The cyclin B1 gene is actively transcribed during mitosis in HeLa cells

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In mammalian cells, the expression level of the cyclin B1 gene plays a critical role in the progression through mitosis. Here we demonstrate that the transcriptional activity of the human cyclin B1 promoter, as well as the rate of gene transcription, is high during mitosis. Indeed, the cyclin B1 promoter maintains an open chromatin configuration at the mitotic stage. Consistent with this, we show that the cyclin B1 promoter is occupied and bound to NF-Y during mitosis in vivo. Our results provide the first example of RNA polymerase II-dependent transcription during mitosis in mammalian cells.

INTRODUCTION

Repression of transcription during mitosis has been known in mammalian cells for more than 30 years. It has been demonstrated that specific RNA polymerase II (pol II) factors, and several transcription factors, are displaced from the chromosomes during mitosis (Gottesfeld and Forbes, 1997). However, mitotic dispersal was not observed for some transcription factors such as the serum response factor p67SRF, and 10–20% of the TFIID complex (Gauthier-Rouviere et al., 1991; Segil et al., 1996).

In mammalian cells, the G2/M transition is controlled by the mitotic cyclins A and B1 (Jackman and Pines, 1997). The expression of cyclin B1 is cell-cycle regulated, peaking at the G2/M transition. It has been described that the transcriptional activity of the human cyclin B1 promoter is induced during the G2/M transition and repressed in G1 (Cogswell et al., 1995; Hwang et al., 1995; Piaggio et al., 1995; Farina et al., 1996; Katula et al., 1997). However, it has not been demonstrated yet whether the cyclin B1 promoter retains transcriptional activity during mitosis. Here we provide evidence for cyclin B1 transcription at the mitotic stage in mammalian cells. Indeed, we show that the cyclin B1 promoter is accessible to restriction endonucleases and interacts with transcription factors during mitosis in vivo. In addition, we demonstrate by formaldehyde cross-linking and immunoprecipitation assays that NF-Y is bound to the cyclin B1 promoter during mitosis. Our results show that the RNA pol II-transcribed gene cyclin B1 is actively transcribed during mitosis where the promoter retains open conformation and factor occupancy.

RESULTS

Transcriptional activity of the exogenous cyclin B1 promoter is high in the G2/M phases of the cell cycle

To investigate the transcriptional activity of the cyclin B1 promoter during mitosis, we stably transfected HeLa cells with a chloroamphenicol acetyltransferase (CAT) reporter gene driven by a human cyclin B1 promoter fragment (–150 to +182 bp) (Piaggio et al., 1995). We synchronized these cells by a double thymidine block. After release from the block, the cells were collected by mitotic shake-off (MSO). Flow cytometric analysis (Figure 1A) and mitotic index (see Supplementary data available at EMBO reports Online) of synchronized cells showed that in the MSO population, 92% of cells were mitotic. The levels of CAT mRNA in cycling and mitotic cells were analyzed by northern blot. The results showed that the activity of the exogenous cyclin B1 promoter fragment was high during mitosis of HeLa cells (9.2-fold higher than asynchronous cells), in agreement with the expression of the endogenous gene.
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Since the half-life of CAT mRNA is longer than the length of mitosis in HeLa cells, it was impossible to determine whether the CAT mRNA levels detected in our MSO population were transcribed in G2 or in mitosis.

The cyclin B1 promoter is accessible to restriction endonucleases in vivo

To analyze cyclin B1 promoter chromatin conformation during mitosis, we performed in vivo restriction site accessibility assays (Bhattacharyya et al., 1997) on a region spanning the promoter and the first intron of the cyclin B1 gene. Nuclear preparations from asynchronous cells and permeabilized mitotic cells were partially digested with BsaAI (Figure 2A) and Sau96I (Figure 2B), cutting at the E-box region and at the Sp1 site of the cyclin B1 promoter, respectively. DNA was then purified and fully digested with XbaI and NcoI generating fragments of 697 and 746 bp, respectively. The resultant fragments were detected by Southern blot analysis using a probe spanning 401 bp of the 5’ region of the cyclin B1 promoter. In mitotic cells the accessibility to BsaAI and Sau96I was comparable to that in asynchronous nuclei, demonstrating that the core promoter region is accessible to the endonucleases during mitosis. In contrast, accessibility to the enzymes that cut outside the core promoter was much lower even in the asynchronous nuclei, indicating an inaccessible chromatin structure in both tested regions.
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(Figure 2C and D). Taken together, these results demonstrate that the cyclin B1 core promoter region is accessible to restriction enzymes, indicating that it maintains an open configuration at the mitotic stage.

**The cyclin B1 promoter interacts with transcription factors during mitosis in vivo**

To test whether the open chromatin structure leads to transcription factor–DNA interactions, we performed in vivo genomic footprinting of the cyclin B1 promoter during mitosis. Asynchronous and mitotic cells were treated with the DNA alkylating reagent dimethyl sulfate (DMS), and methylated G residues were identified using the ligation-mediated polymerase chain reaction technique (LM–PCR) (Dey et al., 1992). In asynchronous cells, four regions on the cyclin B1 promoter non-coding strand were hypersensitive to or protected from DMS methylation: the E box (–124 to –119 bp), the GC box (–80 to –71 bp), the upstream CCAAT box (–17 to –12 bp) and the downstream CCAAT box (+15 to +20 bp) (Figure 3, lane 2). Genomic footprinting of mitotic cells showed the same methylation pattern as in cycling cells, thus indicating a persistent interaction of sequence-specific transcription factors with the cyclin B1 promoter at the mitotic stage (Figure 3, lane 3). It has been demonstrated that the hsp70 promoter is devoid of sequence-specific transcription factor interactions during mitosis in HeLa cells, while it shows protection of the main cis-acting elements in asynchronous cells (Martinez-Balbas et al., 1995). As a control, we performed genomic footprinting experiments on the hsp70 promoter (Martinez-Balbas et al., 1995). As expected, this promoter was devoid of sequence-specific transcription factor interactions in MSO cells (Figure 3, lane 5). Consistent with the restriction site accessibility data shown in Figure 2, these results demonstrate that, during mitosis, the cyclin B1 promoter is occupied by sequence-specific transcription factors.

**NF-Y binds the cyclin B1 promoter during mitosis in vivo**

Genomic footprinting experiments demonstrate that the CCAAT boxes of the cyclin B1 promoter are occupied during mitosis in vivo. We have demonstrated previously that these two elements are key sequences for the cyclin B1 promoter activity and NF-Y binds these sequences in vitro (Farina et al., 1999). Thus, we asked whether NF-Y interacted with the cyclin B1 promoter during mitosis in vivo. Cycling and mitotic cells were treated with formaldehyde to cross-link proteins to DNA. After sonication, the cross-linked chromatin from equivalent numbers of asynchronous and mitotic cells was immunoprecipitated using anti-NF-YA and NF-YB antibodies. As negative controls, we included a reaction lacking the primary antibody and one containing anti-E2F1 antibody. E2F1 is a nuclear transcription factor that does not have a binding site on the cyclin B1 promoter. After immunoprecipitation, enrichment of the endogenous cyclin B1 promoter fragment in each sample was monitored by PCR amplification using primers amplifying the cyclin B1 promoter region from –57 to +183 bp. The results show that both anti-NF-Y immunoprecipitates from cycling and mitotic chromatin contained the cyclin B1 promoter (Figure 4B). To investigate the ability of NF-Y to bind the cyclin B1 promoter in G1, we performed chromatin immunoprecipitation experiments on G1 synchronized cells (Figure 4A). As shown in Figure 4B, anti-NF-Y immunoprecipitates from G1 chromatin contained the cyclin B1 promoter. To evaluate the specificity of the NF-Y interaction with the cyclin B1 promoter, we used the hsp70 promoter. Indeed, a CCAAT box is also required for the transcriptional activation of this promoter in proliferating cells, and NF-Y binds this sequence in vitro and in vivo (Imbriano et al., 2001). NF-Y antibodies immunoprecipitated the hsp70 promoter from asynchronous and G1 cells, but not from mitotic cells (Figure 4B). The non-specific antibody E2F1 was unable to immunoprecipitate the cyclin B1 or hsp70 promoters. Taken together, these results further provide in vivo evidence for a specific retention of NF-Y to the cyclin B1 promoter during mitosis.

**Transcriptional activity of the endogenous cyclin B1 promoter is high during mitosis**

To investigate whether the endogenous cyclin B1 gene is transcribed at the mitotic stage, we analyzed the rate of gene...
NF-Y binds to the *cyclin B1* promoter *in vivo* during mitosis. (A) DNA distribution analysis of propidium iodide-stained MSO and G1 HeLa cells. (B) Formaldehyde cross-linked chromatin was prepared from the same number of asynchronous (Async.), mitotic and G1 HeLa cells. Cross-linked chromatin from each sample was incubated with antibodies to NF-Y (lanes 2, 3, 8, 9, 13 and 14) and E2F1 (lanes 4, 10 and 15), or in the absence of antibody (none). Immunoprecipitates from each antibody were aliquoted and then analyzed by PCR with specific primers for the *cyclin B1* or *hsp70* promoters. A sample representing 0.02% of the total input chromatin (input) was included in the PCR analysis.

**Fig. 5.** Transcriptional activity of endogenous *cyclin B1* promoter is high during mitosis. Asynchronous (Async.), mitotic and G1 phase HeLa cells were tested by a whole-cell run-on assay. Probes were cDNAs from the murine *cyclin B1*, *A*, *E* and *D1* genes.

transcription by whole-cell run-on assays (Parsons and Spencer, 1997). In these experiments a constant cell number of asynchronous, MSO, nocodazole-treated and G1 phase cells have been employed. The RNA labeled during run-on transcription assays was hybridized to filters containing denatured double-stranded cDNA probes for *B1, A, E* and *D1* cyclins. Transcription was detected for all tested genes in the asynchronous cell population. Transcription in mitotic cells was reduced for *A, E* and *D1* cyclins genes, while the *cyclin B1* gene showed a high level of transcription in mitotic cells (Figure 5). Interestingly, although not completely repressed, *cyclin B1* gene transcription was down-regulated in the G1 phase. Taken together, our data demonstrate that the human *cyclin B1* gene is actively transcribed during mitosis in HeLa cells.

**DISCUSSION**

We investigated the occurrence of transcription during mitosis on an RNA pol II-transcribed gene. We have found that the human *cyclin B1* gene is actively transcribed at the mitotic stage. This result is surprising, since it is widely accepted that transcription is repressed during mitosis in higher eukaryotes. Interestingly, in fission yeast the rate of RNA synthesis is maintained during passage through mitosis (Baum et al., 1998). In mammalian cells, until now, no RNA pol II-dependent transcription has been reported in mitotic cells, although there is evidence showing that 10–20% of the TFIIID population remains associated with the condensed mitotic chromatin (Segil et al., 1996). Whether the transcription of the *cyclin B1* gene occurs during all the four mitosis phases remains to be elucidated. The *cyclin B1* protein is quickly degraded at the metaphase. Whenever a spindle checkpoint is imposed during metaphase, there is a reappearance of *cyclin B1* protein due to a loss of *cyclin B1* destruction (Clute and Pines, 1999). Our results suggest that transcription could also contribute to ensure adequate *cyclin B1* levels, which could be immediately available whenever a spindle checkpoint is activated. Whether the mitotic transcription of the *cyclin B1* gene is an event occurring in transformed cells and not in non-transformed cells remains to be elucidated.

Our results not only demonstrate that the *cyclin B1* promoter is accessible to restriction endonucleases, but also show that this promoter is occupied by transcription factors during mitosis. In particular, we demonstrate that NF-Y binds the *cyclin B1* promoter during mitosis. This result is in agreement with the evidence for retaining the TFIIID on mitotic chromatin (Segil et al., 1996). Interestingly, the single-stranded DNA-binding proteins, heterogeneous nuclear ribonucleoprotein K and far upstream binding protein, which are both able to transactivate the c-myc gene in asynchronous cells, remain bound to the c-myc promoter during mitosis (Michelotti et al., 1997). The results presented here indicate strongly that the *cyclin B1* gene transcription occurring during mitosis is due to a functional NF-Y complex bound on its promoter. Importantly, we did not find NF-Y binding to the *hsp70* promoter during mitosis, indicating that specific sequences, other than the CCAAT boxes per se, are required for the recruitment of NF-Y on mitotic chromosomes. Several cell cycle-regulated promoters contain more than one CCAAT box, and not in non-transformed cells remains to be elucidated.

Interestingly, NF-Y also binds the *cyclin B1* promoter in the G1 phase. Since our ChIP experiments are not quantitative, we cannot exclude that NF-Y binds in G1 the *cyclin B1* promoter with a lower affinity than in mitosis. However, this result is consistent with our genomic footprinting data demonstrating that the two CCAAT boxes of the *cyclin B1* promoter bind...
proteins in the G1 phase (S.Sciortino, unpublished observations). Run-on experiments indicate that cyclin B1 transcription, although not completely repressed, is down-regulated in G1. One possibility is that the cell cycle-dependent transcription of the cyclin B1 gene is due to a recruitment of different protein complexes by NF-Y on the promoter in the different cell-cycle phases. We are currently investigating this issue.

In conclusion, although there is a general repression of transcription during mitosis in mammalian cells concomitant with dispersal of transcription factors from the mitotic chromatin, the cyclin B1 gene retains transcriptionally active complexes on its promoter and is actively transcribed at the mitotic stage.

METHODS

HeLa cells synchronization and transfections. For the double thymidine block, HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (Gibco BRL) to 60–70% confluence and incubated with 2.5 mM thymidine (Sigma). After 14 h, fresh medium was replaced for 8.5 h. The second block was performed with 2.5 mM thymidine for 14 h. Cells were incubated with fresh medium for 10 h and the mitotic cells were detached by tapping the flasks 10 times (MSO). These cells were repleted, and after 3 h G1 phase synchronized cells were collected. For the nocodazole block the cells were treated for 12 h with 4 μg/ml of nocodazole (Sigma) and prometaphase cells were detached by tapping the flasks 10 times. For DNA distribution analysis of propidium iodide-stained cells, 104 events were analyzed by an Epics cytofluorimeter (Coulter) using computer-assisted analysis (see Supplementary data). For the mitotic index, cells were cytospin centrifuged, fixed and stained with a 1 mg/ml solution of Hoechst 33258 dye (see Supplementary data). Stable DNA transfections were performed using the calcium phosphate precipitation technique (Graham and van der Eb, 1973).

Northern blot analysis. Total RNA was extracted as described previously (Manioli and Schneider, 1988). The probes were cDNA fragments of the CAT, cyclin B1 and GAPDH genes.

Whole-cell run-on transcription assays. Run-on transcription assays were performed as described previously (Parsons and Spencer, 1997). Probes were cDNAs from the murine B1, A, E and D1 cyclin genes.

Endonuclease restriction accessibility assays. Nuclei from asynchronous cells were prepared and stored as described previously (Bhattacharyya et al., 1997). MSO cells were collected and an amount of cells corresponding to 10–20 μg of DNA was pelleted, permeabilized with 0.5% NP-40 and resuspended in endonuclease buffer. These cells were homogenized gently 10 times in a Dounce homogenizer and brought to a final volume of 100 μl with endonuclease buffer. All the samples were digested at 30°C for 20 min. The following enzymes were used: BsaAI, Sau96I and Xbal from New England Biolabs, and SalI from Boehringer Mannheim. Purified deproteinized HeLa genomic DNA was isolated, digested to completion with the same enzymes and used as controls. Digested samples, resolved on a 1% agarose gel, were hybridized with a probe spanning the first 401 bp of the 5′ region of the cyclin B1 promoter, and detected by autoradiography. Percent cut was calculated as the intensity of digested bands/intensity of the undigested plus digested bands × 100.

In vivo DNA footprinting. The in vivo DNA footprinting was performed using LM–PCR as described previously (Dey et al., 1992). For cyclin B1, the following oligonucleotides were used: first primer, TGGGATTGCAACGCACT (Tm = 54°C); second primer, ACTGGCTTCACTGCTCTCCACTGGCTGCGCT (Tm = 63°C); third primer, CATGGCTTCCTTCCACGAG (Tm = 69°C).

For hsp70, oligonucleotides described previously were used (Martinez-Balbas et al., 1995).

Formaldehyde cross-linking and chromatin immunoprecipitation. Formaldehyde cross-linking and chromatin immunoprecipitation were performed as described previously (Boyd et al., 1998). Immunoprecipitation was performed with protein G. The chromatin solution was precleared by adding protein G for 15 min at 4°C, aliquoted and incubated with 2 μg of affinity-purified rabbit polyclonal antibodies (α-NF-YB was a gift from R. Mantovani, α-NF-YA was a mix of an antibody from Rockland and a gift from R. Mantovani, and α-E2F1 was from Santa Cruz) for 3 h at 4°C with mild shaking. Before use, protein G was blocked with 1 μg/ml sheared herring sperm DNA and 1 μg/ml bovine serum albumin for 2.5 h at 4°C. Cells were incubated with chromatin and antibodies overnight. Immunoprecipitates were eluted and, after reverse cross-linking, ethanol precipitated. Recovered material was treated with proteinase K, extracted with phenol/chloroform/isooamyl alcohol (25:24:1) and precipitated. The pellets were resuspended in 30 μl of H2O and analyzed using PCR. The total input sample was resuspended in 100 μl of H2O and diluted 1:100 before PCR analysis. For PCR the following oligonucleotides were used: cyclin B1 (+183): GGCTTCCTGCTTCCAGCAGCA; cyclin B1 (−57): CGGCGATCCCTGGAATAAACGAG; hsp70 (−75): AGCCCTGGGACAAACGGGAG; hsp70 (−117): GGCAGA-AACCCTGGAAATATCCTCCGA.

Supplementary data. Supplementary data are available at EMBO reports Online.

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