A CDE/CHR tandem element regulates cell cycle-dependent repression of *cyclin B2* transcription

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Abstract Cyclin B is an important regulator of progression through the cell division cycle. The oscillating appearance of cyclin B1 and B2 proteins during the cell cycle is in part due to fluctuating mRNA levels. We had identified earlier a tandem promoter element named cell cycle-dependent element (CDE) and cell cycle genes homology region (CHR) which regulates cell cycle-dependent transcription of *cdc25C*, *cyclin A* and *cdc2*. Here we describe that *cyclin B2* transcription is repressed through a novel CDE/CHR element in resting and G_1 cells. By relief of this repression in S and G_2 oscillating expression of *cyclin B2* mRNA is achieved during the cell cycle.

Key words: Cell cycle; CDE; CHR; E2F; Transcriptional repression; Cancer

1. Introduction

Cyclin B is a central molecule regulating progress through the cell division cycle. B-type cyclins associate with the cdc2 protein kinase to form the maturation-promoting factor (MPF) [1–4]. The MPF complex is essential for the transition from G_2 to mitosis [5,6]. The importance of B-type cyclins was also shown by creating mice with deletions in the genes for the two identified mammalian proteins, cyclin B1 and B2. While knockout mice with deletions in the *cyclin B1* gene die in utero, *cyclin B2*-null mice are viable. Therefore it seems that *cyclin B1* can compensate for loss of *cyclin B2*, but not vice versa. Although *cyclin B2* (−/−) mice appear to be normal they seem to be less fertile and tend to be slightly smaller [7]. However, when human B-type cyclins are expressed in a *cln*$^-$ yeast background cyclin B2 displays a stronger growth-promoting capability than cyclin B1 [8]. Other results also suggest distinct roles for the different forms of cyclin B. It has been shown that subcellular distribution is dramatically different. While cyclin B1 co-localizes with microtubules cyclin B2 is associated with the Golgi apparatus [9].

One general feature of cyclins is that their protein levels oscillate during the cell cycle [10]. B-type cyclins appear in S-phase and accumulate in G_2 and mitosis before disappearing at transition from metaphase to anaphase [11]. The two ways by which these oscillations are maintained are by regulating synthesis and degradation. In regard to their degradation in recent years great progress has been made in understanding ubiquitin-mediated proteolysis leading to the dramatic drop in cyclin B protein levels in the dividing cell at the metaphase–anaphase transition [11–15].

It has been shown that mammalian *cyclin B1* and *cyclin B2* are regulated at the transcriptional level during the cell cycle. Experiments addressing cell cycle-dependent transcription of *cyclin B1* excluded some candidate promoter elements from being relevant for regulation [16]. Part of the cell cycle-dependent transcriptional regulation might be due to an E-box element in this promoter [17]. We have cloned the mouse *cyclin B2* promoter and started to analyze its regulation [11,18]. The mechanism by which *cyclin B2* transcription is regulated during the cell cycle has still to be elucidated.

Transcriptional regulation during the mammalian cell cycle has so far been mostly associated with the function of the E2F family of transcription factors and the retinoblastoma tumor suppressor protein pRb [19–23]. Heterodimers formed by E2F and DP components are able to activate transcription of cell cycle promoters in late G_1- and S-phase. In G_0 and early G_1 complex formation of pRB with E2F/DP represses transcription. In a normally dividing cell transition from this repression in the early phase of the cell cycle to activation at later times is controlled by phosphorylation of pRb [19–23].

More recently we have identified another mechanism by which gene transcription can be regulated in a cell cycle-dependent manner. This mechanism employs a tandem transcriptional element composed of the 'cell cycle genes homology region' (CHR) and the 'cell cycle-dependent element' (CDE). CDE/CHR sites regulate the promoters of *cyclin A*, *cdc25C* and *cdc2* genes by repression in G_0 and G_1 and release from repression later in the cell cycle [24]. We have described earlier that the expression of *cyclin B2* is largely driven by activation through three CCAAT promoter elements which bind NF-Y transcription factors [18]. Here we show that transcription from the *cyclin B2* promoter during the cell division cycle is dependent on a novel CDE/CHR element.

2. Materials and methods

2.1. Cloning and mutation of cyclin B2 promoter luciferase constructs

A genomic DNA fragment was amplified from a plasmid as template which was described earlier [11] with the primers 5′-CGG GGT

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ACC CAA GAA AGA GAA AGC TAT GC-3′ and 5′-AGA AGA TCT GGC CCC GCC GCT CCG CGT C-3′. This created a DNA segment −69 bp to −1189 bp 5′ from the translational start codon of the mouse *cyclin B2* with restriction sites for *Kpn*I and *Bgl*II. These sites were used to clone the fragment into the pGL3 basic vector (Promega) to create the B2-Luci construct driving the expression of firefly luciferase.

Mutations were introduced by employing the transformer site-directed mutagenesis kit (Clontech) and a primer (5′-CAA TAG TGC GTC AGC ATT ACG GTA TTT GAA TCG CGG ACC GG-3′) for mutating the CDE and another oligonucleotide (5′-CAG CGG CGC GGT ATG CAT ATC GCG GAC CGG GCG GTG G-3′) to change the CHR. Nucleotide sequences of all constructs were confirmed by sequencing both strands.

2.2. Cell culture and DNA transfection

Omega E mouse fibroblasts (DSMZ, Braunschweig, Germany) are derived from NIH3T3 cells. They were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO_2. For transfection 4×10^4 cells were plated per well in 0.5 ml medium in 24-well plates. Cells were cultured overnight before transfection. Transfection of cells was done by using 1.5 μl Pfx-6 (Invitrogen) with 0.5 μg plasmid of B2-Luci, its derivatives or a *cyclin A* construct and 10 ng of pRL-SV40 (Promega) in 0.25 ml Optimem transfection medium (Gibco) per well. The *cyclin A* reporter construct PALUC was described by Henglein et al. [25]. For transfection, cells were incubated for 5 h before replacing transfection medium with DMEM containing 10% FCS. Cells were cultured for 1 day in this medium. For cell cycle analyses cells were then starved in DMEM with 0.2% FCS for 60 h. At this time cells were harvested for 0 h time points. To analyze cells during their passage through the cell cycle the remaining mouse fibroblasts were stimulated with 20% FCS in DMEM for the given times.

2.3. Luciferase assays and FACS analyses

Two kinds of luciferase activities were determined with the dual luciferase assay (Promega), firefly luciferase and luciferase from *Renilla reniformis*. Luciferase activities were normalized by comparing firefly luciferase with *Renilla* luciferase activities expressed from the pRL-SV40 (Promega) cotransfected control plasmids in a Turner Design TD 20/20 luminometer. Averages of 12 assays were included in the cell cycle experiments for each point and standard deviations derived. Stimulation factors for each construct were calculated by dividing maximal luciferase activities after serum stimulation by values before addition of serum. Deregulation is the change of the stimulation factor in the mutants versus the stimulation of expression from the wild-type *cyclin B2* plasmid.

Fixation of cells and propidium iodide staining was performed according to a published procedure [26]. Cell sorting was done on a FACScan analyzer with the CELLQuest and ModFit programs (Becton Dickinson).

3. Results and discussion

3.1. Cell cycle-dependent transcription from a cyclin B2 promoter construct

A genomic DNA fragment covering a region of 1.1 kb upstream of the coding region in the mouse *cyclin B2* gene, originally identified by us [11], was cloned into a luciferase expressing reporter plasmid resulting in the B2-Luci construct. Sequencing of the *cyclin B2* fragment and comparison with known transcriptional elements revealed three CCAAT consensus boxes spaced at a distance of 33 bp from each other (Fig. 1). Two transcriptional start sites have been mapped [11]. The genomic fragment does not contain any obvious TATA consensus element (Fig. 1).

In transient transfection assays the *cyclin B2* promoter in B2-Luci confers regulation like the chromosomal gene [11,27]. No significant transcription is observed in G_0 and G_1 cells. Expression starts in S-phase and increases into G_2 before dropping again (Fig. 2A and Table 1). Also timing during the cell cycle relative to *cyclin A* appears consistent with previous observations. Comparison of the expression of B2-Luci to the luciferase activity from a *cyclin A* reporter shows that the two reporters behave like their cellular gene counterparts (Fig. 2A and B) [11,27].

3.2. CDE and CHR elements are regulating cyclin B2 cell cycle-dependent transcription

The *cyclin B2* promoter carries a number of elements potentially relevant for cell cycle-dependent transcription. We mutated some of these elements on the basis of the B2-Luci construct and found their regulation essentially unchanged compared to the wild-type construct (data not shown). Further inspection of the sequence in the 5′-region of the *cyclin B2* gene revealed five sequences identical to the consensus sequence 5′-TTGAA-3′ (Fig. 1) which we had earlier identified as a CHR element in the *cdc2*, *cyclin A* and *cdc25C* promoters [24]. There is only one potential CHR element that is located downstream from a DNA segment that displays some simi-

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-1189 CCCAAGAAAGAGAAAGCTATGCGTAACCTCTGTGATTGCAAGGATGATGGACCAAGAGTCAGCAGCCACAGAACTTTCAT
-1109 TTCCTTTAGCAGGTACTTGAGAGAACTTAATGGGATATAAATAGTTATTTCTGCTCCTTTGGTTTCCAATATCCATGTGT
-1029 AATTTAGGTCGAGGGGTAGTACTGCCACCTTGTCTTTTCAGGTGGGAGTGTTGGACAACACTGGTTCTTCCTTTAGACTC
-949  AGATTGGAAATGTCAGATTTGGGCGAAGGGGGAGGGAGCATTTATTATTTCCCAATAGAAGGGACTTGAACAAGACTGAT
-869  GGGGTAGCCTGATCTCAAGTCGTTCTGCTGGCACTGCACCATTTTCTGTGTTGTTACCTAGTCTTTTCCCCAGGGTAATA
-789  TTCTGGTTTTGTTTTTTGTTTTTGTTTTTGTTTTCTGTCTTGTCTCTCACTGTTCACAACTTGGGATACTCATTATTTTT
-709  CTTTTGAAATCTTGTGCACATCACACCGTCATTTGACAAATAGTTTCTGAGTTCTACTGTATTGTGCTGGAAGTCACACA
-629  AATCACGTCTAGTTTGAATTATGACTCTGCTGGGGATCTGTTTTAGGAAGACCACATACAGTACACATCCGAGTCACAAT
-549  CATCTTGTAGCCCTGAACGACCTGGAGACGATGGCTTCATTTGCCAGACCATTCCAATCCAAATTCCCAGGTAGACGTTT
-469  CCGGAAGAGGAAGTAAGGTCAGAAGTAGCATTTCCAGGAAGTTCTCTGGAACCCACTTCTACCTAACCCAGAACAAACTT
-389  TTAAAGACATATGTCCTTAGGTGATGGCTCCCCACTCCTACCCCTAAAATTAAGACAATCTTTTCCAGTAGGAGGAAAAA
-309  TTATTTTATTTAATATCAGGGACTAGAATTTGAAAATAAGACTGTAGACAAGGAAACAACAAAGCCTGGTGGCCTCGCTG
                                  NF-Y                                  NF-Y
-229  GTTGCTATGACAAGCAAATACAAGCCAGCCAATCAACGTGCAGAAAGGCCTTCCAGTCTAGCCAATGGGTTGCGCGGCCC
                    NF-Y          ┌► CDE        CHR                     ┌►
-149  CTGCGTGCGTCTACCCAATAGTGCGTCAGCGGCGCGGTATTTGAATCGCGGACCGGGCGGTGGACGCGGAGCGGCGGGGC
                                                                        +1
-69   CCTGACCCTCCCAACGGTGTCGCAGACCGGAGTGGCTGTGCCTCGTCCGCACTTGCCAGGGCGGCCCTCATGGCGCTGCT
                                                                        M  A  L  L
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Fig. 1. Nucleotide sequence of the mouse *cyclin B2* promoter. Numbering is relative to the translational start codon. The elements responsible for activation through NF-Y transcription factors are printed in bold. Two transcriptional start sites are indicated by arrows. Two elements which display some homology to cell cycle-dependent elements (CDE) and the cell cycle genes homology region (CHR) are boxed.

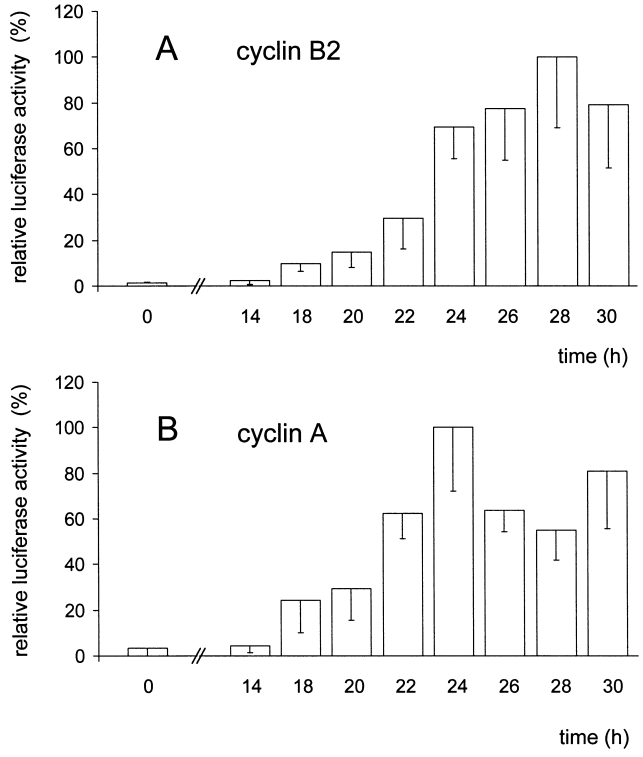

Fig. 2. Expression of luciferase reporter constructs during the cell cycle in resting and restimulated NIH3T3-derived Omega E mouse fibroblasts. Cells were cultured 24 h after transfection and serum starved for 60 h before restimulation. Time after restimulation is indicated. Activity from firefly luciferase reporter assays is given as percent of the maximum value. Values are averages of 12 assays per time point and standard deviations are given. A: Transcription from the mouse *cyclin B2* promoter in the B2-Luci construct. B: *cyclin A* transcriptional activity from the PALUC plasmid.

larity to CDE sites which are also required to form a functional CDE/CHR repressor element (Fig. 1).

We tested the function of the two putative cell cycle elements in transfection assays after mutating each half of the tandem site separately. Both mutations lead to a deregulation of cell cycle-dependent transcription (Fig. 3 and Table 1). This shows that cell cycle-dependent transcription of *cyclin B2* is contingent upon intact CDE and CHR elements in its promoter. Stimulation of activity for the wild-type *cyclin B2* construct comparing resting and G_2 cells is about 68-fold in the experiment shown. By mutation of the CDE or the CHR this factor is reduced to 14- or 4.5-fold, respectively. From these results factors for deregulation of the mutants can be calculated as about 5-fold for the CDE mutant and 15-fold for the CHR mutant (Fig. 3B). This indicates that the CHR has a higher impact than the CDE on the cell cycle-dependent regulation of the *cyclin B2* promoter and that the CDE and CHR elements are responsible for most of the cell cycle-dependent transcription. Comparing the transcriptional activity in resting and in G_2 cells the remaining regulation in the CHR mutant is only similar to that of the *SV40* promoter which served as a control (Fig. 3A). The observation that such cell cycle-dependent regulation is not completely lost has been made earlier and could be due to a rise in general transcriptional activity during the cell cycle [24].

At the end of the cell cycle both mutant constructs show similar activities compared to the wild-type *cyclin B2* promoter. To normalize for transfection efficiency we calculated ratios of luciferase activities from relative light units of firefly luciferase expressed from *cyclin B2* plasmids and light units of *Renilla* luciferase transcribed from an *SV40*-driven control plasmid. The ratios at 26 h were 0.26, 0.21 and 0.39 for B2-Luci, B2-CDE-mut and B2-CHR-mut, respectively. Thus, the three *cyclin B2* constructs are expressed at a similar level at G_2/M. The two mutant reporters are already expressed at significant levels in resting cells whereas in G_0 the wild-type *cyclin B2* plasmid drives expression only at the detection limit (Fig. 3A). Therefore, maximal activities reached are not affected by the mutations and activator elements do not seem to be compromised in the mutant plasmids. This indicates that deregulation is due to a release of repression instead of a decrease in activation, which is similar to results with other promoters [24]. Furthermore this is consistent with our observation that most of the transcriptional activation originates from three CCAAT boxes in the *cyclin B2* promoter activated by binding the transcription factor NF-Y [18]. For the *p130* gene Claude Sardet's group recently described that promoter activity in respective CDE/CHR mutants does not change significantly during the cell cycle but displays clear activation of basal activity [28]. In this promoter the distance between CDE and CHR is different to that in *cyclin B2*. We suggest that there are different types of CDE/CHR tandem elements and that the one in *cyclin B2* belongs to the 'classical type' which confers cell cycle-dependent transcription (Fig. 4). A detailed definition of the different types of elements will only be possible once we identify the protein complexes and mechanisms regulating through these elements.

3.3. A simian virus 40 promoter-driven control reporter is itself cell cycle regulated

A reporter driven by an *SV40* promoter expressing luciferase from *Renilla* is often used to standardize experiments with cell cycle-regulated promoters. We find that this standard is itself influenced during the cell cycle. *Renilla* luciferase expression from the *SV40* promoter is upregulated under the experimental conditions about 5-fold during the cell cycle comparing values from resting cells versus cells with maximum expression in S/G_2 (Figs. 2 and 3A). The degree of this upregulation very much depends on the culture density of plated

Table 1
Cell cycle distribution of mouse fibroblasts examined with DNA staining followed by FACS analysis

Time (h)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	93.4	6.1	0.5
14	90.6	9.4	0.0
18	44.6	55.5	0.0
20	40.6	52.1	7.4
22	34.6	33.9	31.6
24	66.7	15.8	17.6
26	69.4	23.1	7.6
28	60.9	38.3	0.8
30	61.7	36.4	1.9

Relative cell numbers for different cell cycle phases are given. DNA content of mouse fibroblasts cultured in parallel to cells used in the experiment for Figs. 2 and 3 were analyzed by FACS. Cells were transfected and treated the same way as cells used for Figs. 2 and 3. They were harvested at the same time points. Cells were DNA stained with propidium iodide and FACS analyzed. Relative cell numbers giving the distribution of cells in various cell cycle phases were obtained with the ModFit analysis software.

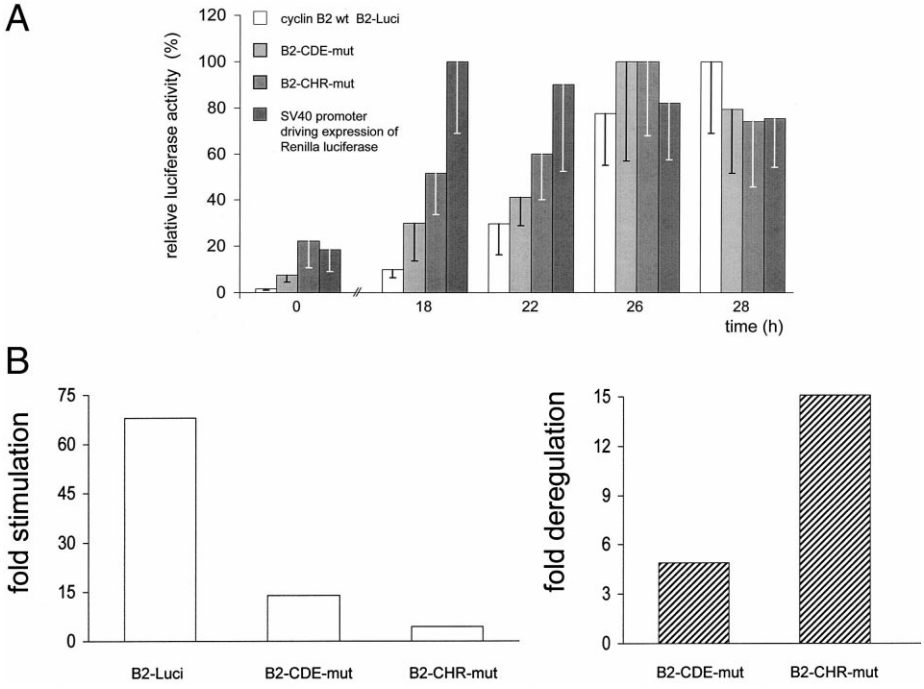

Fig. 3. Deregulation of *cyclin B2* transcription by mutation in the CDE and CHR elements. A: Comparison of activities from *cyclin B2* wild-type and mutant promoter constructs between quiescent and serum stimulated cells. In all experiments mouse fibroblast cells were transiently transfected with B2-Luci (*cyclin B2* wild-type), B2-CDE-mut or B2-CHR-mut plasmids driving expression of firefly luciferase. Cells were co-transfected with a simian virus 40 promoter-driven construct which regulates expression of *Renilla* luciferase. After transfection cells were serum starved for 60 h. At this time cells for the 0 h time point were harvested. Later times refer to cultures analyzed after restimulation with FCS for the respective times. The ratios of activities from firefly *cyclin B2* reporters and the *Renilla SV40*-driven controls at 26 h were 0.26, 0.21 and 0.39 for B2-Luci, B2-CDE-mut and B2-CHR-mut, respectively. The maximal expression for each plasmid was set at 100%. All other activities are given relative to this value. Averages from 12 assays with standard deviations are presented. B: Stimulation factors for each construct were calculated by dividing maximal luciferase activities after serum stimulation by values before addition of serum. Deregulation is the change of the stimulation factor in the mutants versus the stimulation of expression from the wild-type *cyclin B2* plasmid. Experiments shown in Fig. 2 and this figure were done in parallel and results for the wild-type *cyclin B2* construct represent the same assays.

cells. Similar results with even stronger regulation were obtained with a cytomegalovirus promoter (data not shown).

3.4. The cyclin B2 promoter is regulated by a novel CDE/CHR element

A comparison of the CDE in *cyclin B2* reveals that it is different from known CDE and E2F elements (Fig. 4). E2F and CDF-1, a protein whose molecular identity has not yet been unveiled, have so far been implicated in regulating through the CDE in the *cdc25C*, *cyclin A* and *cdc2* promoters [24,29]. We have not observed any complex with *cdc25C* CDE/CHR oligonucleotides in EMSAs that could also bind to a *cyclin B2* probe (data not shown). In order to test a potential involvement of E2F in activating *cyclin B2* transcription we cotransfected E2F-1 and DP-1 expressing plasmids together with reporter constructs. In these experiments the *cyclin B2* promoter remained unaffected by E2F while an E2F-responsive promoter was strongly stimulated (data not shown).

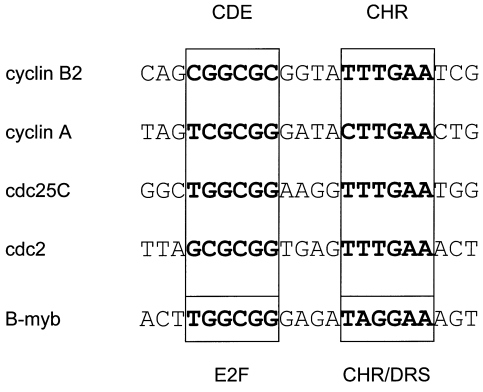

Fig. 4. Alignment of regulatory elements of cell cycle-regulated promoters. Cell cycle-dependent transcription of *cyclin B2*, *cyclin A*, *cdc2* and *cdc25C* is regulated by the 'classical type' CDE and CHR tandem elements. The *B-myb* gene expression is dependent on an E2F site in conjunction with a CHR element (or downstream repression site, DRS). DNA segments of the genes for mouse *cyclin B2* and human *cyclin A*, *cdc25C*, and *cdc2*, and mouse *B-myb* are shown [24,30–33].

We have shown that cell cycle-dependent expression of the *cyclin B2* promoter results from counteracting the activation by NF-Y through repression by a CDE/CHR tandem element in resting cells and the beginning of the cell cycle. Relief of this repression later during the cell cycle allows the accurate expression of the cyclin B2 protein to form MPF by complexing with the kinase cdc2 and controlling transition from G_2 to mitosis. Future experiments are aimed at identifying the protein components involved in cell cycle-dependent transcriptional regulation through the CDE/CHR.

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