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The proteolysis of mitotic cyclins in mammalian cells persists from the end of mitosis until the onset of S phase

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We have studied how the cell cycle-specific oscillations of mitotic B-type cyclins are generated in mouse fibroblasts. A reporter enzyme comprising the N-terminus of a B-type cyclin fused to bacterial chloramphenicol acetyl transferase (CAT) was degraded at the end of mitosis like endogenous cyclins. Point mutations in the destruction box of this construct completely abolished its mitotic instability. When the destructible reporter was driven by the cyclin B2 promoter, CAT activity mimicked the oscillations in the level of the endogenous cyclin B2. These oscillations were largely conserved when the reporter was transcribed constitutively from the SV40 promoter. Pulse–chase experiments or addition of the proteasome inhibitors lactacystin and ALLN showed that cyclin synthesis continued after the end of mitosis. The destruction box-specific degradation of cyclins normally ceases at the onset of S phase, and is active in fibroblasts arrested in G_0 and in differentiated C2 myoblasts. We were able to reproduce this proteolysis *in vitro* in extracts of synchronized cells. Extracts of G_1 cells degraded cyclin B1 whereas $p27^{Kip1}$ was stable, in contrast, cyclin B1 remained stable and $p27^{Kip1}$ was degraded in extracts of S phase cells.

Keywords: cell cycle control/lactacystin/$p27^{Kip1}$/proteasome/ubiquitin

Introduction

Mitotic B-type cyclins activate the $p34^{cdc2}$ protein kinase to form the maturation-promoting factor (MPF) which is required for cells to undergo mitosis (Draetta *et al.*, 1989; Gautier *et al.*, 1989; Labbé *et al.*, 1989). They were the first members of this family of proteins to be discovered (Evans *et al.*, 1983), and owe their name to the saw tooth oscillations of their levels during rapid cleavage cell cycles. B-type cyclins accumulate during S phase, reach maximal levels in G_2 and mitosis and disappear during the metaphase–anaphase transition, remaining undetectable throughout G_1 phase (Pines and Hunter, 1989; Jackman *et al.*, 1995).

The importance of these oscillations for normal cell cycles is widely recognized (Nasmyth, 1993), but many aspects of their regulation remain poorly understood. The most rigorously studied and relatively well characterized point of these oscillations is the rapid degradation of B-type cyclins during mitosis, which is brought about by ubiquitin-mediated proteolysis (Glotzer *et al.*, 1991; Hershko *et al.*, 1991). This specific proteolysis depends on a conserved nine amino acid 'destruction box', which typically is located ~50 residues from the N-terminus of the cyclin. When this region of *Xenopus* cyclin B2 was fused to c-Mos, the resulting chimeric protein was degraded when cells were released from nocodazole-induced mitotic arrest (Okazaki *et al.*, 1992). The introduction of cyclins rendered indestructible by mutations in, or deletion of, their destruction boxes, into HeLa cells (Gallant and Nigg, 1992), frog oocytes (Murray *et al.*, 1989; Holloway *et al.*, 1993), clam oocyte extracts (Luca *et al.*, 1991) or yeast cells (Ghiara *et al.*, 1991; Surana *et al.*, 1993; Yamano *et al.*, 1996) arrests cells in late mitosis. Activation of the proteolytic machinery requires MPF and, at least in cell extracts, can be maintained by addition of the phosphatase inhibitor, okadaic acid (Felix *et al.*, 1990; Lorca *et al.*, 1991; Hershko *et al.*, 1994; Lahav-Baratz *et al.*, 1995). Recent biochemical studies in clam and frog egg extracts have identified several major components of the elaborate machinery which regulates this process. The E1 and E2 components of the ubiquitination process involved in mitotic cyclin degradation are constitutively active. In contrast, the large multisubunit E3 component referred to as 'cyclosome' (Sudakin *et al.*, 1995) or 'anaphase-promoting complex' (APC; King *et al.*, 1995) is active only when prepared from mitotic extracts, and can be activated *in vitro* by phosphorylation with cdc2 (Lahav-Baratz *et al.*, 1995).

In cleaving clam embryos, whose cell cycles lack appreciable G_1 and G_2 phases, the mitotic cyclin protease is activated ~1 min before the chromosomes are seen to undergo anaphase, and remains active for a total of ~5 min (Hunt *et al.*, 1992). A very important discovery was made in budding yeast by Amon *et al.* (1994), who made the unexpected observation that cyclin degradation persists throughout G_1. The activities of the Cln cyclins and Cdc28 were required to turn proteolysis off at START. Clb proteolysis requires the activity of components of the APC/cyclosome, Cdc16 and Cdc27, as well as the product of another gene, *CSE1* (Xiao *et al.*, 1993), whose molecular function is as yet unknown; the human homologue seems to be associated with kinetochores (Brinkmann *et al.*, 1995; Irniger *et al.*, 1995).

Compared with the detailed picture gained from studies on yeast, much less is known about how the oscillations of mitotic cyclin levels are generated in higher eukaryotes. Studies of the cyclin B1 promoter (Cogswell *et al.*, 1995; Hwang *et al.*, 1995; Piaggio *et al.*, 1995; Thatcher *et al.*, 1995) have shown that this cyclin is transcriptionally regulated during the cell cycle, and we show here that the same is true for the mouse cyclin B2 gene. This is in line with findings concerning regulated transcription of several other cell cycle-specific genes, notably cyclin A (Henglein *et al.*, 1994), CDC25C (Lucibello *et al.*, 1995) and cdc2

(McGowan *et al.*, 1990; Dalton, 1992). It has also been found that cyclin B1 mRNA is considerably less stable in G_1 than in G_2, which contributes to its disappearance after mitosis (Maity *et al.*, 1995). While this regulation of transcription and mRNA stability explains why B-type cyclin mRNA levels oscillate during the cell cycle (Pines and Hunter 1989; this work), they fail to explain the apparent absence of the B-type cyclins shortly after the end of mitosis and throughout G_1, for B-type cyclin mRNA persists for several hours in G_1. This discrepancy suggests either that the translation of the mRNA is repressed or that the proteins are highly unstable, as observed in yeast (or both). Here we present evidence that B-type cyclins are synthesized, but remain highly unstable during G_1, until the onset of DNA replication.

We also describe a cell-free system prepared from G_1 phase mouse NIH 3T3 cells that specifically degrades B-type cyclins, but not the cdk inhibitor $p27^{Kip1}$. In contrast, extracts from cells arrested in S phase did not degrade B-type cyclins but rapidly degraded $p27^{Kip1}$. This cell-free system should prove to be a useful tool for further studies of cell cycle phase-specific proteolysis.

Results

Cyclin B2 is transcriptionally regulated

A genomic library from Swiss 3T3K cells was screened with a cDNA probe corresponding to the 5′ end of mouse cyclin B2. A fragment starting from a *Hin*dIII site located 1.1 kb upstream of the transcription start site to an *Apa*I site in the 5′ untranslated region (UTR), was cloned and sequenced. Two main transcription start sites were identified by an S1 nuclease protection assay. One of them coincides with the 5′ end of the published cDNA sequence X66032 (Chapman and Wolgemuth, 1993) starting at nucleotide 15, the second is 32 nucleotides further upstream and coincides with the 5′ end of a cDNA clone isolated by Dr Mark Carrington (University of Cambridge). The promoter lacks a TATA box but has two CCAAT boxes, spaced 34 bases apart and located ~75 bp upstream of the corresponding start sites. Sequence analysis of the promoters of cyclin A, CDC25C and cdc2, which are specifically transcribed in S and G_2, revealed two conserved sequences termed 'cell cycle-dependent element' (CDE) and 'cell cycle genes homology region' (CHR) exactly six nucleotides apart. These elements were shown (Zwicker *et al.*, 1995) to be important for transcriptional repression in G_0. We found these elements in the cyclin B2 gene where they are located within the 5′ UTR of the longer mRNA (Figure 1).

We fused the cyclin B2 promoter to a CAT reporter gene and prepared a series of deletions, to identify the minimal region necessary for transcription. We transfected these constructs into NIH 3T3 cells and established that as little as 110 nucleotides (50 bp upstream and 60 bp 5′ UTR), including at least one CCAAT box, were sufficient for normal levels of transcription (data not shown).

CAT mRNA has a short half-life and can be used to reveal rapid changes in transcription. To establish that the mouse cyclin B2 promoter drives cell cycle-specific transcription like the B1 promoter (Cogswell *et al.*, 1995; Hwang *et al.*, 1995; Piaggio *et al.*, 1995; Thatcher *et al.*, 1995), we co-transfected cells with either the 1.1 kb cyclin

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GAAAATTATTTTATTTAATATCAGGGACTAGAATTTGAAAATAGAC
TGTAGACAAGGAAACAACAAAGCCTGGTGGCCTCGCTGGTTGCTAT
                    caat box
GACAAGCAAATACAAGCCAGCCAATCAACGTGCAGAAAGGCCTTCC
     caat box
AGTCTAGCCAATGGGTTGCGCGCGCCCTGCGTGCGTCTACCCAATA
   Start 1  CDE        CHR              Start 2
GTGCGTCAGCGGCGCGGTATTTGAATCGCGGACCGGGCGGTGGACG

CGGAGCGGCGGGGCCCTGACCCTCCCAACGGTGTCGCAGACCGGAG

TGGCTGTGCCTCGTCCGCACTTGCCAGGGCGGCCCTC ATGGCGCTG
                                        M  A  L
CTCCGACGCCCGACG intron 1
 L  R  R  P  T
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Fig. 1. The sequence of the promoter region and the first exon of mouse cyclin B2. The two transcription start sites are indicated in bold. They correspond to cDNA clones provided by M.Carrington (Start 1) and D.Wolgemuth (Start 2). The CDE and CHR elements discovered in the promoters of CDC25C, cyclin A and cdc2 (Zwiger *et al.*, 1995) are underlined, as are the two consensus CCAAT boxes. Each of these CCAAT boxes is ~75 bases upstream of one of the transcription start sites.

B2 promoter or a constitutive SV40 promoter fused to a CAT gene, together with a plasmid conferring resistance to neomycin. The transfected cells were selected with G-418, and resistant colonies were pooled and expanded. Mitotic cells were obtained by nocodazole arrest and shake off. After washing, they divided and entered G_1 synchronously. Cells were harvested at intervals throughout the cell cycle, which lasted ~16 h, and analysed by FACS (Figure 2A), or extracted for RNA and analysed by Northern blotting with cyclin B2- or CAT-specific radiolabelled probes. Figure 2B shows that the endogenous cyclin B2 mRNA levels oscillated throughout the cell cycle, peaking at mitosis and gradually declining during G_1. Figure 2C shows that CAT mRNA levels oscillated similarly when transcribed by the B2 promoter. In fact, the oscillations of the CAT mRNA were even sharper, probably because CAT mRNA is less stable than cyclin B2 mRNA. While it could be argued that the oscillations of cyclin B2 mRNA might be due to differential stability, the same seems unlikely to be the case for CAT mRNA, for when CAT was transcribed by the constitutive SV40 promoter mRNA levels were constant throughout the cell cycle (Figure 2D).

Only the CAT mRNA levels and not the CAT enzymatic activity could be used to monitor the cell cycle-specific oscillations in transcription, due to the high stability of the CAT enzyme. CAT has a half-life of ~40 h in mammalian cells (Thompson *et al.*, 1991) and thus, once it has accumulated, it cannot be used to monitor rapid changes within a cell cycle of 16 h.

Addition of cyclin B N-termini to CAT specify its degradation in G_1 cells

To generate a more suitable reporter, which ideally would be 'reset' each cycle, we fused the N-terminal 105 residues of mouse cyclin B1 that contain the nine residue 'destruction box' (Glotzer *et al.*, 1991) to the CAT coding sequence. This B1–CAT fusion gene (Figure 3) was placed downstream of the constitutively active SV40 promoter and stably transfected into NIH 3T3 cells. CAT activity was high in cells growing asynchronously, and when blocked in mitosis by nocodazole, indicating that the

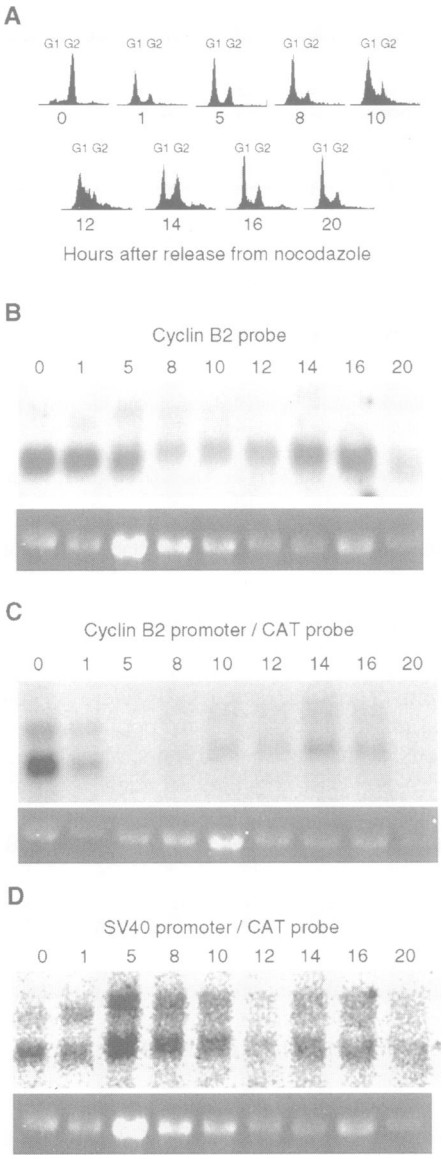

Fig. 2. Cyclin B2 mRNA levels oscillate during the cell cycle. (**A**) NIH 3T3 mouse fibroblasts were blocked in mitosis with nocodazole and then shaken off the plates, washed, released into fresh medium and harvested for FACS at the indicated time points. (**B**) RNA extracted from synchronized cells was analysed by Northern blotting with a cyclin B2-specific radiolabelled probe. The ethidium bromide-stained gels were photographed prior to blotting and the 28S rRNA shown here represents the total RNA loaded in each lane. Cells stably transfected with a CAT reporter gene transcribed by the 1.1 kb cyclin B2 promoter (**C**) or by the SV40 promoter (**D**) were synchronized and analysed by Northern blotting with a CAT-specific probe.

enzymatic activity was not impaired by the addition of the cyclin N-terminus. However, activity dropped rapidly to background levels after nocodazole-arrested mitotic cells were washed and replated in nocodazole-free medium, which allowed them to enter G_1. In contrast, the activity of a wild-type CAT remained unchanged during this procedure (Figure 3). The analogous construct prepared by fusing the N-terminal 86 residues of mouse cyclin B2 to CAT (B2–CAT) was degraded in a similar manner (data not shown). This was somewhat unexpected in view of previous observations that *Xenopus* cyclin B2 was required to be associated with $p34^{cdc2}$ in order for it

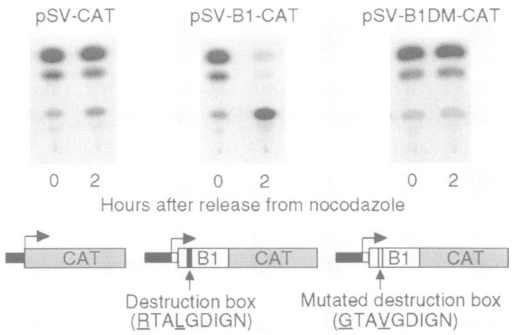

Fig. 3. A cyclin B1–CAT fusion construct is specifically degraded in G_1 phase cells. NIH 3T3 cells were stably transfected with pSV–CAT, pSV-B1–CAT or pSV-B1*DM*–CAT. Cells were blocked with nocodazole (time = 0), released for 2 h (2) into G_1 and analysed for CAT activity.

to be destroyed (Stewart *et al.*, 1994; van der Velden and Lohka, 1994).

To confirm that the degradation of B1–CAT used the destruction box pathway, we mutated the only two conserved amino acids, from RTALGDIGN to GTAVGDIGN, which normally renders cyclins indestructible (Glotzer *et al.*, 1991; Lorca *et al.*, 1992; Stewart *et al.*, 1994). Figure 3 shows that this B1*DM*–CAT was not degraded in G_1, persisting after cells were released from nocodazole. The corresponding B2*DM*–CAT protein (destruction box mutated from RAVLEEIGN to GAVSEEIGN) was similarly stable.

When colonies expressing B1–CAT were established and pooled, expression levels were maintained at steady levels over many cell passages without the need for antibiotic selection. Thus it appears that the B1–CAT reporter did not have any deleterious effect on cells, and could be used as a probe of the cyclin proteolysis machinery.

The cyclin B2–CAT fusion protein mimics cyclin B2 oscillations

In order to study how the oscillations of cyclin B2 were generated, we placed the B2–CAT reporter downstream of the cyclin B2 promoter. This plasmid (B2prom-B2–CAT) was co-transfected with a neomycin resistance plasmid into NIH 3T3 cells. After selection with G-418, cells were synchronized in mitosis by nocodazole arrest, shake off and washing. Cells were harvested at the indicated times and assayed for CAT activity. Figure 4A shows that the high CAT activity seen in mitotic cells rapidly disappeared upon release into G_1, and remained undetectable for ~10 h. Low CAT activity was detected in cells 10 and 12 h after release from nocodazole, and reached maximal levels by 14 h, corresponding to the time when cells were in G_2 (see the FACS analysis in Figure 2A). The levels of endogenous cyclin B2 were determined by immunoblotting extracts of synchronized cells harvested in parallel. They closely followed the CAT activity (Figure 4B). The slight discrepancy between the earliest detectable CAT activity at 10 h and the endogenous cyclin B2 at 12 h is probably due to the higher sensitivity of the CAT assay. This is consistent with the data of Figure 2C, which show that transcription of the cyclin B2 promoter started at 10 h after release from nocodazole block.

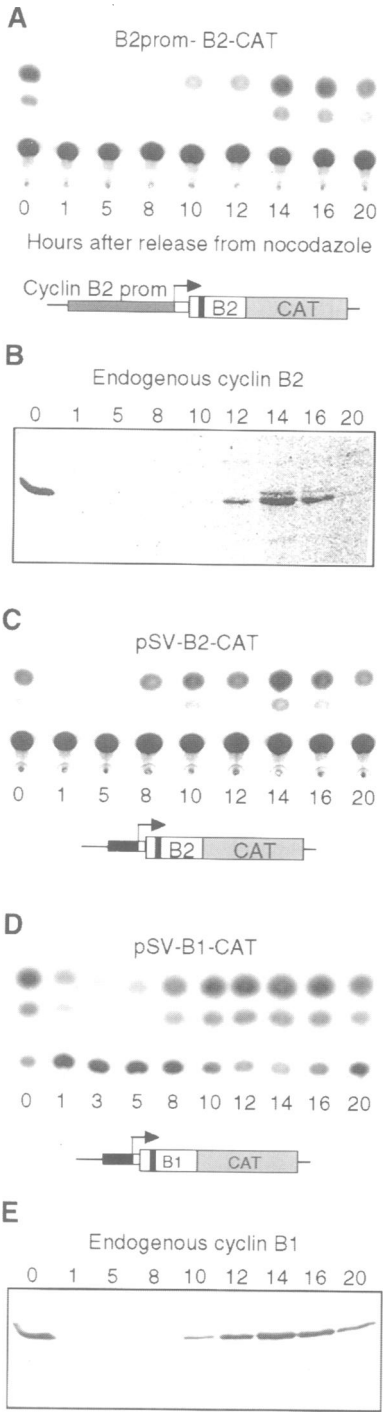

Fig. 4. B-type cyclin–CAT fusion genes mimic endogenous cyclin oscillations. Cells stably expressing a cyclin B2–CAT fusion protein under the control of the endogenous cyclin B2 promoter (see Materials and methods) were synchronized by nocodazole block and harvested at the given time points after release. Levels of the reporter were analysed by CAT assay (**A**) and levels of the endogenous cyclin B2 by immunoblotting (**B**). The experiment was repeated with either a cyclin B2–CAT (**C**) or cyclin B1–CAT (**D**) fusion protein expressed by the constitutive SV40 promoter. Levels of endogenous cyclin B1 were detected by immunoblotting (**E**).

In order to establish how much of the regulation of cyclin B2 was due to transcriptional control, and how much due to protein instability or translational control, we replaced the B2 promoter with the constitutive SV40 promoter (SV-B2–CAT) and repeated the experiment shown in Figure 4A. Strikingly, the cell cycle-specific oscillations of CAT activity generated by this constitutively transcribed B2–CAT were largely conserved, as can be seen in Figure 4C. The main difference was that the SV40 promoter drove an increase in the level of CAT activity 2–4 h earlier than the B2 promoter.

A similar experiment using B1–CAT under the control of the SV40 promoter gave essentially the same results, with undetectable CAT activity for several hours after release from mitosis (Figure 4D). Comparing the levels of endogenous cyclin B1 (Figure 4E) with the B1–CAT activity, it appears that cyclin B1 starts to be transcribed earlier in the cell cycle than cyclin B2. This is consistent with our observations that cyclin B1 mRNA appears several hours earlier than cyclin B2 mRNA (data not shown).

Cyclin B1 and B2 proteins are synthesized in G_1 but are degraded rapidly

The oscillations of the constitutively transcribed cyclin B1–CAT and cyclin B2–CAT fusion proteins indicated that their levels were regulated in G_1 by a post-transcriptional mechanism. If B-type cyclin degradation were a transient event at the metaphase–anaphase transition and confined to a narrow time window as observed in early embryos, we should have observed a steady rise in CAT activity in G_1 when the SV40 promoter was used to express the mRNA. This was clearly not the case: CAT activity remained undetectable for 8 h and then rose sharply (Figure 4C and D). We therefore performed further experiments to check whether this failure to accumulate mitotic cyclins in G_1 was due to control of translation of the mRNA, or to proteolysis that persisted after the end of mitosis.

First, we tested the effects of the proteasome inhibitors lactacystin (Fenteany *et al.*, 1995) and *N*-acetyl-leu-leu-norleucinal (ALLN; Sherwood *et al.*, 1993) on the accumulation of B-type cyclins in G_1. Cells were synchronized by nocodazole block, and released into G_1 in four plates. After 2 h, one of the plates ($t = 0$) was harvested, the second was kept as an untreated control, the third was treated with lactacystin and the fourth with ALLN. These last three plates were harvested after an additional 3 h ($t = 3$). Protein prepared from these cells was analysed by immunoblotting with anti-cyclin B1, anti-cyclin B2 and anti-$p34^{cdc2}$ (Figure 5A). Cyclin levels in the $t = 0$ cells and in the untreated controls were undetectable. In contrast, cells treated with either of the proteasome inhibitors had readily detectable levels of both cyclin B1 and B2. As expected, the inhibitors did not affect the levels of $p34^{cdc2}$, which previously has been reported to be a relatively stable protein (McGowan *et al.*, 1990). This result suggests that significant synthesis of B-type cyclins occurs during G_1 phase, but they are degraded rapidly.

To confirm this hypothesis, synchronized cells were labelled for 15 min with [^{35}S]methionine and then washed and incubated in non-radioactive medium. Cell extracts were immunoprecipitated with anti-cyclin B2 antibodies and analysed by SDS–PAGE. Both G_1 and G_2 cells contained a labelled band of similar intensity corresponding to cyclin B2, but this band disappeared after a 5 min 'chase' in G_1 cells, whereas it was stable in the G_2 cells (Figure 5B). This difference in stability was also reflected

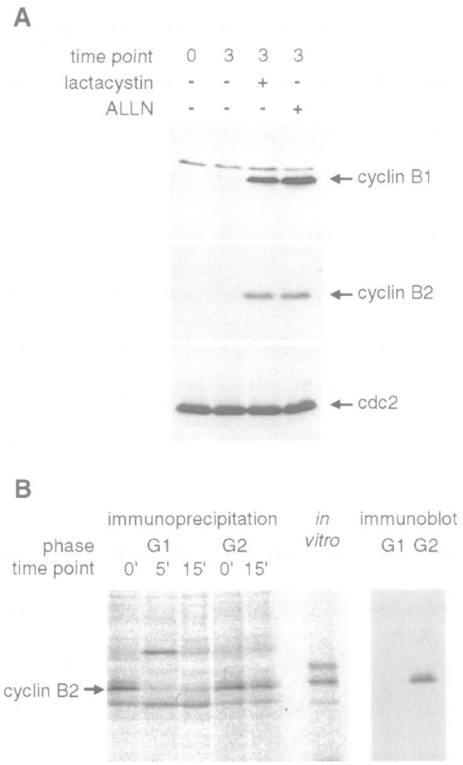

Fig. 5. B-type cyclins are synthesized in G_1 but are degraded rapidly by proteolysis. (**A**) Cells were synchronized by nocodazole block and shake off and released into G_1. Two hours later, cells were either harvested (0) or grown for a further 3 h (3) with or without lactacystin or ALLN, as indicated, and harvested. Proteins extracted from the cells were immunoblotted with anti-cyclin B1, cyclin B2 or cdc2 antibodies. (**B**) Cells were synchronized by nocodazole block and shake off and grown for 2 (G_1), or 14 h (G_2). After 30 min of methionine deprivation, cells were labelled with [^{35}S]methionine for 15 min and either harvested immediately or washed and 'chased' with medium containing unlabelled methionine for 5 or 15 min. Cells were extracted and immunoprecipitated with anti-cyclin B2 antibodies and resolved by SDS–PAGE. An *in vitro* transcribed and translated cyclin B2 was run in parallel as a marker. Total protein extracted from the above cells was also loaded on the same gel and immunoblotted with anti-cyclin B2.

in the immunoblot performed on proteins extracted from these cells. While the G_1 and G_2 cells synthesized comparable amounts of cyclin B2 (as indicated by the intensity of the $t = 0$ band in the labelling experiment), it accumulated to detectable levels only in the G_2 cells, whereas in G_1 cells steady-state levels were undetectable due to its rapid turnover (Figure 5B). We conclude that B-type cyclin mRNA is present in G_1 cells (see also Figure 2B) where it is actively translated, and that cyclin accumulation is prevented by continuous rapid proteolysis.

Cyclin B proteolysis is turned off at the beginning of S phase

The results of the previous experiments showed that the B-type cyclin degradation system was active throughout G_1. We next tested when this proteolysis was switched off in relation to the start of DNA replication. Cells stably transfected with the constitutively transcribed pSV-B1–CAT were synchronized by a nocodazole block in M, released into G_1, and 2 h later [^{3}H]thymidine was added to the cells. Cells were sampled at intervals from mid-G_1 until early S phase (4–8 h after release from nocodazole).

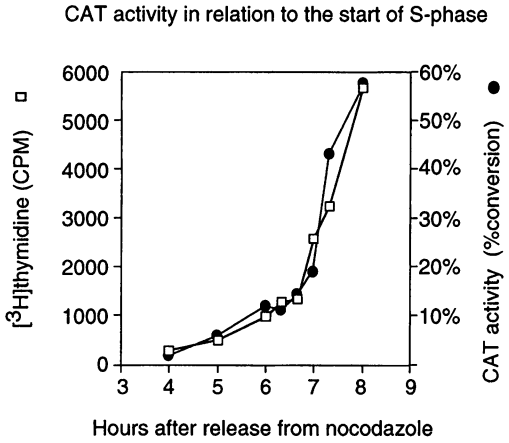

Fig. 6. Cyclin stabilization coincides with the beginning of DNA replication. Cells stably expressing the constitutively transcribed pSV-B1–CAT were synchronized by nocodazole block and shake off and released into G_1 in multiple 35 mm tissue culture plates. Two hours after release, cells were labelled with [^{3}H]thymidine. After an additional 2 h, cells were sampled at short intervals for 4 h. Cell extracts were analysed for incorporation of [^{3}H]thymidine to monitor DNA synthesis, and CAT activity to measure cyclin accumulation.

DNA replication was monitored by [^{3}H]thymidine incorporation, and the time of onset of cyclin stabilization was deduced from the accumulated CAT activity. Both the incorporation of [^{3}H]thymidine and the CAT activity started to rise sharply 7 h after release from the nocodazole block, suggesting that cyclin-specific proteolysis is inactivated around the time of the beginning of S phase (Figure 6).

Cyclin B proteolysis persists in G_0-arrested fibroblasts and in terminally differentiated myoblasts

The data presented so far show that cyclin B proteolysis in rapidly growing cells is turned off only at, or close to, the start of DNA replication. We next asked whether this proteolysis is a time-limited G_1-specific process, or an indefinite state that only ends at the G_1–S transition. To answer this question, we transfected the SV40-driven cyclin B1–CAT fusion gene or its destruction box mutant B1*DM*–CAT into cells arrested in G_0 by serum starvation and analysed them after 48 h. Figure 7A shows that very low CAT levels were detected when the B1–CAT construct was used, whereas high CAT levels were achieved with the indestructible B1*DM*–CAT construct under identical conditions. We conclude that proteolysis can be prolonged considerably, and probably remains active in G_0 as well as in normal G_1.

As the fibroblasts used in this experiment cannot be arrested by serum starvation for >48 h before showing deleterious effects, we tested C2 myoblasts, which can be cultured *in vitro* and induced to terminally differentiate into muscle fibres. Once differentiated, these cells undergo indefinite cell cycle arrest. We established C2 lines stably and constitutively expressing B1–CAT, B1*DM*–CAT or wild-type CAT from the SV40 promoter and induced them to differentiate (see Materials and methods). As shown in Figure 7B, CAT activity expressed by all three cell lines was comparable when they were arrested in S phase with hydroxyurea. Extracts of cycling B1–CAT cells displayed about half the B1–CAT activity compared with the hydroxyurea-arrested cells, presumably because only half

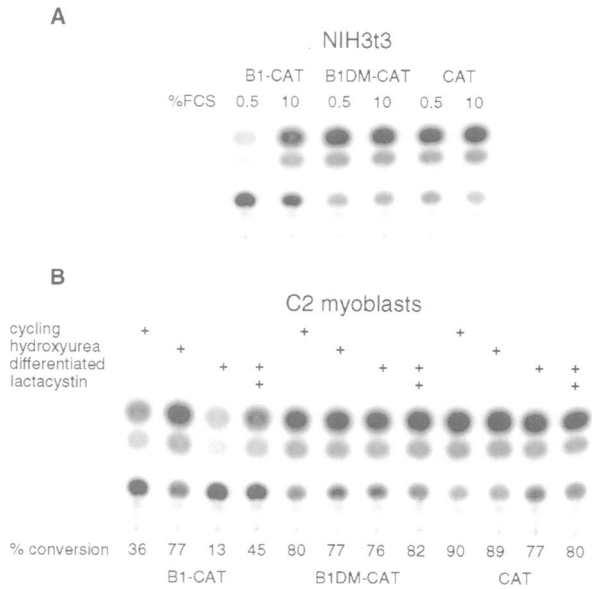

Fig. 7. B-type cyclin proteolysis is active in G_0-arrested fibroblasts and in differentiated myoblasts. (**A**) NIH 3T3 cells were transiently transfected with the constitutively transcribed destructible pSV-B1–CAT, its destruction box mutant pSV-B1*DM*–CAT or wild-type pSV–CAT. Cells were incubated in 0.5% FCS to arrest growth or in 10% FCS to permit growth. The cells were harvested and analysed for CAT activity 48 h after the transfection. (**B**) Mouse C2 myoblasts were stably transfected with pSV-B1–CAT, pSV-B1*DM*–CAT or pSV–CAT, and induced to differentiate by growing them in 2% horse serum + insulin for 7 days. Four conditions were tested, as indicated by (+): 1, undifferentiated cycling myoblasts; 2, hydroxyurea-arrested myoblasts; 3, differentiated cells; and 4, differentiated cells treated with lactacystin for 5 h. The cells were harvested and assayed for CAT activity.

of the cells are in the S, G_2 and M phases where this protein is stable, and the rest are in G_1 where this reporter is highly unstable. In B1–CAT cells that were induced to differentiate, only very low CAT activity could be detected. CAT activity could be increased by treating these differentiated cells with lactacystin, indicating that the lack of CAT activity was primarily due to proteolysis. Cells expressing the destruction box mutant B1*DM*–CAT and the wild-type CAT expressed the same levels of activity under all conditions.

Extracts prepared from G_1 cells specifically degrade cyclin B1

The results described above clearly indicate that B-type cyclins are continuously degraded during G_1 by a destruction box-specific proteolysis pathway. Further characterization of this degradation pathway is very difficult to perform in intact cells, and we therefore tested whether cell cycle-specific proteolysis could be reproduced in a cell-free system that would permit biochemical analysis. We prepared extracts from synchronized cells and tested their ability to degrade specific substrates. G_1 extracts were made from cells blocked in mitosis with nocodazole, shaken off, released into G_1 and harvested after 4 h, corresponding to mid-G_1 phase. The S phase extracts were prepared from cells treated with hydroxyurea for 24 h, which blocks cells in S phase by inhibiting ribonucleotide reductase. These extracts were incubated with a [^{35}S]-methionine-labelled substrate prepared by *in vitro* transcription and translation of a mixture of full-length cyclin B1 and cyclin Δ110-B1. The latter is an N-terminal truncated version of cyclin B1 lacking a destruction box, and was included as an internal control for loading and non-specific degradation. Full-length cyclin B1 was degraded with a half-life of ~45 min in the G_1 extracts, whereas it was stable in the S phase extract (Figure 8A and C).

Recent reports suggest that the cyclin-dependent kinase inhibitor $p27^{Kip1}$ is unstable in S phase (Hengst and Reed, 1996) and it is apparently degraded by the ubiquitin–proteasome pathway (Pagano *et al.*, 1995). We therefore transcribed and translated mouse $p27^{Kip1}$ *in vitro* and tested its stability in both the G_1 and S extracts. We found that $p27^{Kip1}$ was stable in G_1 and was degraded in S phase extracts. This result verifies that the S phase extracts are capable of specific proteolysis, and suggests that this experimental system is applicable to a range of proteins other than cyclins.

To begin to characterize the biochemical pathway of cyclin B1 destruction in G_1 extracts, we tested a number of inhibitors. Cyclin B1 degradation in these extracts was completely inhibited by 10 mM *N*-ethylmaleimide and by 300 μg/ml tosyl-lysine chloromethyl ketone (TLCK), but was unaffected by pepstatin, chemostatin and leupeptin. Protein ubiquitination as well as the subsequent proteolysis by the proteasome require ATP (Ganoth *et al.*, 1988), ATP depletion of the G_1 extracts by added hexokinase and glucose blocked cyclin proteolysis (data not shown).

Addition of an N-terminal fragment of sea urchin cyclin B fused to protein A (13–66pA) to *Xenopus* egg extracts inhibited cyclin degradation, whereas a destruction box-mutated version of the same protein (13–66pA R/A) had no such effect (Holloway *et al.*, 1993; D.Harrison unpublished results). We observed the same inhibition in our extracts when we added 5 μM 13–66pA to the G_1 extracts, while the same concentration of 13–66pA R/A had no effect on the destruction (Figure 8B and C).

Discussion

Here we show that transcription of mouse cyclin B2 is regulated similarly to that of cyclin B1 during the cell cycle, taking place mainly from late S phase until mitosis, although transcription of cyclin B1 may commence somewhat earlier in S phase than we observed for B2 (Hwang *et al.*, 1995). The mouse cyclin B2 promoter shares the CDE–CRE elements identified in mammalian CDC25C, cyclin A and cdc2 (Zwicker *et al.*, 1995), all of which are transcribed primarily in the S and G_2 phases. This transcriptional regulation can account for the rise of B-type cyclins in S and G_2 phases. These proteins are degraded at the end of mitosis, but it remained unresolved as to why the levels of the B-type cyclins do not start rising again immediately after the end of mitosis, for while no new cyclin mRNA appears to be transcribed in G_1 cells, the levels of their mRNAs decline only gradually and persist for several hours (see Figure 9). This discrepancy between the presence of cyclin mRNA and lack of the cognate protein implied either translational repression of the mRNA, or rapid continuous degradation of the protein or a combination of the two mechanisms.

The evidence presented here suggests that continuing rapid proteolysis accounts for the low levels of cyclins in

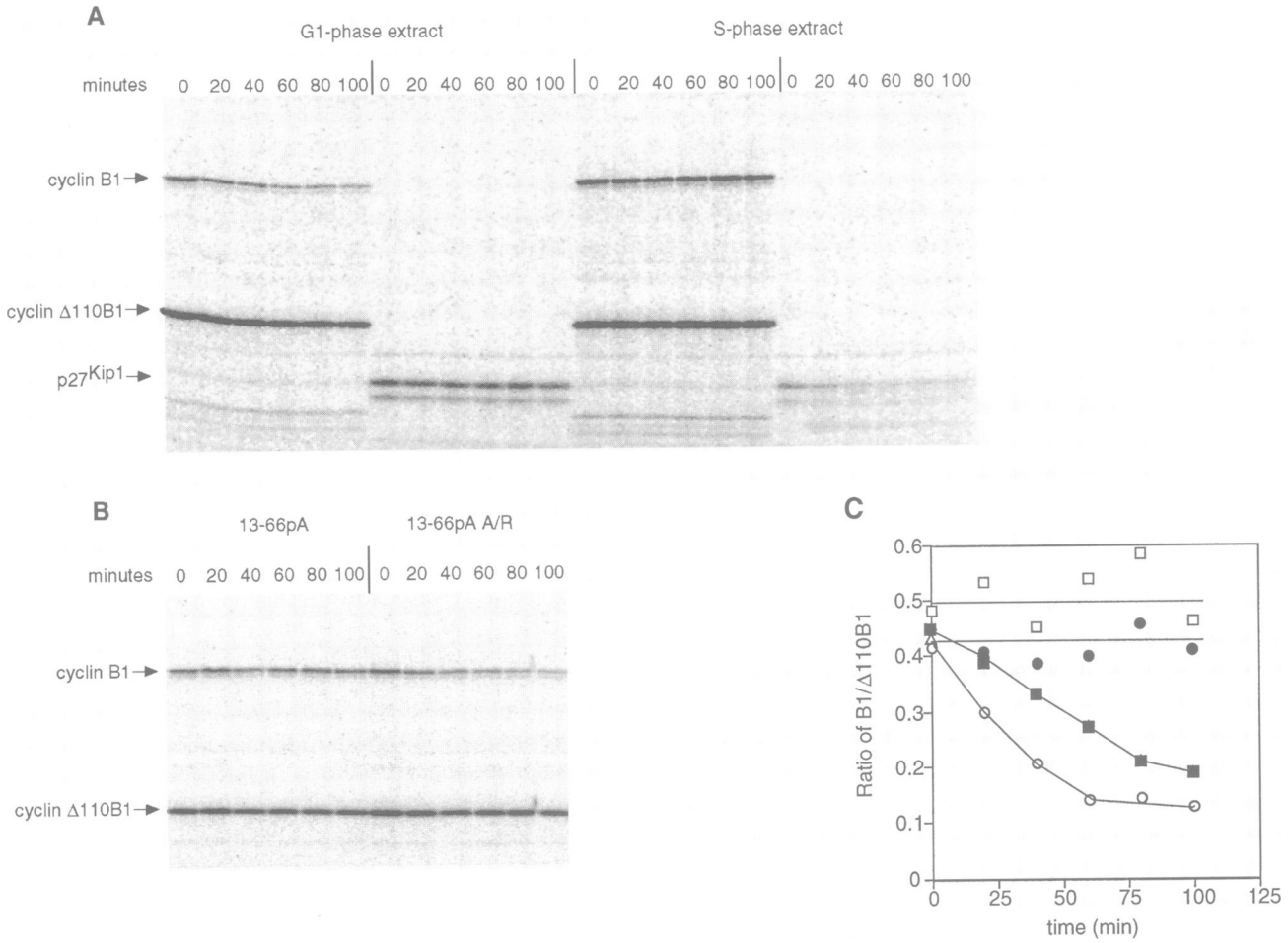

Fig. 8. Cell-free extracts specifically and differentially degrade cyclin B1 and $p27^{kip1}$. (**A**) Cell extracts were prepared from mid-G_1 or S phase arrested cells, and destruction assays were performed on *in vitro* transcribed and translated full-length cyclin B1 and cyclin Δ110B1, as well as on $p27^{Kip1}$ (see Materials and methods). (**B**) Destruction assays were performed in G_1 extracts supplemented either with the N-terminal sea urchin cyclin B fused to protein A (13–66pA) or a destruction box mutant version of it (13–66pA A/R). (**C**) Stability of the full-length cyclin B1, compared with the N-terminal deleted cyclin Δ110B1, was quantified by a phosphorimager and plotted. G_1, ○; S, ●; 13–66pA, □ and 13–66pA A/R, ■.

G_1 phase. A reporter construct containing the N-terminus of either cyclin B1 or B2 fused to bacterial CAT had normal CAT activity but, unlike wild-type CAT, it was degraded at the end of mitosis. When we mutated the cyclin destruction box of this reporter it was no longer degraded. The dependence of the reporter's turnover on an intact destruction box suggested that it was degraded by the same pathway that degrades the endogenous mitotic cyclins.

When we expressed the mRNA encoding this protein from the cyclin B2 promoter in mouse fibroblasts, CAT activity measured through the cell cycle mimicked the cycling behaviour of the endogenous cyclin B2 levels. When we replaced the regulated cyclin B2 promoter with a constitutive SV40 promoter, however, the cell cycle-specific oscillations were largely conserved and no CAT activity was detected in G_1. This result could also be explained by translational control but, when G_1 cells were treated with either lactacystin or ALLN, two proteasome inhibitors, both cyclin B1 and B2 accumulated to readily detectable levels, indicating that they are synthesized in G_1. The synthesis and rapid degradation of cyclin B2 during G_1 was shown by immunoprecipitating cyclin B2 from cells briefly pulsed with [^{35}S]methionine and chased with unlabelled medium. The degree of labelling during the pulse suggested that the synthesis of cyclin B2 was similar in G_1 and G_2, but the cyclin disappeared in G_1 within 5 min. Transfection of the SV40-driven B1–CAT reporter into G_0-arrested cells or differentiating myoblasts suggested that the destruction box-dependent proteolysis pathway remains active in quiescent cells, although the generality of this finding needs to be explored further.

We have not yet shown that the cyclin degradation we observe in these cells (and in extracts prepared from them) employs the ubiquitin-mediated proteolysis pathway that is found in yeast and *Xenopus*, but the following evidence strongly supports such a notion. Above all, the degradation is dependent on an intact destruction box, which is essential for the ubiquitination and degradation of cyclins in mitosis (Glotzer *et al.*, 1991). Moreover, as the degradation is sensitive *in vivo* to treatment with lactacystin and ALLN, the proteasome is most likely responsible for its final stages. Proteolysis in cell-free extracts requires ATP and is inhibited by the same reagents that inhibited the degradation of mitotic cyclins in clam extracts (Luca and Ruderman, 1989), which is known to follow the ubiquitination pathway (Hershko *et al.*, 1991).

What terminates the destruction box-specific proteolytic

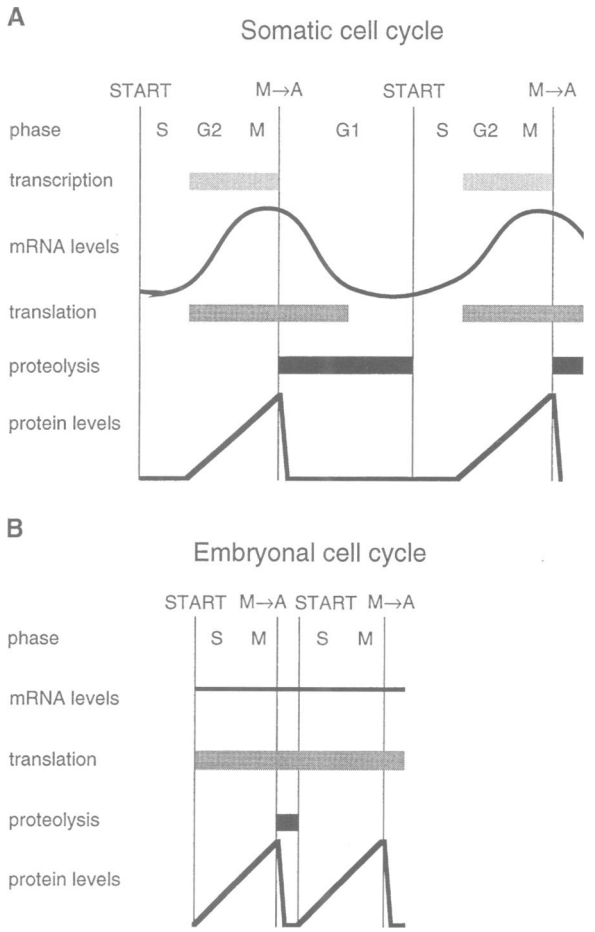

Fig. 9. Mitotic cyclin oscillations are generated by proteolysis lasting from the metaphase transition to START. (**A**) In somatic cells, mitotic cyclin genes are transcribed during S and G_2 phases, giving rise to periodic accumulation of cyclin mRNA. As long as this mRNA is present in the cells it is constitutively translated. Proteolysis of cyclins is initiated at the metaphase to anaphase transition (M→A) and switched off at START. This proteolysis ultimately defines the saw tooth pattern of cyclin levels. If the cell cycle is arrested prior to the onset of START, as occurs in differentiating cells, cyclin proteolysis remains active. (**B**) No transcription takes place during the frog and clam early embryonic cell cycles, which depend on maternal mRNA for their protein synthesis. Both the cyclin mRNA level and its translation are constant. The cell cycle of these cells comprises a rapid succession of mitotic divisions and DNA replication with a proteolysis window that lasts only a few minutes. Again, this proteolysis gives rise to the observed saw tooth pattern.

machinery at the end of G_1? In budding yeast, the activity of Cdc28 promoted by Cln1 and 2 seems to be required (Amon *et al.*, 1994). It has been suggested that cyclin E might play such a role in *Drosophila* embryos (Knoblich *et al.*, 1994), but the evidence is somewhat indirect and does not necessarily imply that cyclin E is directly involved in turning off degradation. We have made preliminary explorations of this point in *Xenopus* egg extracts. We added $p21^{cip1}$, a cyclin E–CDK2 inhibitor, to cyclin destruction assays, but did not observe any effect on cyclin destruction, which shut off as usual ~45 min after addition of Ca^{2+}. If cyclin E–CDK2 were required to turn the destruction off, we should have observed destruction persisting, as it does when extracts are treated with okadaic acid. We also found no effects of added cyclin E–CDK2 in the G_1 cell extracts (M.B. and P.Descombes, unpublished data). Guadagno and Newport (1996) also did not observe impaired cyclin accumulation in frog extracts treated with $p21^{Cip1}$.

Evidence linking the progression of G_1 to protein stability gave rise to the definition of the restriction point (Medrano and Pardee, 1980). Since we show here that G_1 cells contain a highly specific protease that is switched off at the onset of S phase (when it would appear that another system with a different specificity turns on), we wonder whether other substrates besides cyclins, which might be important for the G_1 to S transition, might also be regulated by this system. Understanding the mechanism and regulation of the destruction box-dependent proteolysis machinery in higher eukaryotes seems very important, and we hope that the cell-free system described here may allow a biochemical approach to the problem.

Materials and methods

Plasmids

pSVCAT was prepared by cloning the SV40 promoter from pSVβgal (Promega) into pCAT basic (Promega). pSV-B1–CAT was prepared by cloning a fragment encoding the 5′ UTR and the first 105 amino acids of mouse cyclin B1 by PCR into the *Hin*dIII–*Xba*I sites of pSV–CAT downstream of the SV40 promoter and upstream and in-frame with the CAT open reading frame. pSV-B1*DM*–CAT was prepared from B1-DM by PCR mutagenesis.

B2prom-B2–CAT was prepared by first fusing the cDNA of mouse cyclin B2 (a gift of D.Wolgemuth; Chapman and Wolgemuth, 1993) to a 1.1 kb *Hin*dIII–*Apa*I fragment of mouse B2 upstream sequence including promoter and 5′ UTR sequences isolated from a mouse 3T3K genomic library. A 1.4 kb *Hin*dIII–*Nhe*I fragment of this plasmid comprising the B2 promoter, 5′ UTR and coding sequence of the first 86 amino acids of cyclin B2 was cloned into pCAT basic linearized with *Hin*dIII–*Xba*I. The constitutively transcribed pSV-B2–CAT was prepared by replacing the endogenous cyclin B2 promoter with the SV40 promoter.

Cell cultures, transfections and analysis of gene expression

NIH 3T3 cells were obtained from Dr R.Treisman and C2 myoblasts from Dr M.Raff. They were grown in E4 medium supplemented with 10% fetal calf serum (FCS). C2 myoblasts were induced to differentiate by feeding confluent plates for 5 days with E4 medium containing 2% horse serum (Gibco) and 10 μg/ml insulin.

Co-transfections of 20 μg of the relevant plasmids together with 1 μg of pMC1NeoPolyA (Stratagene) were performed by calcium phosphate co-precipitation in BES buffer (Ausubel *et al.*, 1994). Cells were selected with 600 μg/ml of Geneticin (Gibco) and several hundred colonies were pooled and expanded for each transfection.

For mitotic arrest, cells were treated with 2 μM nocodazole (Sigma) for 6–16 h and recovered by tapping the dishes and rinsing them several times with medium. Cells were then suspended in a large volume of medium without nocodazole, recovered by centrifugation, resuspended and transferred onto plates with fresh medium. Lactacystin (R.J.Corry) was used at 10 μM and ALLN (Sigma) at 150 μg/ml.

DNA replication was monitored by adding [^{3}H]thymidine (Amersham) to the cells 2 h after release from nocodazole arrest and fixing them at the given time points with 10% trichloracetic acid (TCA). The fixed cells were applied to GFC filters, washed with 1% TCA and EtOH. The dried filters were placed in scintillation mix and counted in a Packard scintillation counter.

CAT assays were performed by standard methods (Ausubel *et al.*, 1994) with [^{14}C]chloramphenicol (Amersham) and acetyl-CoA (Sigma). CAT activity was calculated as the percent of the mono- and diacetylated fractions of the total counts by quantitation on a Molecular Dynamics Phosphorimager.

Immunoblots and immunoprecipitations were performed by standard methods (Harlow and Lane, 1987) using mouse anti-cyclin B1 and anti-$p34^{cdc2}$ monoclonal antibodies, prepared in our laboratory by Dr J.Gannon, and rabbit anti-cyclin B2 polyclonal antibodies, the kind gift of Dr Mark Carrington.

The synthesis and stability of cyclin B2 during the cell cycle were analysed as follows: ~10^7 synchronized cells, 2 (G_1) or 14 h (G_2) after

release from mitotic arrest, were deprived of methionine and cysteine for 30 min and then labelled for 15 min with 100 μCi of [^{35}S]methionine and cysteine. They were then either washed and harvested immediately (0 min) or chased with complete unlabelled medium for 5 or 15 min. Incorporation in the extracts was determined by TCA precipitation, and 20 000 c.p.m. of each sample were immunoprecipitated with anti-cyclin B2 antibodies and analysed by SDS–PAGE.

Destruction assays in cell extracts

Cells were synchronized either in mid-G_1 by the nocodazole block and release method, or in early S phase by incubation with 2 mM hydroxyurea for 24 h. Extracts were prepared as described by Li and Kelly (1984); plates were washed twice with phosphate-buffered saline (PBS) and twice with cold hypotonic extraction buffer [20 mM HEPES pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. The buffer was completely aspirated, cells were scraped off and disrupted with several strokes of a Dounce homogenizer. The cell debris and nuclei were pelleted by centrifugation in a cooled Eppendorf centrifuge for 15 min, and aliquots of the cleared lysate were snap frozen in liquid N_2 and stored at –70°C. For destruction assays, extracts were thawed and supplemented with an energy-regenerating system (25 mM phosphocreatine and 10 μg/ml creatine kinase) and 1 mM ATP. Assays were performed in 10 μl of extract mixed with 0.5 μl of radiolabelled substrate (cyclin B1 + Δ110B1, or $p27^{Kip1}$), at 30°C, taking samples for analysis by SDS–PAGE at 20 min intervals.

The [^{35}S]methionine-labelled substrates for the destruction assays were prepared by a coupled transcription and translation reaction (Craig *et al.*, 1992) in nuclease-treated rabbit reticulocyte lysates supplemented with 10% nuclease-treated *Xenopus* egg interphase extracts. Cyclin B1 was expressed from plasmid 1213 (M.Carrington) which is a full-length cDNA clone in pT3T719U. The cyclin Δ110B1 construct was prepared by deleting a *Hin*dIII fragment encoding the N-terminal part of the cyclin. Mouse $p27^{Kip1}$ was expressed from a cDNA cloned into pGEM1.

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