

Transcription of the human cell cycle regulated BUB1B gene requires hStaf/ZNF143

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ABSTRACT

BubR1 is a key protein mediating spindle checkpoint activation. Loss of this checkpoint control results in chromosomal instability and aneuploidy. The transcriptional regulation of the cell cycle regulated human BUB1B gene, which encodes BubR1, was investigated in this report. A minimal BUB1B gene promoter containing 464 bp upstream from the translation initiation codon was sufficient for cell cycle regulated promoter activity. A pivotal role for transcription factor hStaf/ZNF143 in the expression of the BUB1B gene was demonstrated through gel retardation assays, transient expression of mutant BUB1B promoter-reporter gene constructs and chromatin immunoprecipitation assay. Two phylogenetically conserved hStaf/ZNF143-binding sites (SBS) were identified which are indispensable for BUB1B promoter activity. In addition, we found that the domain covering the transcription start sites contains conserved boxes homologous to initiator (Inr), cell cycle dependent (CDE) and cell cycle genes homology regions (CHR) elements. Mutations within the CDE and CHR elements led to diminished cell cycle regulation of BUB1B transcription. These results demonstrate that BUB1B gene transcription is positively regulated by hStaf/ZNF143, a ubiquitously expressed factor, and that the CDE-CHR tandem element was essential for G2/M-specific transcription of the BUB1B gene.

INTRODUCTION

The spindle checkpoint functions to prevent premature anaphase entry until all chromosomes have completely aligned at the metaphase plate. BubR1 is a key protein mediating spindle-checkpoint activation during which it is phosphorylated. A loss of spindle-checkpoint function inevitably results in chromosomal instability and

aneuploidy (1,2). Given that aneuploidy is prevalent in many types of cancers, it is believed that spindle-checkpoint failure may be at least partly responsible for the development of cancer (3). Mouse genetics study showed also that haplo-insufficiency of BubR1 resulted in enhanced genomic instability and development of lung and colon cancer (4,5). In synchronized cells, expression of the BUB1B gene, which encodes BubR1, is undetectable in G1 but it peaks in G2/M (6,7). This cell cycle dependent expression explains the tissue distribution and the abundance of BubR1 mRNA in cells with a high mitotic index (8). In this work, which represents the first report concerning the regulation of BUB1B gene expression, we localized the promoter of this gene to positions $-464/-107$ relative to the translation initiation codon. We found that the BUB1B promoter contains three positively *cis*-acting motifs: one Inr-like motif and two elements recognized by the hStaf/ZNF143 transcription factor. This factor can up-regulate the BUB1B promoter activity, and chromatin immunoprecipitation assays demonstrated that the endogenous hStaf/ZNF143 is bound to the BUB1B promoter *in vivo*. The ZNF143 protein is the human ortholog of Staf, the *Xenopus* selenocysteine tRNA^{Sec} gene transcription factor (9,10). The human Staf (hStaf/ZNF143) is a seven C2-H2 zinc finger protein capable of enhancing transcription of the tRNA^{Sec} but also of the snRNA and snRNA-type genes (10,11). hStaf/ZNF143 can also stimulate transcription from an mRNA-type pol II promoter (10,12,13). To date, only seven protein-coding genes have been described to be regulated by hStaf/ZNF143: the cytosolic chaperonin containing t-complex polypeptide 1 (TCP1) (14); the interferon regulatory factor (IRF3) (15); the neuronal nitric-oxide synthase (NOS1) (16); the transaldolase (TALDO1) (17); the aldehyde reductase (AKR1A1) (18); the mitochondrial ribosomal protein S11 (MRPS11) (19) and the synaptobrevin-like 1 (SYBL1) (20). In addition, vertebrates contain the ZNF76 protein which constitutes a ZNF143 paralog (13,21). ZNF76 and ZNF143 are basically considered to play the same role even though their relative expression levels differ in various tissues (13). However, recent results suggested

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that ZNF76 functions as a transcriptional repressor through its interaction with TBP and that sumoylation modulates its transcriptional properties (22). Very recently, genome-wide analysis led us to identify 1175 hStaf/ZNF143-binding sites distributed in 938 mammalian promoters in protein-coding genes. By extrapolating these values to the full sizes of the genomes, we can infer the existence of at least 2500 Staf-binding sites (SBS) distributed in 2000 promoters. This large number suggests that the SBS constitutes one of the most widespread transcription factor binding sites in mammalian promoters (23).

In G1/S, the transcriptional repression of many genes such as CDC25C, CDC2, CCNA2 (coding for cyclin A), PLK1 (polo-like kinase) and RB6K (rabkinesin 6) is regulated by two repressor elements known as CDE (cell cycle dependent element) and CHR (cell cycle genes homology region). In these genes, mutation of the CDE and CHR elements allowed elevated transcription during G1 and the consequent loss of cell cycle regulated expression (24–27). In the present study, we demonstrate that the cell cycle regulation of BUB1B gene transcription is also achieved through the presence of two elements homologous to the repressor elements CDE and CHR.

MATERIALS AND METHODS

Reporter constructs and expression vectors

The human BUB1B promoter fragment –1185/–31 was PCR amplified from human genomic DNA using direct and reverse primers incorporating SacI and BamHI sites, respectively. The amplified product was cloned directly at the 5'-end to the luciferase reporter gene into the SacI/BamHI digested pFLASH I vector (SynapSys). The 5'-end truncated derivatives of the –1185/–31 fragment (–864/–31, –585/–31, –464/–31, –314/–31, –305/–31, –236/–31 and –107/–31) were PCR amplified from construct –1185/–31 and ligated to SacI/BamHI cut pFLASH I. Mutant constructs were generated by using the QuickChange II XL site-directed mutagenesis kit (Stratagene). All constructs were verified by automated DNA sequencing. Constructs –464/–31 mCHR contain the –176ATTTGAA-170 to –176CGGGTCC-170 substitution, –464/–31 mCDE contain the –166TGGCGG-161 to –166GTTATT-161 substitution. The *Drosophila* expression vectors pPAC-ZNF76 and pPAC-ZNF143 containing the human ZNF76 and ZNF143 cDNAs were described in (13). The oligonucleotide sequences used in this study are available on request.

Transfection and luciferase assays

COS-7 cells were transfected by the calcium phosphate co-precipitation procedure with 1 µg of reporter construct, 0.5 µg of pCH110 plasmid as the internal control, and carrier DNA to bring up the total DNA content to 10 µg/plate. SL2 *Drosophila* cells were transfected as described in (12) with 25, 100 or 500 ng of pPAC-hStaf/ZNF143 or pPac-ZNF76, and 200 ng of pACH110 as internal control. After 48 h, cells were lysed and the β-galactosidase activity was measured as previously described (12). The luciferase

assay was performed as recommended by the manufacturer (Promega). The luciferase activity was normalized to the β-galactosidase activity. Each transfection experiment was done in triplicate.

hStaf/ZNF143 protein preparation and DNA-binding assay

The hStaf/ZNF143 DNA-binding domain was produced using the glutathione S-transferase (GST) gene fusion system. Briefly, the DNA containing the Staf zinc finger coding region between A264 and E472 was excised from E10 (10) as a HindIII/EcoRV fragment, blunted and inserted in the direct orientation into the SmaI site of pGEX-2TK (GE Healthcare). The fusion protein was purified using glutathione-sepharose beads. The glutathione S-transferase moiety was cleaved with thrombin. Full-length hStaf/ZNF143 was synthesized by *in vitro* coupled transcription-translation with the TnT system (Promega) as described in (13). Fifty microliter reactions were programmed with 1 µg of pSK(-)-ZNF143 (13). Gel retardation assays were performed essentially as described in (28) with 20 fmol of the labeled probe in the presence of either the hStaf/ZNF143 DBD (1, 2, 4 and 20 pmol), 2.5 and 5 µl of programmed lysate or 10 µg of HeLa cells nuclear extracts. The various probes containing the wild-type and mutant versions of the SBS in the BUB1B promoter were generated by PCR amplification of regions –342/–196, –342/–265, –281/–196 using ³²P-labeled oligonucleotides.

ChIP assay

The rabbit polyclonal antipeptide antibody against a C-terminal epitope of the *Xenopus* Staf (29) was used for ChIP as essentially described in (23,30). Purified DNA was analyzed by PCR with the test primer pair TAAGTGTTCCTCGCTCGGCTCAGA and CTCAGAGCACCCCTTCCTTCTTC specific for the BUB1B promoter and complementary to positions –427/–404 and +12/+35, respectively. The BUB1B control primers CCACTGTGGGGTGCTGATGTCTGG and CGGGA TGCGGGGGTTGC hybridized to sequences 2647–2623 and 2439–2422 bp, respectively, upstream of the BUB1B ATG initiation codon. The human tRNA^{Sec} gene test primer pair hybridized to sequences –391/–365 and –205/–181 of the human tRNA^{Sec} gene promoter. The human tRNA^{Sec} control primer pair recognizes sequences located at 2555/2530 and 2346/2321 bp upstream of the human tRNA^{Sec} gene. Cycling parameters were 95°C for 3 min, 35 cycles at 95°C for 30 s, 55–65°C (depending on each primer pair) for 30 s, 72°C for 30 s and 72°C for 5 min.

Cell culture, synchronization and cell cycle analysis

Transiently transfected COS-7 cells were arrested at the G1/S boundary by a single or double thymidine block. To block them at the M phase, cells were treated with nocodazole. In brief, for thymidine block and nocodazole treatment, thymidine or nocodazole were added 18 h after transfection to 2 mM or 0.17 mM final concentration, respectively. After 22 h incubation, cells were harvested and used for luciferase and flow cytometry assays. For the

double thymidine block, thymidine was added to 2 mM as the first block. After a 16 h incubation, cells were washed twice with PBS and incubated in complete growth media for an additional 8 h. Thymidine was then added as the second block for 16 h. Subsequently, cells were washed twice with PBS, and complete growth media was added to release them from the block. This time point was set as 0. Cells were harvested at various time points and used for luciferase and flow cytometry assays. For flow cytometry, cells were briefly trypsinized, pelleted by centrifugation at 200 g, resuspended in PBS and fixed in 75% ethanol. After centrifugation and rehydration in PBS for 15 min at room temperature, cells were pelleted and resuspended in 1 ml of staining buffer containing propidium iodide (0.5 mg/ml), 0.1% Triton-X100, 0.1 mM EDTA and RNase A (25 mg/ml). Cell cycle distribution was determined by analyzing their DNA content on a Becton Dickinson FACScalibur flow cytometer.

RESULTS

Characterization of the BUB1B promoter

A search at the database of transcription start sites (DBTSS) (31,32) revealed that transcription of the BUB1B gene is directed from multiple transcription start sites (TSS) located in a 75-bp long region. In this report, promoter numbering starts from the first nucleotide of the translation initiation codon. The TSS region is located between positions -198 and -124, with a major transcription start site at -178 (Figure 1A). To identify the regions responsible for transcriptional regulation of BUB1B, we transiently transfected COS-7 cells with several luciferase reporter constructs containing progressively deleted 5'-flanking regions of the BUB1B gene and then measured the luciferase activity of the resulting cell extracts (Figure 1A and B). The parental construct -1185/-31 contains the region -198/-124 covering the transcription start sites. A deletion from -1185 to -586 resulted in ~5-fold decrease of luciferase activity (Figure 1B, compare constructs -864/-31 and -585/-31 with -1185/-31). Further deletions from -584 to -306 did not significantly affect the transcriptional activity (Figure 1B, compare the activity of constructs -464/-31, -314/-31 and -305/-31 with construct -585/-31). An additional deletion to -237 resulted in ~10-fold decrease of activity. Strikingly, a further deletion to -107 created a construct unable to drive transcription of the reporter gene (Figure 1B, compare -236/-31, -107/-31 and the empty vector Luc with -1185/-31). We concluded from the luciferase assays that transcription of the BUB1B gene was positively regulated by two regions, one located between -1185/-585 and the other between -305/-107.

Transposable elements and potential regulatory motifs in the 5'-upstream region

Sequence comparison and analysis of upstream regions of the BUB1B gene that contain orthologs in the mouse, rat and dog genomes, revealed the presence of transposable elements in regions homologous to -1185/-585 in the

four genomes. Table 1 shows the occurrence of two short interspersed nuclear elements (SINE). In addition, a long-terminal repeat sequence (LTR) is present upstream of the mouse and rat BUB1B genes. Computational analysis of the -1185/-585 region with the MatInspector software (33) did not reveal the presence of interspecies conserved sequences for transcription factor binding sites. Upon computational analysis of the first 464 bp of the BUB1B promoter, no TATA box could be found in the vicinity of the transcription start site. The main transcription start site at -178 is likely to function as an initiator region (Inr) since the sequence TTAAATT located at positions -180 to -174 is very similar to the Inr consensus sequence YYAN(T/A)YY (34). No downstream promoter element (DPE) was found at proximity of the main transcription start site. Interestingly, however, two consensus binding sites for the transcription factor hStaf/ZNF143 (10,11,13) were found in the region -305/-107 which positively regulates BUB1B gene expression. They are called hereafter SBS for simplicity's sake. The first SBS1, located at positions -305/-288, and the second SBS2 at positions -256/-239, were found to be interspecies conserved at 72 and 88%, respectively (Figure 2A). In Figure 2B, the 18-bp SBS1 and SBS2 were aligned with the sequence of the human tRNA^{Sec} SBS (11) and with the consensus sequences determined by binding site selection (11,35). Immediately, upstream of the two SBS is found an interspecies conserved 7 bp ACTACAA motif (Figure 2A), which does not correspond to any binding site for known transcription factors. Furthermore, Figure 2A shows that the -305/-107 region contains blocks of high sequence identity to the CHR (positions -174/-170) and the CDE (positions -165/-161) (see also Figure 6A). These elements are known to be involved in the cell cycle regulated transcriptional repression of many genes (24-26,36-39). In the promoter of these genes, the CDE is generally adjacent to or 1-5 bp upstream of the CHR element (Figure 6A). This contrasts with the BUB1B promoter where the CDE homologous motif precedes the CHR by 4 bp. Finally, the upstream 5'-flanking region of the BUB1B gene contains one CpG island extending from -415 to -50.

hStaf/ZNF143 binds to the BUB1B basal promoter

In the first place, gel retardation assays were performed to determine whether hStaf/ZNF143 does bind the BUB1B promoter. To do this, the ³²P-labeled DNA fragment (-342/-196) encompassing the two putative SBS (probe I in Figure 1A) was incubated with increasing amounts of the purified hStaf/ZNF143 DNA-binding domain (hStaf/ZNF143 DBD). Figure 3A shows that the hStaf/ZNF143 DBD bound with high yield to the BUB1B promoter. Increasing amounts of the protein generated two retarded complexes (C1 and C2 in Figure 3A, compare lane 1 with lanes 2-5). The C1 and C2 complexes were specific because they were competed out by an excess of unlabeled SBS of the *Xenopus* tRNA^{Sec} gene (Figure 3A, lane 6) but not of an unrelated oligonucleotide (Figure 3A, lane 7). We next examined the binding capacities of the hStaf/ZNF143 DBD to a BUB1B promoter

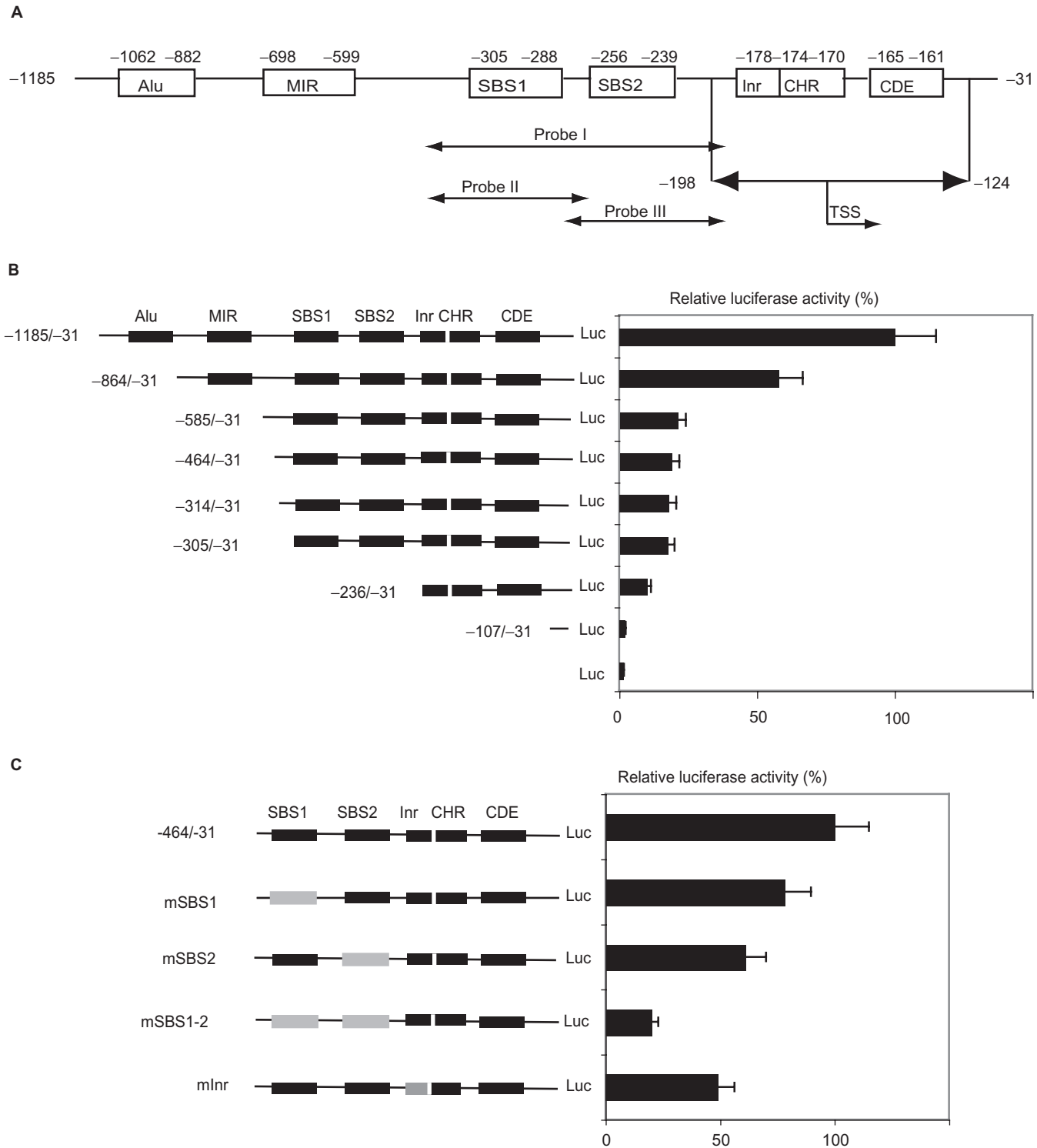


Figure 1. Identification of the human BUB1B gene promoter and mutation analysis of promoter elements. **(A)** Schematic representation of the region located 5' to the BUB1B gene. The different elements identified are boxed. Numbering is relative to the translation initiation codon. The double-headed arrow depicts the region containing the various TSS. Gel shift probes I, II and III are diagrammed below the promoter. **(B)** Schematic representation of the 5'-deleted BUB1B promoter-luciferase constructs and their activities in COS-7 cells. Cells were transiently transfected with the 5'-deleted BUB1B promoter-luciferase (Luc) constructs and assayed for luciferase activity. Values are given as relative luciferase activity normalized to 100 for the longest promoter construct -1185/-31. Data are presented as the mean \pm SD of three separate experiments. **(C)** Schematic representation of BUB1B promoter-luciferase constructs mutated in the Inr-like motif, SBS1 and SBS2 elements and their activities in COS-7 transfected cells. Wild-type and mutant elements are boxed in black and gray, respectively. The relative luciferase activity in % was normalized to 100 with the -464/-31 construct. Data are presented as in (B).

Table 1. Characteristics of the transposable elements localized in the 5' region of the human, dog, mouse and rat BUB1B genes

Organism	Element	Name	Size (bp)	Family	Class	Position/ATG
Human	1	FRAM	180	Alu	SINE	-1062/-882
Human	2	MIRb	115	MIR	SINE	-698/-583
Dog	1	SINEC CF3	166	Lys	SINE	-1256/-1090
Dog	2	MIRb	108	MIR	SINE	-744/-636
Mouse	1	LTR	568			-1609/-1041
Mouse	2	B1_Mus2	69	Alu	SINE	-1040/-971
Mouse	3	PB1	82	Alu	SINE	-929/-849
Rat	1	LTR	541			-1570/-1029
Rat	2	B1_Rn	73	Alu	SINE	-1028/-956
Rat	3	PB1D10	92	Alu	SINE	-921/-830

carrying alterations in the SBS. Three mutant versions of the BUB1B promoter were engineered. In mSBS1, the CCCA sequence at positions 3–6 of SBS1 was replaced by AAAC; the same mutation was introduced in the SBS2 sequence to yield mSBS2. The mSBS1-2 construct combined both mutations simultaneously. It appeared that formation of the retarded complexes was strictly dependent on the SBS integrity. Indeed, whereas mSBS1 and mSBS2 enabled formation of one single retarded complex only (Figure 3B, compare lanes 5–7), the simultaneous presence of both mutations in mSBS1-2 completely abrogated DBD binding (Figure 3B, lane 8). The binding to wild-type or mutant BUB1B promoter of the full-length hStaf/ZNF143, produced from programmed rabbit reticulocyte lysate, was further evaluated. As observed with the DBD, increasing amounts of protein generated the characteristic C1 and C2 complexes (Figure 3C, lanes 2 and 3) which are specific since they disappear in the presence of an excess of the wt SBS but not with an unspecific competitor (Figure 3C, lanes 4 and 5). This binding pattern is strictly dependent on the SBS integrity because the combined presence of the mSBS1 and mSBS2 mutation totally abrogated hStaf/ZNF143 binding (Figure 3C, lane 7).

To ask whether hStaf/ZNF143 is expressed in HeLa cell, we performed gel retardation assays with HeLa cell nuclear extracts and probes II and III containing SBS1 and SBS2, respectively. In such an experiment, we expect finding one single complex with each of the probe. This was effectively the case, as shown in Figure 3D (lanes 2 and 10). The specificity of binding was attested by the competition obtained with an unlabeled hStaf/ZNF143 consensus oligonucleotide (Figure 3D, lanes 3, 4, 11) but not with an unrelated unlabeled oligonucleotide (Figure 3D, lanes 5, 6, 12). The presence of hStaf/ZNF143 in the complex was assessed by the displacement of the complexes observed with an anti-hStaf/ZNF143 (Figure 3D, lanes 7 and 13) but not with a pre-immune antibody (Figure 3D, lanes 8 and 14). To further validate these findings, the association of hStaf/ZNF143 to the human BUB1B promoter *in vivo* was investigated with the chromatin immunoprecipitation assay (ChIP). Chromatin, formaldehyde cross-linked with sheared DNA 0.5–1 kbp in length, was prepared from HeLa cells and incubated with an antipeptide antibody directed against the C-terminal part of hStaf/ZNF143 (29) or with a control

pre-immune antibody. The recovered DNA was analyzed by semi-quantitative PCR with primers spanning the SBS of the BUB1B promoter (test sequence) or a region located 2.5 kbp upstream of it (control sequence). The analysis was performed with two dilutions of the DNA obtained from anti-hStaf/ZNF143 and pre-immune ChIP. We also tested a serial dilution of the input material to demonstrate that the PCR was quantitative within a linear range of concentration. A specific signal, absent with the pre-immune antibody, was obtained with the DNA immunoprecipitated with the hStaf/ZNF143 antibody (Figure 4A, compare lanes 1, 2 and 3, 4 of the test). In contrast, no specific signal could be obtained with the control primer pair (Figure 4B, lanes 1–4 in the control). As an additional control, the same DNA samples were used to show the binding of hStaf/ZNF143 to the human tRNA^{Sec} gene promoter, known to be targeted by hStaf/ZNF143 (11) (Figure 4B). As expected, a specific signal was obtained only for the PCR reaction using the DNA immunoprecipitated with hStaf/ZNF143 and performed with the test primer pair (compare lanes 2 and 3 of the test and control reactions). Collectively, these results demonstrate the presence of two SBS and the association of hStaf/ZNF143 to the BUB1B promoter *in vivo*.

Effects of hStaf/ZNF143-binding site and Inr mutations on BUB1B promoter activity

The effect of the debilitating mutations mSBS1, mSBS2 and mSBS1-2 was assessed *in vivo* by introducing them into construct -464/-31 (Figure 1C). The mutant constructs were then transfected into COS-7 cells and promoter activities were reported by the luciferase activity. Mutation of the SBS1 and SBS2 resulted in a slight decrease to 78 and 61% of the wild-type level, respectively (Figure 1C). The simultaneous mutation, however, induced a much more pronounced effect since the activity dropped to 19% of the wt level (Figure 1C), indicating that SBS1 and SBS2 are of prime importance to BUB1B promoter activity. We also observed that the TTAAATT sequence (positions -180 to -174) is very similar to the Inr consensus sequence YYAN(T/A)YY (34). To test the functional importance of this interspecies conserved motif, the TTAAATT sequence was changed to TTCCCTT. The activity of the luciferase reporter decreased to 49% of the wild-type level (mInr in Figure 1C). From this data, we

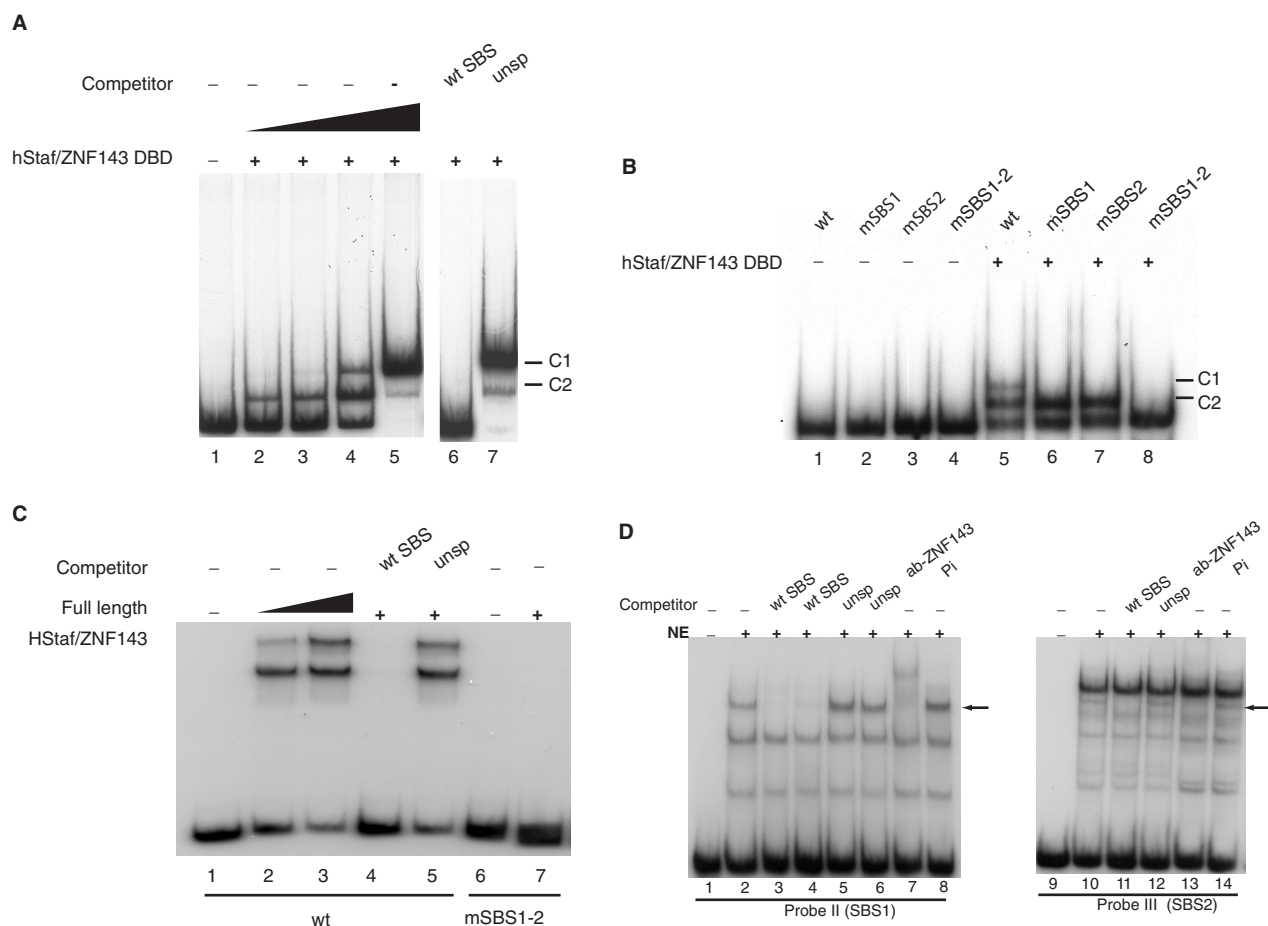


Figure 3. hStaf/ZNF143-binding assays on wild-type and mutant versions of the BUB1B promoter. (A) Gel retardation assay with a fragment encompassing positions $-342/-195$ of the BUB1B promoter. The ^{32}P labeled DNA (probe I) was incubated in the absence (lane 1) or presence of increasing amounts of hStaf/ZNF143 DBD (lanes 2–5). The reactions in lanes 6 and 7 were performed with the same amount of protein as in lane 5 but in the presence of a 1000-fold excess of unlabeled specific (wt SBS) and unspecific competitors (unsp). Binding assays in lanes 1–5 and 6, 7 were performed in separate experiments. C1 and C2: complexes containing one and two proteins, respectively. (B) A 148-bp 5' end-labeled fragment containing the wild-type or mutant versions of the SBS was used in the binding studies. Lanes 1–4, no protein added. Lanes 5–8 binding assays with the same amount of DBD. Probes are indicated above the lanes. (C) The wt (lanes 1–5) or the SBS1 and SBS2 mutant version borne by probe I (lanes 6 and 7) were incubated in the absence (lanes 1 and 6) or presence of increasing amounts of rabbit reticulocyte lysate containing full-length hStaf/ZNF143 (lanes 2 and 3). Lane 7 contained the same amount of protein as in lane 3. Lanes 4 and 5 were performed with the same amount of protein as in lane 3 but in the presence of a 1000-fold excess of unlabeled specific (lane 4, wt SBS) and unspecific competitor (lane 5, unsp), respectively. C1 corresponds to both sites of the probe saturated with the protein; the protein binds to one of the sites only in C2. (D) Gel retardation assay with the wt probes II (lanes 1–8) or III (lanes 9–14) in the absence (lanes 1 and 9) or presence (lanes 2–8 and 10–14) of HeLa cell nuclear extracts (NE). The reactions in lanes 3, 4 and 11, 5, 6 and 12, 7 and 13, 8 and 14 were performed in the presence of unlabeled specific competitor (wt SBS), unspecific competitor (unsp), anti-hStaf/ZNF143 and pre-immune antibody, respectively. The specific competitor was added at a 500-fold (lane 3) and 1000-fold molar excess (lanes 4, 11). A 500 and 1000-fold molar excess of unspecific competitor was contained in lanes 5 and 6, 12 respectively. The arrow points to the complexes mentioned in the text.

using probe II (Figure 1A) containing the SBS1 and nuclear extracts from synchronized COS-7 cells. Figure 6B shows that the binding of hStaf/ZNF143 was similar with cells in the G1/S and G2/M cell cycle. In the light of these findings, we speculated that hStaf/ZNF143 did not play a central role in the G2/M-specific transcription of BUB1B and that another mechanism must be involved in the G2/M specificity. Indeed, transcription of the TCP1 (14), IRF3 (15), TALDO1 (17), MRPS11 (19) and SYBL1 genes (20) is controlled by hStaf/ZNF143 but is not cell cycle dependent (7). Furthermore, expression of the ZNF143 gene is known not to be cell cycle regulated (7).

Effects of mutations in the CDE and CHR homologous elements on the cell cycle dependent BUB1B transcription activity

The G2/M specific transcription of many genes such as CDC25C, CDC2, CCNA2, PLK1 and RB6K (24,26,27,40) is regulated by a tandem of repressor elements, the cell cycle dependent element (CDE) and the cell-cycle genes homology region (CHR). We found that two sets of sequences at positions $-174/-170$ and $-165/-161$ bear a strikingly high similarity to the CHR and CDE consensus sequences (Figure 2A). The difference resides in their relative organization,

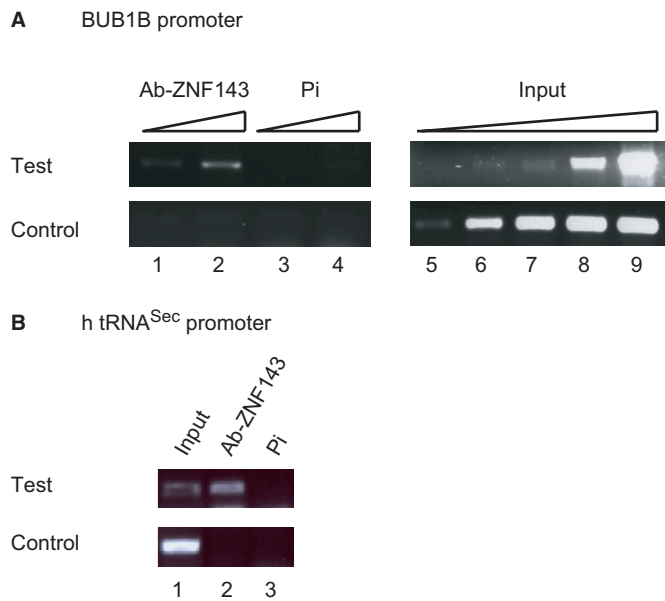


Figure 4. hStaf/ZNF143 is associated to the BUB1B gene promoter in HeLa cells. Genomic DNA fragments, recovered from input material or immunoprecipitated with hStaf/ZNF143 or pre-immune antibodies, were subjected to PCR amplification with primer pairs specific for the promoter or upstream regions. **(A)** PCR analysis of the BUB1B promoter. Lanes 1, 2 and 3, 4: two different amounts (1 and 5%) of immunoprecipitated DNA with hStaf/ZNF143 (Ab-ZNF143) or pre-immune antibodies (Pi), respectively. Lanes 5–9: increasing amounts of input material (0.002, 0.01, 0.05, 0.2 and 1%) were analyzed to demonstrate that the assays were within the linear range of PCR amplification. PCR primer pairs were specific for the promoter region of the BUB1B gene (test assay) or for a unique region located ~2.5 kbp upstream of the BUB1B gene (control assay). **(B)** PCR amplification was performed with a primer pair specific for the human tRNA^{Sec} (htRNA^{Sec}) gene promoter (test assay) or a region located 2.4 kbp upstream of it (control assay). Lanes 1, PCR performed with 0.01% of input DNA. Lanes 2 and 3, PCR performed with 1% of DNA recovered from CHIP.

the CHR motif lying upstream of the CDE in the BUB1B promoter (Figure 7A). These observations suggest that the two putative *cis*-elements can also function as a G1/S-specific repressor, as previously reported for the G2/M-specific genes. To test the hypothesis, we introduced mutations in the putative CHR and CDE motifs (mCHR and mCDE in Figure 7B). After transfection into COS-7 cells, the luciferase activity was measured in cells arrested in G1/S with thymidine or in G2/M phase transition by nocodazole (Figure 7C). Drug treatment resulted in the synchronization of at least 85% of the cells in the various phases as determined by propidium iodine staining and flow cytometry (data not shown). The luciferase activity in extracts of cells transfected with the wild-type -464/-31 and arrested in G1/S was 2.2-fold lower than that from cells arrested in G2/M (Figure 7C and D). In contrast, transfection of the CHR and CDE mutants of the BUB1B promoter-luciferase constructs abolished cell cycle periodicity and resulted in a 2.8- and 3.1-fold enhanced transcription activity relative to the wild-type level in G1/S arrested cells (Figure 7C and D). In G2/M arrested cells transfected with the CHR and CDE mutants, however,

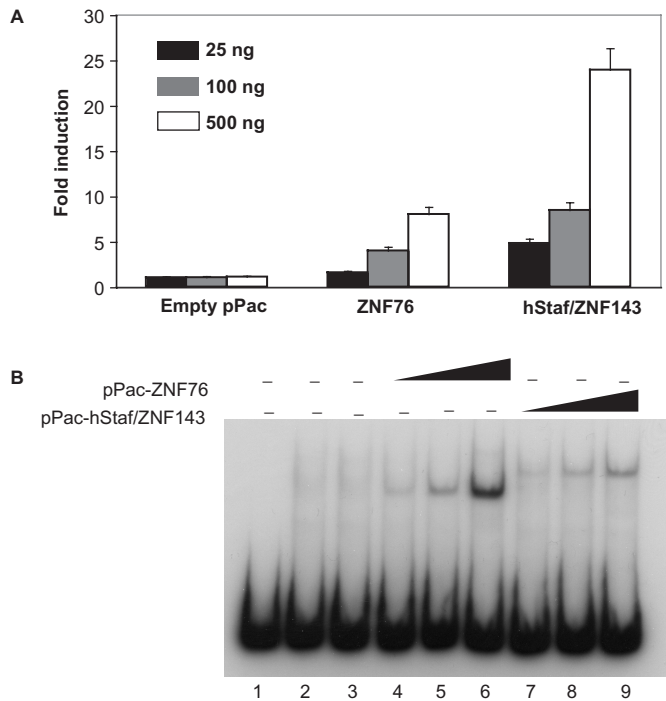


Figure 5. hStaf/ZNF143 and ZNF76 transactivate the human BUB1B promoter in *Drosophila* Schneider cells. **(A)** SL2 cells were cotransfected with the -464/-31 luciferase reporter gene construct and increasing amounts of empty pPac, pPac-hStaf/ZNF143 or pPac-ZNF76 expression vectors (25 ng, solid boxes; 100 ng, gray boxes; 500 ng, open boxes). Data are expressed as the fold of induction of luciferase activity normalized to that obtained following co-transfection of the reporter with the empty vector pPac. Values are the mean \pm SD of at least three independent transfection experiments. **(B)** Expression of ZNF76 and hStaf/ZNF143 in transfected SL2 cells. Expression of the proteins in SL2 cells was confirmed by gel retardation assays. The wt probe II containing SBS1 was incubated with untransfected SL2 extracts (lane 1), transfected SL2 extracts with empty pPac (lane 2), transfected SL2 extracts with pPac-ZNF76 (lanes 4–6) or pPac-hStaf/ZNF143 (lanes 7–9). Lanes 4 and 7, 5 and 8, 6 and 9: probes incubated with extracts from SL2 cells transfected with 25, 100 and 500 ng of effectors, respectively.

the measured activities were similar to the wild-type (Figure 7C and D). These results strongly suggest that CDE and CHR act as G1/S-specific repressor elements in the BUB1B promoter and are essential for the cell cycle expression of this gene.

DISCUSSION

The BUB1B gene, which is highly expressed in cells with a high mitotic index, exhibits a cell cycle dependent expression with an undetectable transcription in G1 and a gene expression peak in G2/M (6,7). In the present study, we investigated the transcriptional regulation mechanism of the human BUB1B gene. We identified that regions -1185/-585 and -305/-107 in the promoter are involved in the transcriptional regulation of the BUB1 gene. Region -1185 to -585, which performs a positive regulation on BUB1B gene expression, contains two transposable elements. Sequence comparison of the regions homologous to -1185/-585 in other mammalian

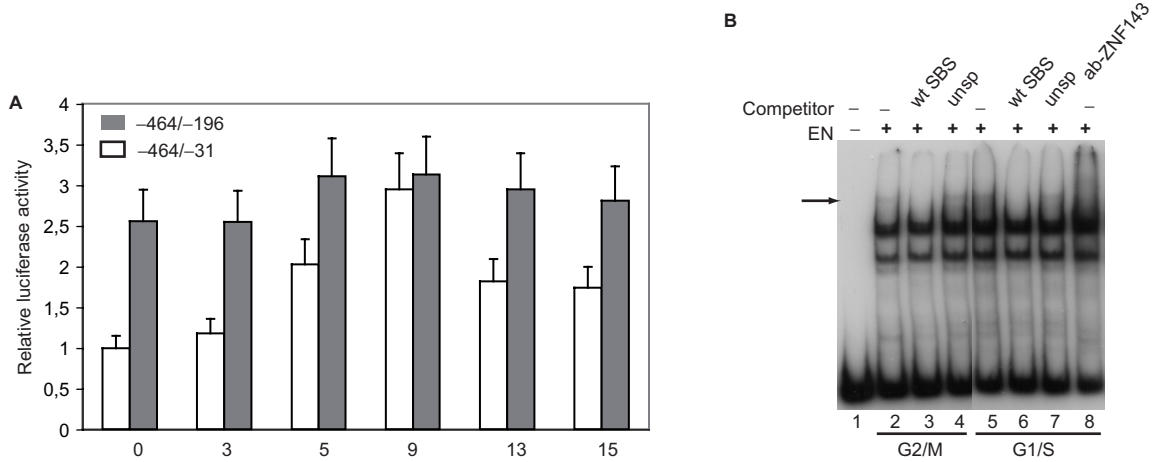


Figure 6. The luciferase activity arising from the construct containing the BUB1B promoter is regulated during the cell cycle. **(A)** COS-7 cells were transiently transfected with -464/-31 (open boxes) or -464/-196 (solid boxes) constructs. Cells were synchronized with a double thymidine block and released (time 0) and harvested at the indicated times for luciferase assays. Data are presented as the mean \pm SD of three independent experiments. **(B)** Gel retardation assay with the wt probe II in the absence (lane 1) or presence of COS-7 nuclear extracts from cells synchronized in G2/M (lanes 2-4) or G1/S (lanes 5-8). Reactions in lanes 3 and 6, 4 and 7, 8 were performed in the presence of unlabeled specific competitor (wt SBS), unspecific competitor (unsp) and anti-hStaf/ZNF143 antibody, respectively. Position of the specific complex is indicated with an arrow.

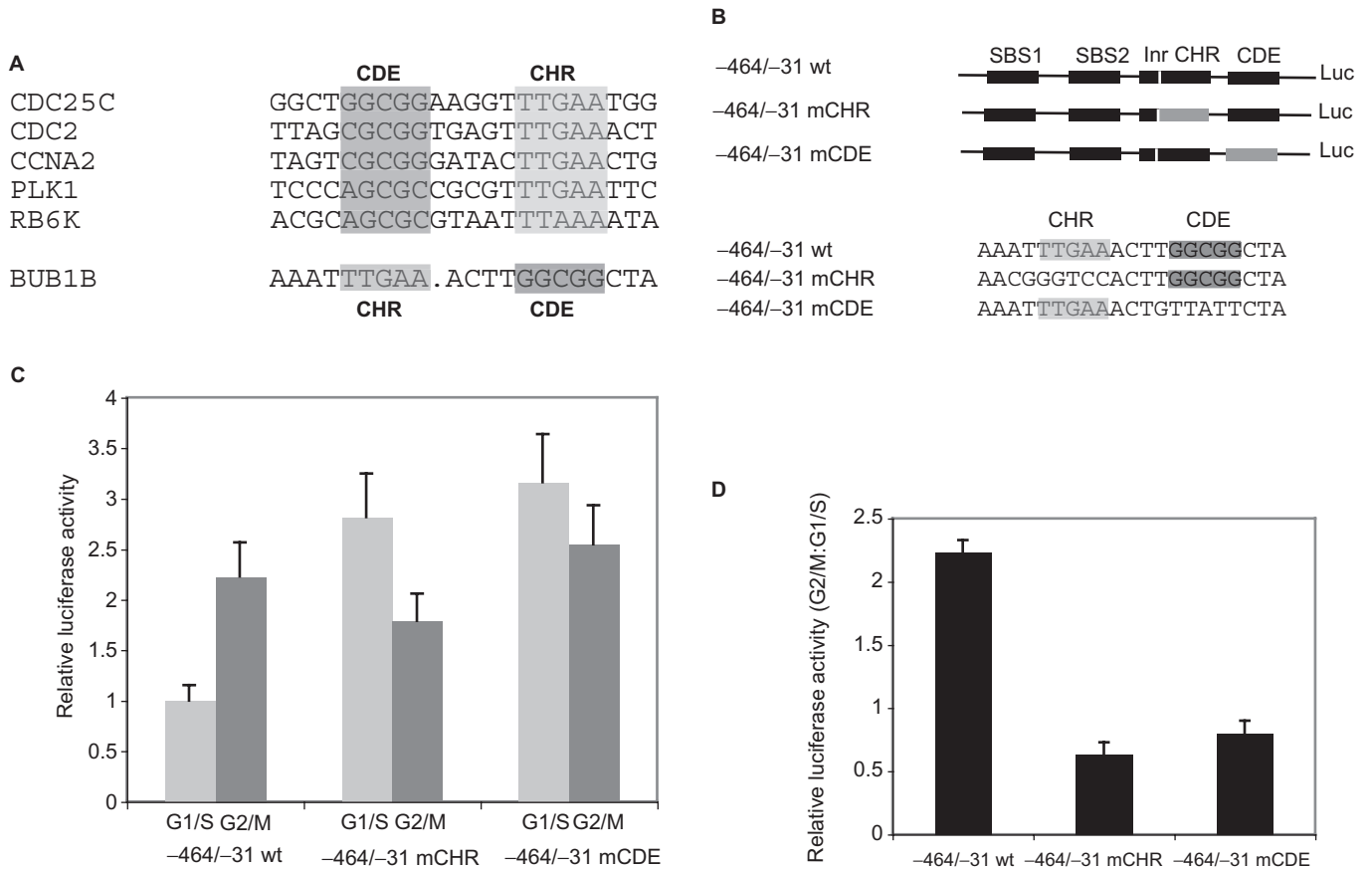


Figure 7. Effects of mutations of the CDE and CHR elements on the BUB1B promoter activity. **(A)** Alignment of the CDC25C, CDC2, CCNA2, PLK1 and RB6K promoter sequences in the region of the CDE and CHR elements (25-27). CDE and CHR elements are highlighted in gray. The bottom line displays the part of the BUB1B promoter sequence harboring high identity with the CDE and CHR motifs. **(B)** Schematic representation of the BUB1B luciferase constructs and sequences of the wild-type and mutant promoters. Here, -464/-31 mCDE and -464/-31 mCHR correspond to substitutions including the conserved GGCGG and TTGAA, respectively. **(C)** Transient transfection experiments into COS-7 cells with the wild-type -464/-31 and mutant -464/-31 mCHR and -464/-31 mCDE constructs. Following transfection, cells were treated with thymidine to block exit from G1/S (open boxes) or with nocodazole to block that from G2/M (solid boxes). Cells were harvested and assessed for luciferase activity. **(D)** The relative luciferase activity of cells in G2/M versus G1/S is given for the wild-type and mutant constructs. Data are presented as the mean \pm SD of three independent experiments.

genomes did not reveal the presence of conserved transcription factor binding sites. This suggests that the positive regulation is performed by species-specific elements. Similar to other cell cycle regulated genes, the BUB1B promoter does not possess a TATA box and, as a consequence, gives rise to many transcription start sites. We identified five interspecies conserved *cis*-acting elements in the 5'-flanking region -305 to -107, and a trans-acting factor involved in the control of BUB1B gene expression. Three *cis*-acting motifs positively regulate transcription and the other two constitute a cell cycle dependent transcriptional repressor. The motif surrounding the major transcription start site is likely to function as an initiator. The other two positively *cis*-acting motifs, SBS1 and SBS2, are specifically recognized by hStaf/ZNF143 which itself was found associated to the BUB1B promoter in HeLa cells. Mutations of SBS1 and SBS2, singly or in combination, led to a sharp decrease of reporter activity. Analysis of the BUB1B promoter activity in insect cells demonstrated that hStaf/ZNF143 and ZNF76 can reconstitute BUB1B promoter activity. Although Staf was originally identified as the transcription factor regulating tRNA^{Sec} gene transcription, it also controls snRNA, snRNA-type and mRNA gene expression (10,11,14-20,41). The present study extends the role of hStaf/ZNF143 to BUB1B gene transcription. The two identified SBS1 and SBS2 sites, distant by 31 bp, were found associated to the 7bp ACTACAA motif which lies immediately 5' to the SBS. This motif, under our conditions, is apparently not involved in transcription activity of the BUB1B promoter since we observed that the 5' deletion eliminating the ACTACAA sequence did not affect promoter activity. Scrutiny of the sequences flanking the SBS in snRNA and snRNA-type genes did not reveal the presence of an ACTACAA-associated motif. In contrast, it is found in the SBS characterized in the SYBL1 promoter (20) and in 58% of the SBS that we recently identified by a genome scale analysis (23). The finding that the SBS are essential for expression of the BUB1B gene raises the interesting question of whether hStaf/ZNF143, the main activating factor of the Staf family, is involved alone in transcription activation of the BUB1B gene. Alternatively, could ZNF76 also play a role in the expression of this gene? Although the ChIP assay was performed with antibodies recognizing specifically hStaf/ZNF143, this does not exclude the possibility that ZNF76 can also be involved in BUB1B expression.

The other two *cis*-acting motifs that we identified are localized in the region covering the transcription start site and we showed that they function as cell cycle dependent repressor elements. They harbor high identity with the consensus sequences of the CDE (G/CGCGG) and CHR (TTGAA) elements identified in the cell cycle regulated promoter genes such as CDC25, CDC2, CCNA2 (coding for cyclin A), PLK1 (polo-like kinase) and RB6K (rabkinesine 6) (24-27). These two elements are known to induce repression of transcription and we established that their mutation led to almost complete impairment of the cell cycle dependent transcription activity of the BUB1B promoter. Worth of note, however, the tandem

Table 2. Characteristics of the SBS identified in the RBL2, PLK1 and BIRC5 gene promoters

Gene	SBS sequence	Position
RBL2 (p130)	TCCCCAGCAGCCCGTGCT	-166
PLK1 (polo-like kinase)	CTCCCATGGTGCCGCGCG	-88
BIRC5 (survivin)	CTCCCGGCACACCCCGCG	-111
BIRC5 (survivin)	CTCCCAGAAGGCCGCGGG	-79
BIRC5 (survivin)	CTCCCGACATGCCCGCG	-35

repressor element is organized in the CHR-CDE configuration in the BUB1B gene whereas the arrangement CDE-CHR occurs in all the other identified repressors.

Careful inspection of the promoter sequences of other genes known to be regulated with a tandem of CDE and CHR repressor elements pointed to the presence of SBS in the promoters of the RBL2 (p130 protein), PLK1 (polo-like kinase protein) and BIRC5 (survivin protein) genes (26,38,42,43) (Table 2). Studies on genes that are cell cycle regulated by the CDE-CHR tandem of repressor elements (CDC25C, CDC2 and aurora A) revealed that ubiquitously expressed transcription factors such as Sp1, NF-Y and E4TF1 act positively by binding to target sequences upstream of the CDE-CHR motifs (36,39,40). The cell cycle dependent repression of upstream activators via the CDE and CHR elements has been established as the major regulatory mechanism (36,40). As shown by genomic footprinting on these promoters, both CDE and CHR elements are protected in a periodic fashion, suggesting involvement of a specific factor. A binding activity termed CDF-1 has been identified and proposed to interact with both CDE and CHR in the CDC25C gene promoter (44). In a further work, a factor (CHF) interacting specifically with CHR has been isolated in the CCNA2 gene promoter, encoding cyclin A (45). In the situation described for the CDC25C promoter, it was proposed that CDF-1 presumably functions by specifically repressing the NF-Y mediated activation (44). However, no further characterization of the CDF-1 and CHF complexes was undertaken. Recently, a very interesting mechanism was reported to involve the CDE and CHR elements in chromatin remodeling of the CCNA2 promoter. In this case, the CDE-CHR tandem which is needed for repression in quiescent cells, was also demonstrated to be required for organizing the chromatin structure specific for the inactive promoter (46). However, this particular nucleosome organization becomes disorganized as a consequence of mutations in the CDE-CHR repressor element. In the case of the BUB1B promoter, we have clearly demonstrated the functionality of the CDE-CHR tandem as a repressor element, but the precise underlying mechanism remains to be investigated.

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