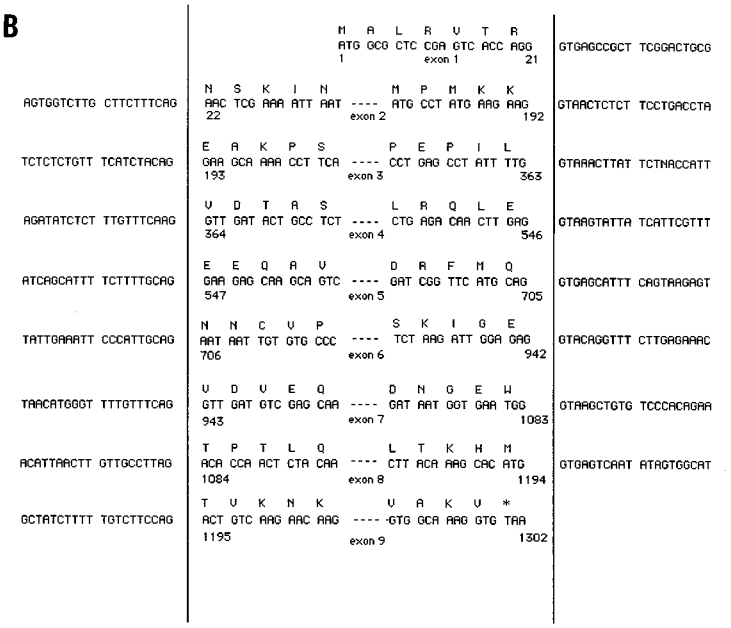
In vitro transcription reactions. In vitro transcription reactions were carried out as described previously (39, 52) with active HeLa nuclear extracts prepared by the method of Herbst et al. (25) or purchased from Promega. pALB286 was included in all transcription reactions as a reference for loading variation and for nonspecific oligonucleotide competition. Oligonucleotide competitors were preincubated with HeLa extract prior to addition of nucleoside triphosphates (NTPs) and *O*-methyl GTP, and the transcription products were separated by electrophoresis through a 6% polyacrylamide gel. The bands were quantitated with a Molecular Dynamics Computing Densitometer (model 300B).

Electrophoretic mobility shift assays. Nuclear extracts, labeled probes, and DNA-protein complexes were prepared and analyzed as previously described (3, 11), except that the binding buffer was modified to 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–1 mM dithiothreitol–DTT 1 mM EDTA (pH 7.5). The following oligonucleotides were used as probes and as competitors (consensus sites are italicized, and mutations are underlined): Sp1-3′, 5′-CGTTCGAGAGGGGCG GGGCCACAGTG-3′; USF/AP-2, 5′-GAGGCAGAC*CACGTG*GAGAGCCTG GCCAGGCCTTCC-3′; Myc, 5′-GTCGACCCCACC*ACGTG*GTGCCTGTCG-3′; MyoD, 5′-GATCCCCCC*AACTG*CTGCCTGA-3′; AP-2, 5′-GATCGAACT *GACCGCCC*GCGGCCCGT-3′; USF-WT, 5′-GGTGTAGGC*CACGTG*ACCG GG-3′; USF-MUT, 5′-GGTGTA<u>T</u>GC<u>T</u>AC<u>A</u>TG<u>G</u>CCGGG-3′; and CRE, 5′-AT TCGCCGTTAA*TGACGTCA*TGCTGATCAGAG-3′. Polyclonal antiserum raised against the C-terminal portion of the 43-kDa form of human USF1 (55, 59), which recognizes all USF family members, was kindly provided by Michele Sawadogo, M. D. Anderson Cancer Center, University of Texas. This antibody was diluted 1:30, and 1 μl was added to the binding reaction.

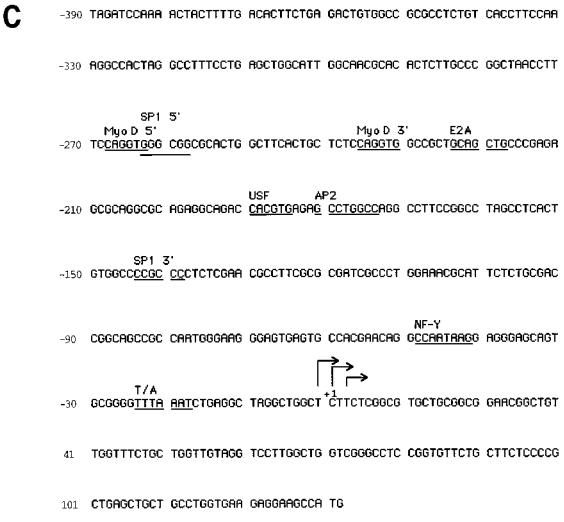
RESULTS

Isolation of the cyclin B1 promoter. PCR primers designed against the 5′ end of the cyclin B1 cDNA were used to screen the DuPont Merck Pharmaceutical Company Human Foreskin Fibroblast P1 Library no. 1 by Genome Systems Inc., St. Louis, Mo. Clones DMPC-HFF1-0657-B11 and DMPC-HFF1-1338-A12 were obtained, and both contained the cyclin B1 gene as determined by Southern blotting (data not shown). Clone 1338-A12 was used for all further subcloning. Southern blotting of restriction enzyme-digested human genomic DNA and P1 clone 1338-A12 was used to identify three overlapping fragments which encompassed the cyclin B1 gene: (i) CB1, a 10-kb *Hin*dIII fragment which contained largely promoter and some 5′ coding sequence; (ii) CB3, an 8-kb *Bam*HI fragment which contained the middle and 3′ end of the gene; and (iii) CB2, a 3-kb *Kpn*I fragment which provided an overlap between CB1 and CB3. All three CB fragments were subcloned, and they were mapped and oriented by restriction enzyme digestion (Fig. 1A). Primers were designed to sequence the intron-exon junctions (Fig. 1B), as well as the promoter region (Fig.





6Py N C/T AG | G SPLICE JUNCTION CONSENSUS C/A AG | GT A/G AGT



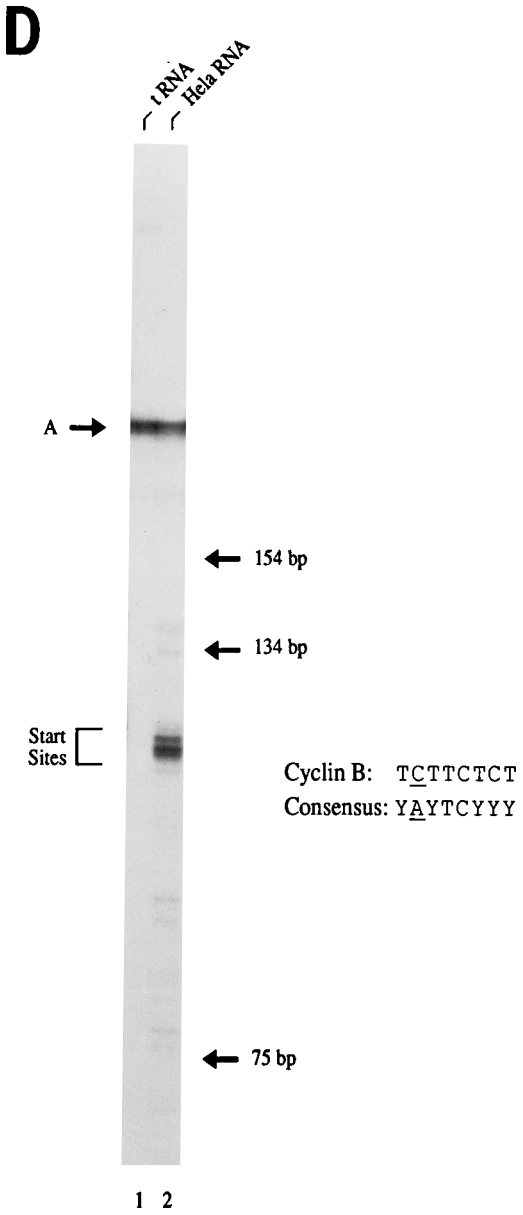


FIG. 1. Schematic of the cyclin B1 gene. (A) Genomic structure of the cyclin B1 gene. This cyclin B1 gene map shows unique restriction enzyme sites determined by digests and by sequencing. Hatched boxes represent exons, and lines represent either introns or promoter sequence. Genomic fragments, CB1, CB2, and CB3, used to map the gene are shown below. (B) Intron/exon structure. Intron/exon junctions are separated by lines, with flanking intron sequence shown outside of the lines and coding sequence shown inside. A consensus splice donor/acceptor sequence is shown at the bottom. (C) Promoter sequence. The cyclin B1 promoter sequence up to bp −318 is shown. Transcription factor-binding sites are underlined. Arrows mark the location of the transcription start sites determined by RNase protection. (D) CAP site mapping. An RNase protection with tRNA control (lane 1) or 50 μg of HeLa RNA (lane 2) is shown. The three closely spaced bands which are present in lane 2 but absent from lane 1 mark the transcription start site(s). Transcription starting from the middle band most closely matches the consensus initiator repeat (INR) sequence as defined by Roy et al. (49). A is an RNase-resistant band seen in all lanes. The actual probe size is 439 bp (data not shown).

1C). The genomic organization of the cyclin B1 gene consists of nine exons and eight introns. The sizes of the introns were mapped by a combination of Southern blotting, sequencing, and restriction enzyme mapping, and they range from 195 bp to 2.8 kb. Most of the intron/exon junctions conform to published consensus (35).

An RNase protection assay was performed to establish the transcriptional start site. A closely spaced set of three bands was detected approximately 119 bp upstream of the ATG (Fig. 1D), which agrees with the transcription start site mapped by primer extension (12). This putative transcription start site consists of a pyrimidine-rich tract which is homologous but not identical to a consensus initiator repeat sequence (14, 49) in that a C residue replaces the conserved A at position +1. The gene lacks a consensus TATA box, although a TA-rich region is appropriately located 20 bp upstream. Additional putative control elements include an NF-Y element, two Sp1 sites, an AP-2 site, and four E-box sequences (Fig. 1C). The E-box sequences fall into three categories: two are a consensus for MyoD binding, one is a near consensus for E12-E47 binding, and the last is a consensus USF-binding site (15).

The cyclin B1 promoter confers G_2-enhanced expression upon a CAT reporter gene. G_2/M-specific expression of the cyclin B1 gene is determined in part by an increase in active transcription starting in late S phase and peaking in late G_2 (41). Although the increase measured by run-on experiments in HeLa cells was modest (2.5-fold), we asked whether the cloned cyclin B1 promoter could confer G_2-specific expression to a CAT reporter gene. A CAT construct containing 3,800 bp of 5′ promoter sequence was constructed and introduced stably into HeLa cells along with the pSV2neo plasmid, which confers neomycin resistance. G418-resistant colonies were picked and expanded into lines. Initially, 24 lines were analyzed for constitutive CAT activity. The six lines with the lowest CAT levels were synchronized in early S phase by thymidine treatment or in G_2/M phase by nocodazole treatment, and G_2-specific CAT activity was measured as the G_2/M-to-S ratio. All of these lines demonstrated an increase in G_2-specific CAT activity, 2.1-fold on average, compared with either cycling or S-blocked cells (which were equivalent because 90% of cycling HeLa cells are in the G_1 and S phases). The CAT assays with two clones which showed the highest (2.8-fold) increase in G_2-specific CAT expression are shown (Fig. 2, lanes 1–6).

To determine whether this G_2-specific increase could be measured in a transient-transfection assay, pCycB(−3800)-CAT was electroporated into HeLa cells, the cells were blocked in S or G_2/M as above, and their G_2-specific CAT activities were compared. Flow-cytometric analysis of bromodeoxyuridine- and propidium iodide-stained nuclei was performed to assess the effectiveness of the blocks. In the experiment shown (Fig. 2, lanes 7 to 12), 80% of the thymidine-blocked cells were in G_1 and S whereas 85% of the nocodazole-treated cells were in G_2/M. Cyclin B1 CAT activity was significant in thymidine (S)-blocked cells but was 4.5-fold enhanced in nocodazole-treated (G_2/M) cells. A CAT construct driven by the constitutive human β-actin promoter was induced only 1.2-fold, demonstrating that G_2-enhanced activity was specific to the cyclin B1 promoter. HeLa cells transfected with the promoterless parent vector, pCAT basic (Promega), did not have CAT activity. In five experiments, the average increase in G_2-specific CAT activity was threefold. These results demonstrate that pCycB(−3800)-CAT has constitutive basal CAT activity in G_1- or S-phase cells but that this activity is further increased as cells traverse through G_2.

The USF-1 transcription factor is required for cyclin B1 gene transcription in vitro and in vivo. To identify *cis*-acting elements important for constitutive expression of the cyclin B promoter, PCR amplification from the pCycB(−1050)-CAT template was used to make 5′-truncated amplimers (Fig. 3A), which were subcloned into the G-less cassette plasmid pC2AT (52). In vitro transcription reactions were carried out with extracts prepared from cycling HeLa cells (Fig. 3B). Transcription from the longest (wild-type) construct containing −1050 bp of promoter was normalized to 100% activity. Percent activity for the 5′ deletion mutants was compared with that for the −1050 construct and was corrected for loading variation with the signal derived from the ALB286 plasmid (which contains the mouse albumin promoter driving the G-less cassette). No significant changes in transcription activity were seen with successive deletions extending to −414 and −258 bp. However, a construct deleting the region between −258 and −184 bp, which contains a CACGTG USF-like site, was only 60% as active as the −1050 promoter. Further deletion to −174 bp, which removes the adjacent AP-2 site, resulted in a further reduction to 29% of the wild-type promoter activity. Sequential deletions had no effect until removal of the −42 to −17 bp

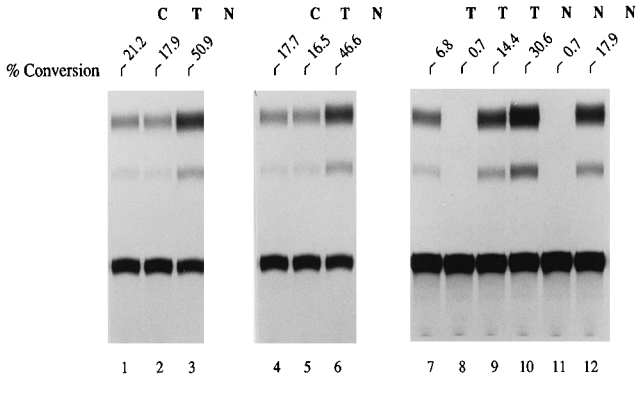


FIG. 2. The cyclin B1 promoter confers G_2-specific expression to a CAT reporter gene. HeLa cells stably transfected with pCycB(−3800)-CAT were either blocked in G_1 with thymidine-aphidicolin (T, lanes 2 and 5), in G_2/M with thymidine-nocodazole (N, lanes 3 and 6), or not blocked (C, lanes 1 and 4) and harvested for CAT assay. Results of a representative experiment from two clones, clone 5 (lanes 1 to 3) and clone 10 (lanes 4 to 6), are shown. HeLa cells transiently transfected with pCycB(−3800)-CAT (lanes 7 and 10), pCATbasic (lanes 8 and 11), or pβ-actin-CAT (lanes 9 and 12) were blocked in early S phase with thymidine (T, lanes 7 to 9) or in G_2/M with thymidine-nocodazole (N, lanes 10 to 12) and harvested for CAT assay as described in Materials and Methods.

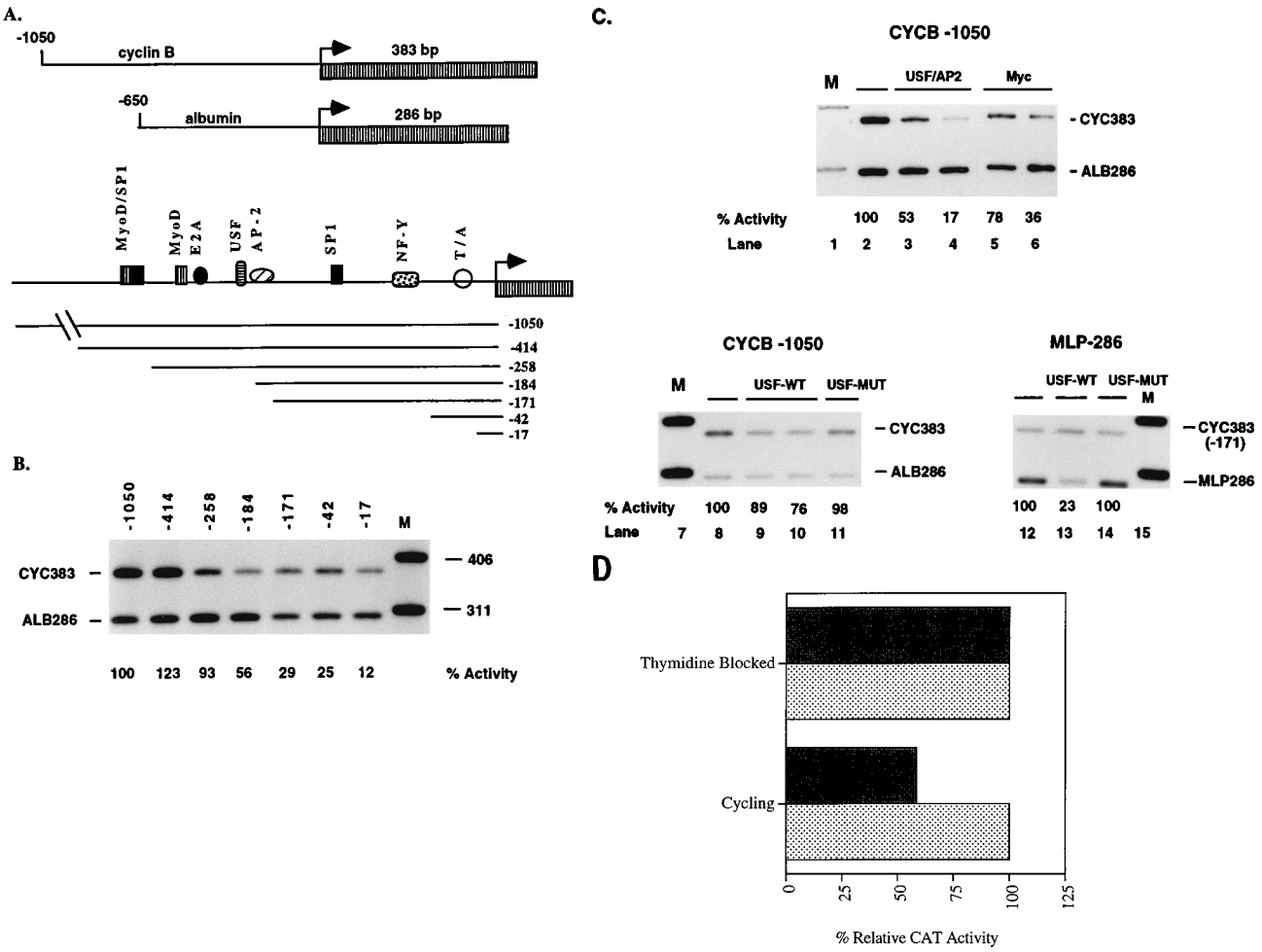


FIG. 3. The USF site is necessary for constitutive cyclin B1 gene transcription. (A) The cyclin B1 promoter (containing bp −1050 to +6 relative to the cap site) and rat albumin promoter (containing bp −650 to +21 relative to the cap site) were positioned upstream of a 383-bp (CYC383) or 286-bp (ALB286) G-less DNA cassette (represented by a striped box), respectively. The locations of putative factor-binding sites relative to the cyclin B1 promoter cap site are also shown, as are the positions of 5′ deletions introduced into the CYC383 G-less cassette vector. (B) Effect of promoter deletions on in vitro transcription. The autoradiogram shows the polyacrylamide gel electrophoresis-separated transcription products from in vitro transcription of the CYC383 and ALB286 plasmids. At the top of each lane is the location of the 5′-most end of the CYC383 promoter constructs. For all assays, 0.3 μg of plasmid DNA was used and the ALB286 plasmid was used as an internal control. The bands are identified according to size (in bases) and are labeled according to the promoter from which they were synthesized. The percent activity represents the relative level of CYC383 promoter activity compared with the −1050 construct and was corrected for loading variations by using the signal derived from the ALB286 plasmid. The percentage shown represents the average of results from four independent experiments. The markers shown are *Hpa*II-digested pBR322 3′-end labeled with Klenow fragment and [^{32}P]dCTP. (C) CACGTG-containing oligonucleotides inhibit the in vitro transcription reaction. Oligonucleotide competitors USF/AP-2, Myc consensus, adenovirus MLP USF site (USF-WT), and its mutant version (USF-MUT) were included in the in vitro transcription reactions. Uninhibited reactions in lanes 2, 8, and 12 were assigned a level of 100% activity. Inhibited reactions were performed with the following molar excess of oligonucleotide: 7.5-fold (lanes 3 and 5); 15-fold (lanes 4, 6, and 9); and 30-fold (lanes 10, 11, 13, and 14). Markers are shown in lanes 1, 7, and 15. (D) Mutagenesis of the USF site ablates transcription in cycling HeLa cells. pCycB(−290)-CAT (stippled bars) and pCycB(−290mutUSF)-CAT (shaded bars) were electroporated into HeLa cells. The transfected cells were allowed to cycle or were blocked with thymidine for 36 h and then harvested for CAT assay 48 h posttransfection. CAT activity from pCycB(−290)-CAT was normalized to 100%, and CAT activity from the mutant was expressed as a percentage of the wild-type activity.

TA-containing region, which reduced transcription to 12% of wild-type activity.

To establish whether the −189 to −184 bp USF site was critical to the loss of activity associated with removal of the −258 to −174 bp region, oligonucleotide competitions were carried out with the identical USF/AP-2 site, a Myc consensus site (5), and a USF consensus site from the adenovirus MLP (22, 47). These oligonucleotides were added to the transcription reactions at 7.5-, 15-, and 30-fold molar excesses, prior to the addition of NTPs and [^{32}P]UTP. At a 15-fold molar excess, USF/AP-2 oligonucleotide was the most potent competitor, decreasing the activity of the transcription reaction to 17% of levels without competitor (Fig. 3C, lane 3), whereas a consensus Myc oligonucleotide had a somewhat lesser effect, decreasing levels to 36% of control levels (lane 6). A 30-fold excess of the MLP USF site had the least effect, reducing levels to 76% of control levels (lane 10). Since all of these sites contain the core CACGTC-binding motif, the data suggest that a protein which binds to this element regulates cyclin B1 transcription. Two experiments were performed to confirm the importance of the CACGTG motif. First, a mutant USF oligonucleotide (USF-MUT) containing three site-specific mutations in the USF-binding site was compared with its wild-type counterpart as a competitor in the in vitro transcription reaction. At a 30-fold molar excess (Fig. 3C), the mutant oligonucleotide had no significant effect on transcription (98%; lane 11) compared with the wild-type oligonucleotide (76%; lane 10). Control reactions demonstrated that the wild-type (USF-WT) oligonu-

cleotide did not inhibit the −171 promoter construct, which lacks the USF site, but was effective against a USF-regulated MLP template (lane 13). Second, the USF site of pCycB (−290)-CAT was mutated and constitutive transcription of pCycB(−290mut USF)-CAT compared with its wild-type parent after transient expression of these constructs in cycling versus thymidine-treated (S blocked) HeLa cells. Mutation of the USF site caused a 41.2% decrease in promoter activity on average in cycling HeLa cells (Fig. 3D), consistent with the effect of its deletion in vitro (Fig. 3B). However, no difference in promoter activity was seen between wild-type and mutant USF in thymidine-treated (S) blocked HeLa cells, suggesting that transcriptional activation through the cyclin B1 USF site depends on cell cycle progression.

USF-1 DNA-binding activity is cell cycle regulated. To identify which proteins bound to the USF and AP-2 sites, a ^{32}P-labeled oligonucleotide probe, USF/AP-2, was incubated with a HeLa nuclear extract and the complexes were separated by electrophoretic mobility shift assay. Proteins binding to the USF/AP-2 probe formed a shifted doublet (bands marked B1 and B2 in Fig. 4A). To help identify the protein(s) in the B1 and B2 bands, competitor oligonucleotides from related sites were included in the binding-reaction mixtures at a 200-fold molar excess (Fig. 4A). The B1 and B2 bands (Fig. 4A) were completely inhibited by an oligonucleotide containing a Myc consensus site (lane 4) and partially inhibited by a related E-box sequence containing a consensus MyoD site (lane 3), whereas Sp1-3′ and AP-2 competitors had little to no effect (lanes 2 and 5). Specific competition by the Myc consensus site but not by the AP-2 site suggested that the proteins were binding to the CACGTG USF site. USF was first identified and purified on the basis of its ability to bind to regulate the adenovirus MLP (14, 22). We therefore determined whether the adenovirus MLP USF site would inhibit the complexes that bind to the related site of the cyclin B1 promoter (Fig. 4A). The wild-type oligonucleotide inhibited the complexes (lane 7), whereas the mutant with three substitutions in the USF-binding site had no effect (lane 8). Second, an antibody directed against human USF1 protein, which recognizes all USF family members, caused a supershift of both complexes (lane 10), whereas normal rabbit serum had no effect (lane 11). Together, these data confirm that the USF site-binding activity is USF or contains a protein antigenically related to USF.

To determine whether the USF site-binding activity was regulated in a cell cycle-specific fashion, HeLa nuclear extracts were prepared from cycling cells or cells blocked in S phase by thymidine treatment or G_2/M by thymidine-nocodazole treatment (Fig. 4B). Binding to the USF site was comparable in both cycling (C, lane 1) and thymidine-treated (T, lane 2) (S phase) cells but was increased 2.5-fold on average in thymidine-nocodazole-treated (N, lane 3) (G_2/M phase) cells, suggesting that this binding activity was increased in a G_2-specific fashion. To confirm that G_2-enhanced binding activity was regulated during cell cycle transit, nocodazole (G_2/M)-blocked cells were released into fresh medium for 3 h (N+3) and 6 h (N+6) and nuclear extracts were prepared. Flow-cytometric analysis confirmed the efficacy of the blocks and release into G_1: 85% of the nocodazole-blocked cells were in G_2/M, whereas at 3 and 6 h after release, 68 and 74%, respectively, were in G_1 (data not shown). When these nuclear extracts were bound to the USF/AP-2 probe, USF binding was increased in nocodazole-blocked cells (lane 4), clearly reduced as early as 3 h postrelease (lane 5), and comparable to that in cycling or thymidine-blocked cells by 6 h postrelease (lane 6). These experiments strongly suggest that the USF-binding activity is cell cycle regulated.

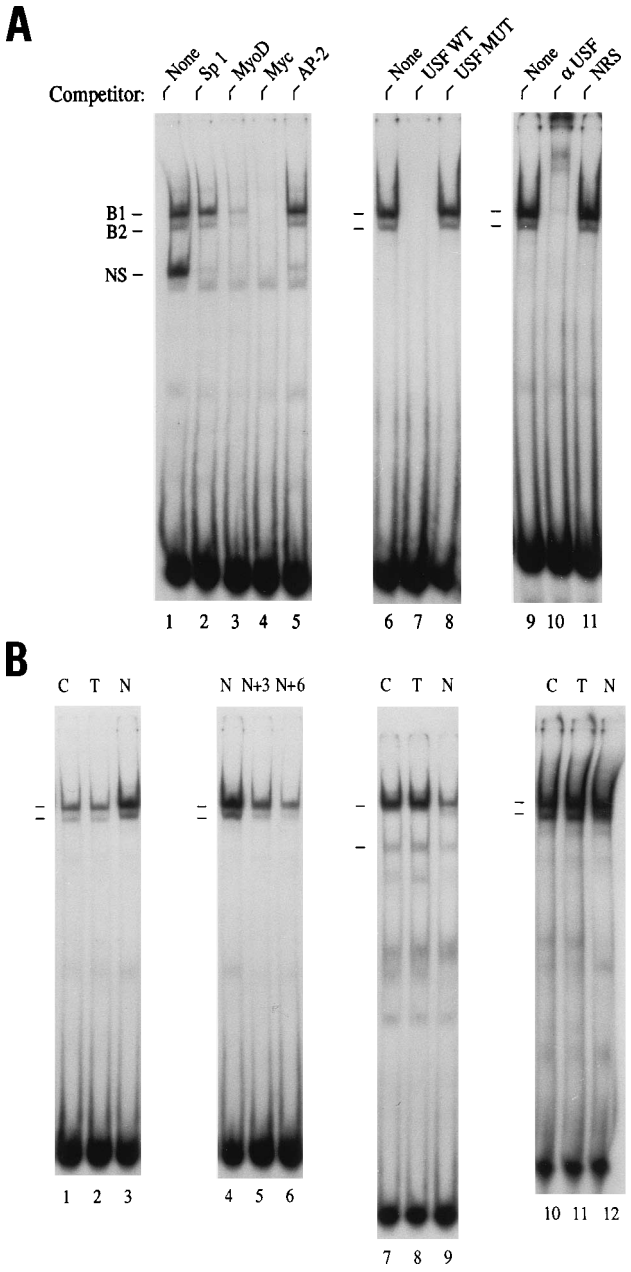


FIG. 4. USF1 binds the CACGTG motif in a cell cycle-regulated manner. (A) Electrophoretic mobility shift assay shows that USF1 binds to the USF site. HeLa nuclear extracts were bound to the USF/AP-2 probe containing the cyclin B1 promoter USF and AP-2 sites (lanes 1 to 8). Two specific bands (B1 and B2) were seen, along with a nonspecific (NS) band, in some extracts. Competitor oligonucleotides included to determine specificity were Sp1-3′ (lane 2), MyoD consensus (lane 3), Myc consensus (lane 4), AP-2 consensus (lane 5), adenovirus MLP USF consensus (lane 7), and mutant USF oligonucleotide (lane 8). Addition of an anti-USF1 antibody (lane 10) supershifts the B1 and B2 bands, while normal rabbit serum (NRS; lane 11) has no effect. (B) USF binds in a cell cycle-regulated fashion. Nuclear extracts were prepared from cycling (C; lanes 1, 7, and 10), thymidine-treated (T; lanes 2, 8, and 11), or thymidine-nocodazole-treated (N; lanes 3, 4, 9, and 12), HeLa cells or thymidine-nocodazole-blocked HeLa cells released for 3 h (N+3, lane 5) or 6 h (N+6, lane 6). These extracts were incubated with USF/AP-2 (lanes 1 to 6), Sp1-3′ (lanes 7 to 9), or consensus CRE (lanes 10 to 12) probes. Specific bands are marked by lines.

To assess whether G_2-specific binding was unique to the USF site, cell cycle-blocked HeLa extracts were bound to the cyclin B1 Sp1-3′ site and an unrelated cyclic AMP response element (CRE) site. Sp1 binding was comparable in cycling and thymidine-treated cells but decreased 60% in nocodazole-treated cells. While the loss of Sp1 in the G_2/M phase may be cell cycle specific, at least 15% of the Sp1-binding activity is lost with micronuclei or metaphase nuclei that do not sediment readily with the rest of the prometaphase nuclei (data not shown). Binding to a consensus CRE site was unchanged regardless of the cell cycle status, suggesting that binding of some transcription factors is unaffected by the cell cycle status. Of the three sites tested, only USF-binding activity increased during the S-to-G_2/M transition, suggesting that a USF protein could be a candidate protein for G_2 transcriptional control.

DISCUSSION

The stage-specific and sequential expression of cyclins ensures that cells will progress through the cell cycle (58). Recent studies have suggested that total expression and/or stage-specific expression of these proteins is altered in some types of tumor cells (20, 29, 31, 53). Overexpression of certain oncogenes and tumor suppressor genes has been shown to deregulate transcription of cyclins in a gene-specific fashion (6, 28, 36, 40, 55, 57, 61). Thus, an understanding of how their transcription is regulated may help us understand how cyclin expression is deregulated in certain tumor lines.

In this paper, we give the first description of the genomic structure of the human cyclin B1 gene and initial characterization of its promoter. By a combination of Southern blotting, restriction digestion, and sequencing, we demonstrate that the cyclin B1 gene encompasses approximately 8.8 kb of sequence and contains nine exons and eight introns (Fig. 1A). All of the splice sites contain consensus splice donor/acceptor sequences (Fig. 1B). An RNase protection assay mapped the transcription start site to a pyrimidine-rich region homologous but not identical to INR sequences from other genes (Fig. 1C). A promoter fragment containing approximately 3,800 bp of 5′ sequence conferred modest increases in G_2-specific expression to a CAT reporter gene in HeLa cells (Fig. 2). The modest levels of G_2-specific transcription conferred to the CAT reporter gene (two- to threefold) either in stable integrants or after transient expression (Fig. 2) are consistent with the induction of the endogenous gene in HeLa cells as measured by nuclear run-on analysis (41). We also identified a promoter-proximal USF site which is necessary for the constitutive activity of this promoter both in vitro and in vivo and required that the cells be actively cycling (Fig. 3). This site appears to bind USF or an antigenically related protein (Fig. 4A), and the protein binding is induced in a G_2-specific fashion (Fig. 4B).

The E-box consensus binding site CACGTG has now been identified in promoters for the cyclin D and A genes, as well as the cyclin B1 gene reported here. The CACGTG motif can bind several proteins, including c-Myc, TFE3, and USF. Recent work has focused on the effects of overexpression of c-Myc because of its links to cell proliferation and cancer; however, the effects of c-Myc overexpression appear to differ with the promoter context. There is general agreement that c-Myc induces expression of the cyclin A gene (27, 28). Another recent report shows that c-Myc represses the cyclin D1 gene or a cyclin D1 promoter linked to a CAT reporter gene (28, 40). Here we do not address the effects of c-Myc overexpression on the cyclin B1 promoter, although there are several indications that it might not be involved. First, anti-c-Myc antibodies did not affect binding to this site in the electrophoretic mobility shift assay (data not shown). Second, the CACGTG motif is flanked by a 5′ T residue (on the noncoding strand), which makes this a less attractive site for c-Myc binding (4, 5). Finally, overexpression of c-Myc does not induce cyclin B1 expression in fibroblast cells (27). Clearly, the −189/−184 bp CACGTG site is important for constitutive cyclin B1 transcription because of the ability of CACGTG-containing but not mutant oligonucleotides to block in vitro transcription and because a mutation of this site blocks in vivo expression from a CAT reporter gene in cycling HeLa cells. Whether c-Myc-binding sites are important for the constitutive transcription of the other cyclin genes has not been addressed.

Our DNA-binding studies suggest that USF or a related protein binds to the −189/−184 USF site on the basis of two results: (i) a USF-binding site from the adenovirus MLP inhibited binding whereas a mutant with three site-specific mutations did not; and (ii) an anti-USF antibody supershifted this complex. USFs are ubiquitously expressed proteins which have been shown to bind the promoters of a number of cellular genes involved in tissue-specific or developmental regulation (10, 32, 44, 51, 54). The only other report suggesting a link to cell cycle control involved its potential regulation of the mitogen and serum-inducible p53 gene (48). The demonstration here that USF1 binds to a site which is important for constitutive expression of the cyclin B1 promoter in cycling HeLa cells suggests another link between USF and cell cycle control. From the demonstration here that USF binding is induced in G_2/M-blocked cells, it is tempting to speculate that USF might be a G_2-specific control protein for cyclin B1. While we could not consistently demonstrate G_2-specific activation of a shorter promoter construct containing the wild-type USF site, we could show that transcriptional activity was USF site dependent in cycling HeLa cells but not in S blocked cells. Because the experiments used a promoter fragment which ended at −290 bp, it seems plausible that elements upstream of −290 bp act in concert with the USF site to mediate the full level of G_2 activation. We have noted three elements between −300 and −1000 bp of the cyclin B1 promoter which are identical to known S-phase-specific activators of histone genes. Perhaps these elements work in concert with the USF site to mediate the higher level of G_2-specific activation seen in the −3800 bp construct.

Although the USF1 element is certainly important for constitutive transcription from the cyclin B1 promoter, other transcription factor-binding sites are clearly involved. First, deletion of the adjacent AP-2 site, in addition to the USF site, caused a further decrease in cyclin B1 transcription in vitro. Likewise, the USF/AP-2 oligonucleotide was the most effective of all the CACGTG-containing competitors, confirming that flanking sequence was important. It has been reported that CACGTG-recognizing proteins also discriminate flanking residues (4, 5), but this would not explain why removal of the 3′ AP-2 site-containing sequence is functionally important, since deletion of the CACGTG motif with the −184 bp truncation mutant (Fig. 3B) should have completely ablated USF binding and function. A protein which binds weakly to the cyclin B1 AP-2 site that is specifically inhibited by a consensus AP-2 oligonucleotide was detected with transcription-active HeLa nuclear extracts in the binding reactions (data not shown). It is tempting to speculate that deletion of the AP-2 site causes a loss of in vitro transcription because the binding site for this AP-2-like protein is lost. Future experiments should address this question. Second, the Sp1-3′ site competes very effectively in the in vitro transcription assay. However, this inhibition appears not to be linked to the Sp1 sites themselves, since their deletion was not functionally important (see −258 and −42

deletions, Fig. 3B) and the Sp1-3′ site is still effective at inhibiting transcription from a deletion construct lacking both Sp1 sites (data not shown). Perhaps the Sp1 oligonucleotide inhibits a critical interaction at the basal promoter. Finally, this paper does not address the potential role of the other MyoD- and E2A-like E-box sequences in control of cyclin B1 transcription. A recent study has shown that cyclin D1 inhibits the ability of MyoD and myogenin to transactivate through cognate recognition sites in the muscle creatine kinase promoter/enhancer (46). Future studies will address the role of these E-box-binding proteins and potentially also of cyclin D1 on regulation of cyclin B1 transcription.

In conclusion, we have cloned and mapped the cyclin B1 gene, sequenced its promoter, and demonstrated that it has the expected G_2-specific regulation. We have identified a USF-binding site which is important for its regulation and have discovered that USF binding is regulated during the G_2 phase of the cell cycle. These studies suggest a novel link between USF and the cyclin B1 gene, which partially explains how maturation-promoting factor complex formation is achieved. Although we cannot exclude the possibility that USF or the cyclin B1 promoter is aberrantly regulated in these transformed HeLa cells, this observation may provide further insight into how misregulation of the cyclin B1 promoter could contribute to cancer and transformation.

ACKNOWLEDGMENTS

We extend special thanks to the members of the Glaxo Genome Analysis Group, whose help was crucial to finishing the cyclin B1 genomic map, and to the members of Cell Biology and Molecular Genetics, who provided valuable input during the course of these studies. We also thank Michele Sawadogo for providing the anti-USF1 antisera, David Taylor and Kevin Roof for providing technical assistance, Duncan Walker and John Gray for critically reading the manuscript, and Mark Furth for supporting this collaboration.

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