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Cell cycle regulation of the human *cdc2* gene

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Transcription of the human *cdc2* gene is cell cycle regulated and restricted to proliferating cells. Nuclear run-on assays show that *cdc2* transcription is high in S and G_2 phases of the cell cycle but low in G_1. To investigate transcriptional control further, genomic clones of the human *cdc2* gene containing 5′ flanking sequences were isolated and shown to function as a growth regulated promoter *in vivo* when fused to a CAT reporter gene. In primary human fibroblasts, the human *cdc2* promoter is negatively regulated by arrest of cell growth in a similar fashion to the endogenous gene. This requires specific 5′ flanking upstream negative control (UNC) sequences which mediate repression. The retinoblastoma susceptibility gene product (Rb) specifically represses cdc2 transcription in cycling cells via 136 bp of 5′ flanking sequence located between −245 and −109 within the UNC region. E2F binding sites in this region were shown to be essential for optimal repression. A model is proposed where Rb negatively regulates the *cdc2* promoter in non-cycling and cycling G_1 cells.

Key words: cdc2 gene/cell cycle/retinoblastoma

Introduction

The *cdc2* gene was first identified in the fission yeast *Schizosaccharomyces pombe* as a temperature sensitive (ts) allele, which at the restrictive temperature arrests cells at the G_1-S and G_2-M transitions of the cell cycle (Nurse, 1985; Hayles and Nurse, 1986). It encodes the 34 kDa catalytic subunit (p34) of a cell cycle regulated protein kinase essential for initiation of DNA replication and entry into mitosis. Like its budding yeast counterpart *CDC28* (see Beach *et al.*, 1982), cdc2 kinase activity is controlled post-translationally (Simanis and Nurse, 1986).

A human homologue of the $cdc2^+$ gene has been cloned by functional complementation in yeast (Lee and Nurse, 1987), revealing substantial conservation of cell cycle control elements at the molecular level. Moreover, the human *cdc2* cDNA encodes a 34 kDa protein ($p34^{cdc2}$) with 63% amino acid identity to fission yeast p34. $p34^{cdc2}$-related protein kinases which can directly substitute for fission yeast p34 have since been identified in plants, marine invertebrates, amphibians and upper vertebrates (Krek and Nigg, 1989; Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990; Hirt *et al.*, 1991). Both biochemical (Gautier *et al.*, 1988; Draetta and Beach, 1988; Labbé *et al.*, 1989; Riabowol *et al.*, 1989; Chou *et al.*, 1990; Lamb *et al.*, 1990; Peter *et al.*, 1990) and genetic (Th'ng *et al.*, 1990) experiments implicate $cdc2^+$ homologues in regulation of mitotic initiation in mammalian cells, however, a clear role at the G_1-S transition has yet to be established (see Blow and Nurse, 1990; D'Urso *et al.*, 1990; Furukawa *et al.*, 1990).

Post-translational regulation is fundamentally important in control of $p34^{cdc2}$ kinase activity in the cell cycle of eukaryotes (reviewed by Draetta, 1990; Nurse, 1990). Unlike the situation in yeast, however, there is evidence that $p34^{cdc2}$ levels in mammalian cells are modulated in response to cellular growth state by regulating levels of *cdc2* mRNA. Following arrest of cell growth, human *cdc2* mRNA and $p34^{cdc2}$ levels generally decline to almost undetectable levels, and increase as cells enter the cell cycle (Draetta *et al.*, 1988; Lee *et al.*, 1988a; D'Urso *et al.*, 1990; Wang *et al.*, 1991). Levels of *cdc2* mRNA and $p34^{cdc2}$ protein also decline during differentiation (Draetta *et al.*, 1988; Lee *et al.*, 1988a; D'Urso *et al.*, 1990) and development (Krek and Nigg, 1989), consistent with $p34^{cdc2}$ being required for cell proliferation. Although levels of $p34^{cdc2}$ appear to be constant throughout the cell cycle (Draetta and Beach, 1988; McGowan *et al.*, 1990) both *cdc2* mRNA levels and rates of $p34^{cdc2}$ synthesis are cell cycle regulated (McGowan *et al.*, 1990). Previous reports, however, have not evaluated the role of transcriptional control in determining *cdc2* mRNA and $p34^{cdc2}$ levels in mammalian cells.

The retinoblastoma susceptibility gene product (Rb) is proposed to be an important regulator of cell growth and proliferation (reviewed by Weinberg, 1990). Inactivation of the Rb gene is correlated with deregulation of cell growth and the ability of several viral proteins such as E1A, SV40 Large T and HPV E7 to bind Rb correlates with their ability to transform cells (see Whyte *et al.*, 1989). Rb undergoes cyclic changes in its phosphorylation state throughout the cell cycle; in S and G_2 phases it is hypophosphorylated in contrast to its hyperphosphorylated state in G_1. Phosphorylation of Rb late in G_1 correlates with its conversion from an active to an inactive form which appears to represent an important control step in the G_1 to S transition (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Thomas *et al.*, 1991). Consistent with this idea, Rb is exclusively hypophosphorylated in non-dividing cells, a feature which is believed to be crucial for its role as a growth suppressor (Stein *et al.*, 1990). Recent evidence suggests that Rb may act at the transcriptional level by modulating the activity of growth regulated genes by forming a complex with the E2F transcription factor (see Wagner and Green, 1991).

This paper presents direct evidence that transcription of the human *cdc2* gene is cell cycle regulated. The human *cdc2* gene is transcribed in S and G_2 phases, but not in G_1 until just before the G_1-S transition. Human genomic *cdc2* clones have been isolated and 5′ flanking sequences characterized to further investigate *cdc2* transcriptional regulation. These studies show that transcription of the human *cdc2* gene is negatively regulated in stationary cells.

Furthermore, the retinoblastoma gene product is discussed as being a candidate negative regulator of *cdc2* transcription which, has implications for its general role in cell cycle control.

Results

Cell cycle regulated transcription of the human cdc2 gene

The observation that human *cdc2* mRNA levels are cell cycle regulated (McGowan *et al.*, 1990) suggested that transcription of *cdc2* is also under cell cycle control. To determine whether human *cdc2* transcription is cell cycle regulated, nuclear run-on assays were performed on nuclei prepared from thymidine−aphidicolin synchronized cells. FACS analysis was used to determine cellular DNA content following propidium iodide staining (see Figure 1A) and [^{3}H]-thymidine incorporation as a measure of DNA synthesis (Figure 1B).

These experiments show clear cell cycle regulation of *cdc2* transcription in synchronized HeLa cells (see Figure 1C). Transcription was highest during S and G_2 phases (0−10 h) and decreased at a time corresponding to late G_2 or early M phase (12−18 h): late in G_1 (18−24 h) of the following cell cycle, levels of nascent transcripts again increased. Transcription of the human *cdc2* gene varied over a 7-fold range throughout the cell cycle compared to β-actin which remained relatively constant. Similar results were obtained using a nocodazole synchronization procedure (data not shown). The pattern of *cdc2* transcription is similar to that of *cdc2* mRNA accumulation (McGowan *et al.*, 1990; S.Dalton, unpublished) indicating that regulation of transcription is an important level of control in determining human *cdc2* mRNA levels during the cell cycle.

Isolation of human cdc2 genomic sequences

To investigate cell growth/cell cycle regulated transcription of the human *cdc2* gene in more detail, human genomic clones containing *cdc2* promoter sequences were isolated by screening a genomic library with two 60mer oligonucleotides, complementary to the sense strand of the published human *cdc2* cDNA sequence (Lee and Nurse, 1987). A phage clone isolated from this screen containing a 17 kb *Sal*I insert, Hg.cdc2-03, was selected for further study. Sequence analysis across and flanking a unique *Sac*II site in the Hg.cdc2-03 insert (see Figure 2A) revealed this to correspond to the region of a unique *Sac*II site in the human *cdc2* cDNA (see Lee and Nurse, 1987). Downstream of the *Sac*II site in the 5′-untranslated region, an intron−exon splice junction was identified followed by complete divergence from the cDNA sequence (Figure 2C). Exons throughout this clone were sequenced using primers complementary to the cDNA sequence in order to verify it as a genomic copy of human *cdc2* sequences (data not shown). This procedure

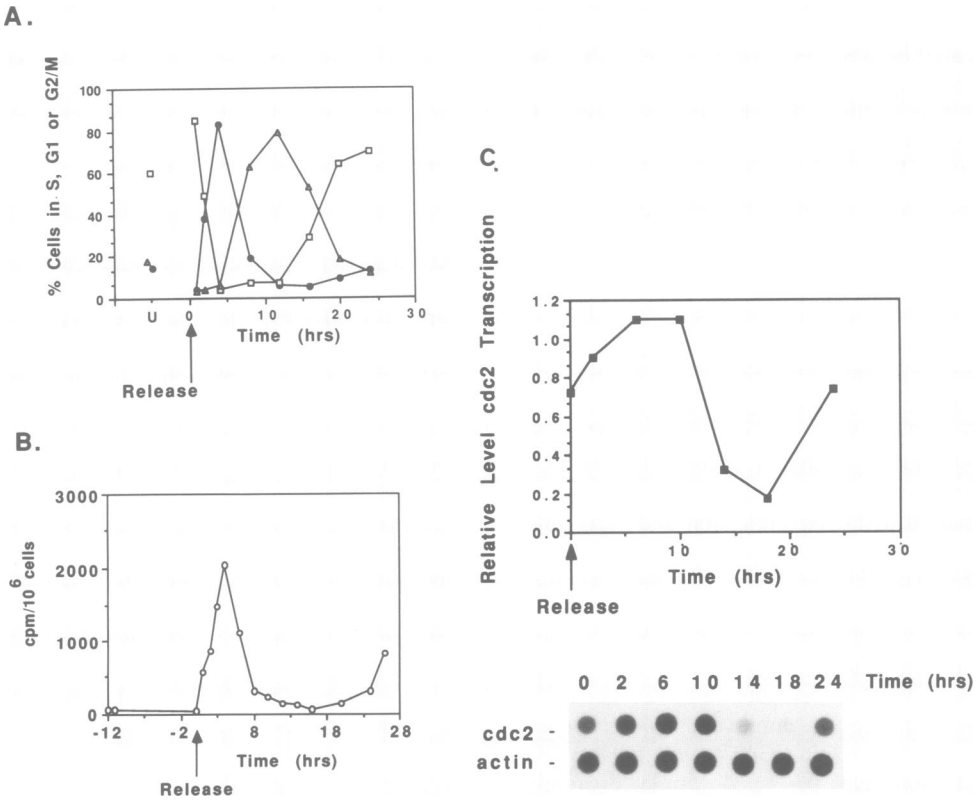

Fig. 1. Cell cycle regulation of h.cdc2 transcription. HeLa cells grown at a density of 0.5 × 10^6/ml in spinner flasks were synchronized at the G_1/S boundary by a thymidine−aphidicolin block and released into S phase. Progression through the cell cycle was monitored by (**A**) FACS analysis; the percentage of (●) S phase cells, (□) G_1 cells and (△) G_2−M cells in the synchronized cell population in addition to an unsynchronized sample (U) are shown and; (**B**) [^{3}H]thymidine pulse labelling (15 min pulse) showing synchronization of HeLa cells; data are expressed as the amount of TCA insoluble material incorporated per 10^6 cells. The time of release from the aphidicolin block is indicated. (**C**) Nuclei were made at various times from cells synchronized by a sequential thymidine−aphidicolin block, pulse labelled with [^{32}P]rUTP and hot nascent transcripts used to probe filter immobilized single stranded human *cdc2* or β-actin cDNA insert and processed as described in Materials and methods. Relative rates of *cdc2* transcription (normalized against β-actin) over the labelling interval for different time points was determined by laser densitometry.

identified several introns which were mapped and sized (Figure 2A). Intervening sequences (IVS) 1,2,3 and 4 interrupt the cDNA sequence at positions corresponding to positions immediately following bases 116, 176, 333 and 458, respectively (see Lee and Nurse, 1987). The Hg.cdc-03 insert does not contain the entire *cdc2* coding region but it extends to codon 106.

To map *cdc2* transcription start sites, a combination of primer extension and RNAse protection analysis using poly(A)$^+$ HeLa RNA was performed (Figure 2B). Two major transcription start sites were mapped 74 and 56 bp upstream of the unique *Sac*II site (Figure 2B) and are designated start site 1 (SS1) and start site (SS2) which generate transcripts with 5′ untranslated regions of 115 and 97 bases, respectively. The major start site (SS1) is hereon assigned +1 as a reference for base positioning in the

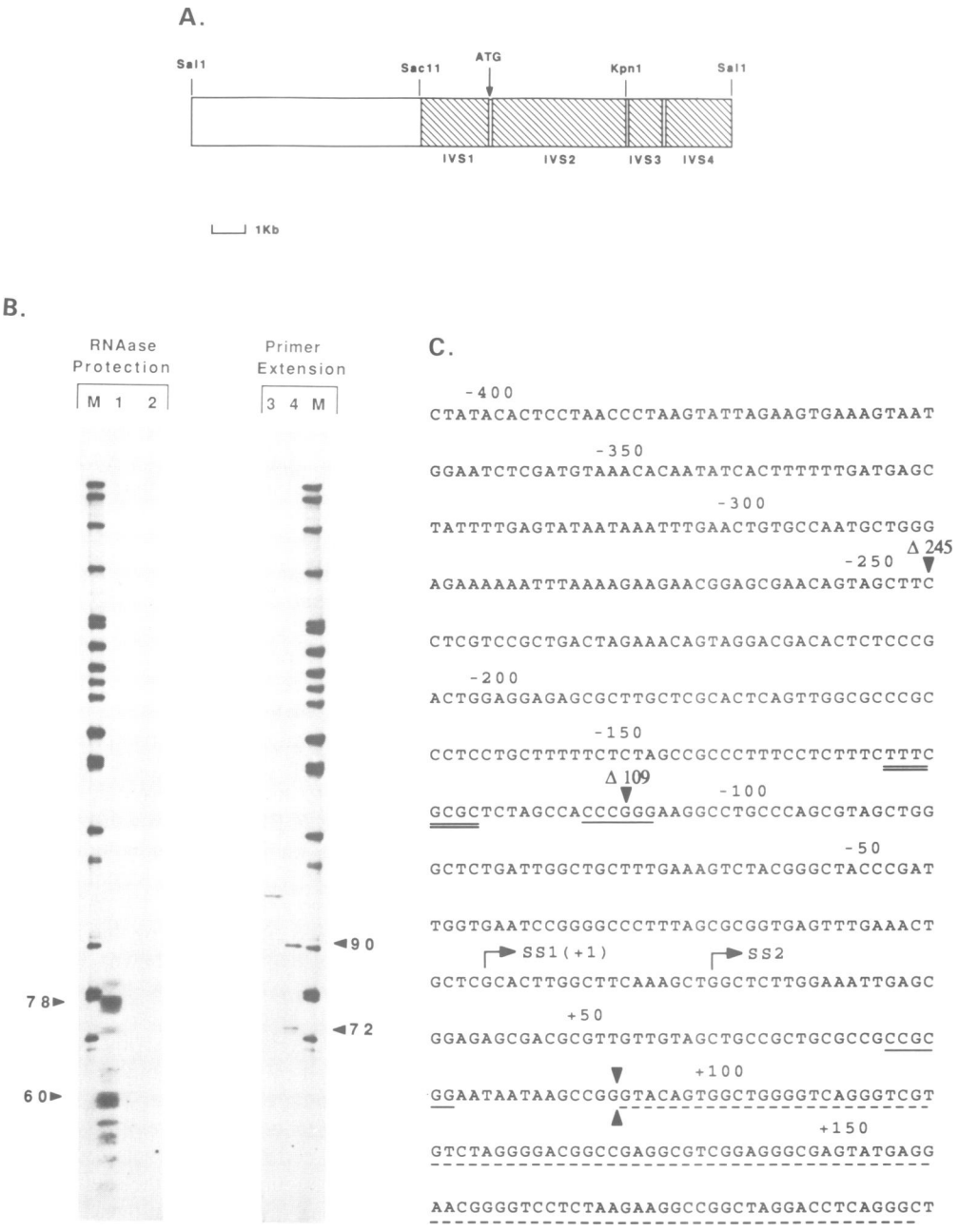

Fig. 2. Identification and characterization of human genomic *cdc2* 5′ flanking sequences. (**A**) Map of Hg.cdc2-03 genomic clone including relative positions of introns (IVS 1, 2, 3 and 4; hatched regions), exons, the initiator methionine codon and 5′ flanking region. (**B**) Mapping 5′ ends of human *cdc2* mRNA by RNAase protection (left panel) and primer extension analysis (right panel); on 6% polyacrylamide−8 M urea sequencing gels; see Materials and methods). Markers (M) are [^{32}P] end-labelled *Msp*I cut pBR322; length (in bases) of primer extension and RNAase products are indicated. Left panel: lane 1, RNAase protection with 2 μg poly(A)$^+$ HeLa RNA; lane 2, 10 μg yeast tRNA. Right panel: lanes 3 and 4 primer extension of 2 μg HeLa poly(A)$^+$ RNA with cyclin B and h.cdc2 primers respectively. Details of the primer sequence and RNA probe are described in Materials and methods. Lane 3 shows a major primer extension product to mark the 5′ end of human cyclin B mRNA (Pines and Hunter, 1989). (**C**) 5′ flanking sequence of human *cdc2* showing two sites of transcriptional initiation (SS1 and SS2) and the junction of the 5′ untranslated region with intron 1 (underlined with broken line). +1 reference is at the major transcription start site (SS1). The *cdc2* primer extends from positions +90 to +61 and the antisense RNA protection probe from +79 to −109. *Sac*II and *Sma*I sites are underlined. −109 and −245 deletion endpoints are indicated by arrows (refer to Figures 3 and 5).

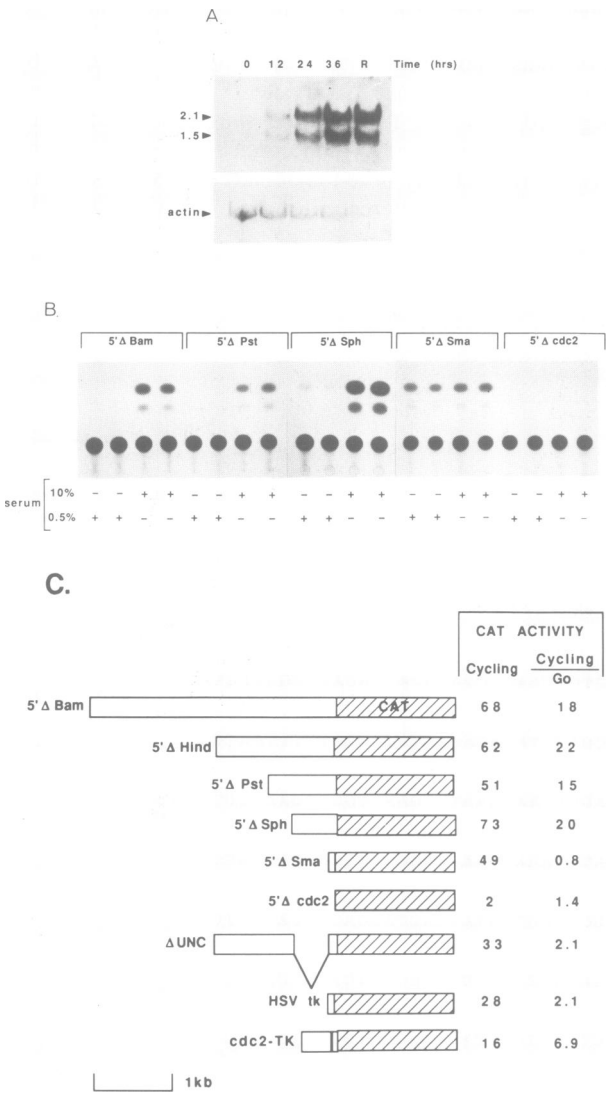

Fig. 3. Growth regulation of *cdc2* mRNA and promoter activity in ICRF23 cells. (**A**) ICRF23 fibroblasts were switched from DMEM plus 10% to DMEM plus 0.5% FCS for 36 h followed by refeeding with DMEM plus fresh 10% FCS. At various times cytoplasmic RNA (10 μg) was prepared and analysed by Northern blot hybridization probing with a human [^{32}P]-labelled *cdc2* cDNA insert. Sizes of the two *cdc2* mRNA species are indicated in kb. R, growing cells; 0, serum starved for 36 h; 12, 24 and 36 h after stimulation with 10% FCS. Lower panel; Northern blot of the same RNA samples probed with [^{32}P]-labelled β-actin cDNA probe. (**B**) Human *cdc2* promoter−CAT reporter constructs (5 μg, see Figure 3C) were co-transfected into ICRF23 fibroblasts with pCH110 (2 μg, LacZ reporter). After removal of calcium phosphate precipitates, cells were cultured in DMEM medium with 10% or 0.5% FCS. After 60 h cells were harvested and CAT assays performed by method 1 as described in Materials and methods. Each experiment was performed in duplicate, growth conditions are indicated as − (DMEM plus 0.5% FCS) or + (DMEM plus 10% FCS). 5′ deletion constructs are designated according to the restriction site end point in the promoter. (**C**) Transfections were performed as described in (b) using the indicated constructs and CAT activities determined by method 2 (see Materials and methods), normalized against β-galactosidase activity (internal control) and expressed as a value (CAT activity/β-galactosidase activity) in cycling cells or as an indication of relative (normalized) CAT activity in cycling cells versus non-cycling (G_0) cells.

genomic sequence (Figure 2C). In the immediate region upstream of the start sites no classical TATA-like elements are evident, although the presence of TTTGAAA motifs 66

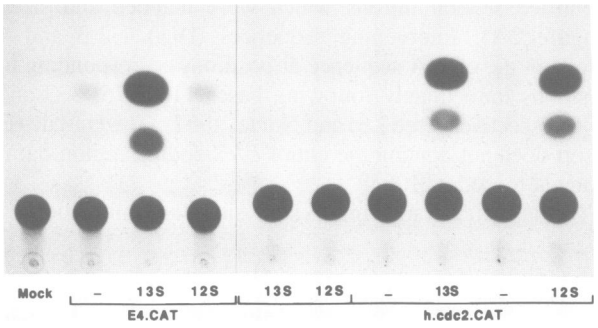

Fig. 4. The human *cdc2* promoter is silent in growth arrested baby rat kidney cells. Baby rat kidney cells were transfected with either a human cdc2−CAT reporter (5′ΔBam endpoint of human *cdc2* promoter; tracks 7−10; 5 μg) or pE4.CAT (tracks 2−4; 5 μg), pCH110 (2 μg) and, 12S(pJF12; tracks 4, 6 and 10) or 13S(pJN20; tracks 3, 5 and 8) E1A expression plasmid (5 μg). 48 h after removal of precipitates, CAT and β-galactosidase activities in extracts were determined by method 1 (see Materials and methods). β-galactosidase activities between extracts varied no more than 2-fold (data not shown).

and 10 bp upstream of SS1 were noted. Two CCAAT motifs (5′-TCAAAGCAGCCAATCAGAG-3′ and 5′-CCGGATT-CACCAATCGGGT-3′) exhibited extended sequence identity with the consensus CAAT box binding site for CP-1/2 on the non-coding strand are located at −76 and −44 respectively.

The human cdc2 promoter is negatively regulated in growth arrested cells

Levels of *cdc2* mRNA substantially decrease as mammalian cells cease to proliferate but increase significantly as cells exit G_0 and re-enter the cell cycle. Thus, *cdc2* mRNA levels are growth regulated (see Introduction). This regulation was investigated further in primary human embryonic lung fibroblasts (ICRF23) by assessing the role of transcriptional control using *cdc2* promoter−reporter fusions.

Arrest of ICRF23 cell growth by serum starvation was routinely monitored by [^{3}H]thymidine incorporation. After 30 h in 0.5% fetal calf serum (FCS), [^{3}H]thymidine incorporation was typically 10- to 20-fold lower than cells grown in 10% FCS indicating that most of these cells were no longer cycling (data not shown). Following a shift to 0.5% FCS serum, *cdc2* mRNA levels decreased to almost undetectable levels within 36 h (T = 0; see Figure 3A). Reversal of growth arrest by serum stimulation was accompanied by elevation of *cdc2* transcript levels which increased progressively after 12, 24 and 36 h in a similar fashion to that described previously (Lee *et al.*, 1988a). In contrast, β-actin mRNA levels remained unchanged.

Growth regulation of the human *cdc2* gene at the transcriptional level was investigated by comparing promoter activity of a *cdc2* promoter (2.5 kb *Bam*HI fragment)−CAT reporter gene fusion in cycling and G_0 cells. In these transient expression assays (see Materials and methods), reporter constructs were transfected into ICRF23 cells and CAT activities assessed under different growth conditions. CAT activity (Figure 3B and C) generated from the *cdc2* promoter−CAT reporter was barely detected in serum-starved, non-cycling (G_0) cells, generally being 15- to 20-fold lower in comparison to cycling cells. Similar changes in CAT mRNA levels in transient assays were also detected by RNAse protection analysis in ICRF23 cells (data not

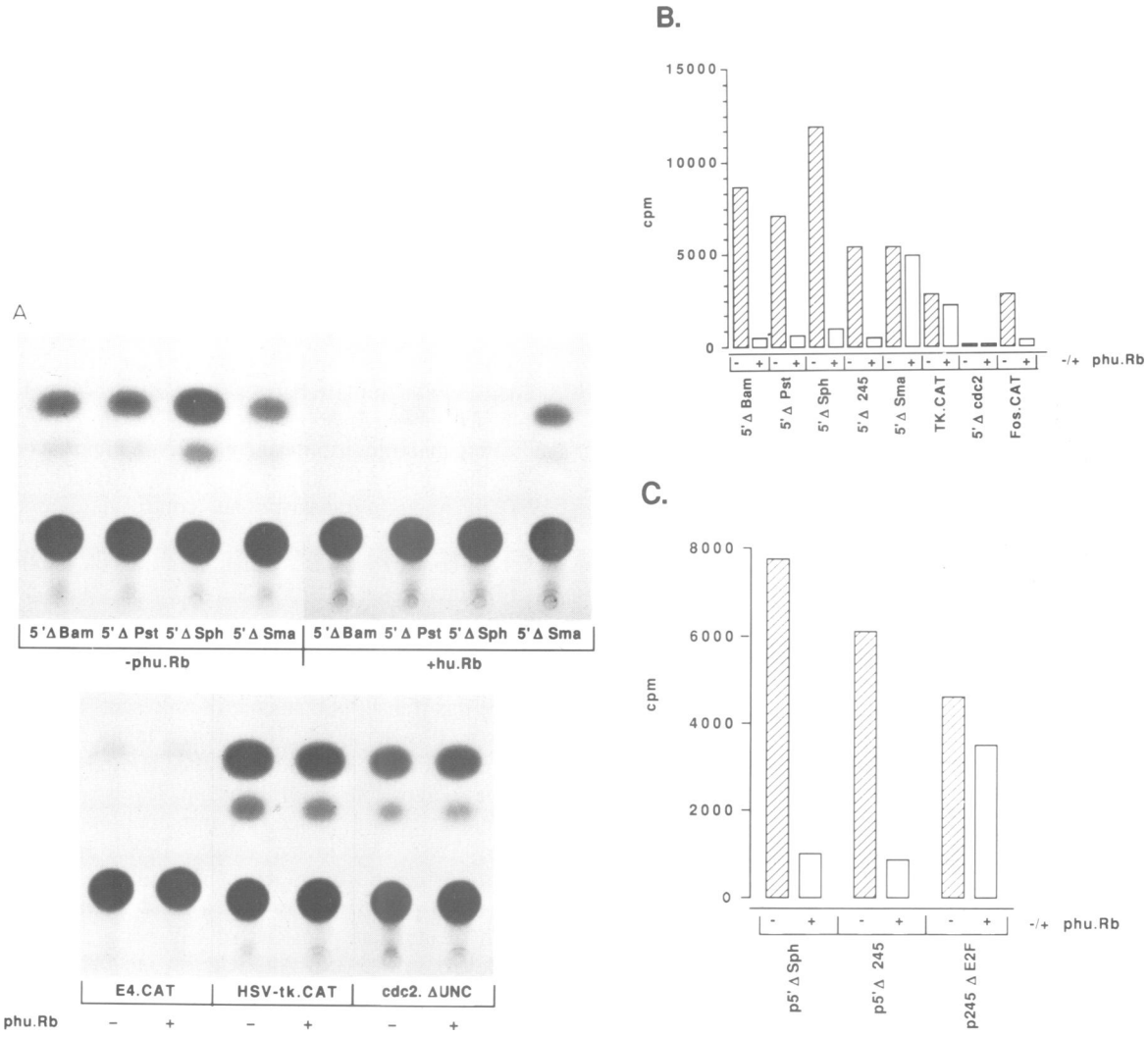

Fig. 5. Negative regulation of the human *cdc2* promoter by the retinoblastoma gene product. (**A**) ICRF fibroblasts in exponential growth phase were transfected with either cdc2−CAT constructs (5 μg) pE4.CAT (5 μg) or pBL.CAT2 (HSV tk-CAT, 5 μg) reporters and where applicable the Rb expression plasmid phu.Rb (2 μg, Robbins *et al.*, 1990). In all experiments pCH110 (2 μg) was included as an internal control. 60 h after removal of precipitates, cells were harvested and CAT activity analysed by method 1 as described in Figure 3. (**B**) Mapping sequences required for repression of the *cdc2* promoter by Rb. Co-transfection assays in ICRF23 cells were performed using cdc2−CAT constructs +/− phu.Rb as in (A) but quantification of CAT activity was determined by method 2 and expressed as c.p.m. after being normalized for β-galactosidase activity. (**C**) E2F sites are required for optimal repression by Rb.cdc2−CAT reporters were transfected +/− phu.Rb as described above and CAT activity assessed by method 2. Data is expressed as described in (C).

shown). These experiments show that 2.5 kb of *cdc2* 5′ flanking sequence is sufficient for regulated transcription in response to growth status of the cell (see Figure 3B and C).

To define in more detail 5′ flanking sequences required for growth regulation of the *cdc2* promoter, a series of 5′ deletion truncations were also tested in ICRF23 fibroblasts under different growth conditions (Figure 3B and C). This analysis revealed several points regarding regulation of the human *cdc2* promoter. In cycling cells, no significant differences were detected in promoter activity until bases −949(Δ*Pst*I) to −722(Δ*Sph*I) were deleted. Deletion of these sequences increased promoter activity in cycling and G_0 cells by ~2-fold compared with other promoter constructs, once corrected for β-galactosidase activity (Figure 3B and C). With the exception of the −109 deletion (Δ*Sma*I, see Figure 3B and C), all fusion genes were tightly regulated by growth status. However, deletion of sequences between −722 and −109 deregulated the *cdc2* promoter as indicated by constitutive expression in cycling and growth arrested cells (Figure 3B and C). These results suggest that the *cdc2* promoter is negatively regulated in non-cycling cells and that this effect is mediated through upstream negative control (UNC) sequences within the −722 to −109 region. Deletion of UNC sequences therefore appears to deregulate the *cdc2* promoter such that transcriptional activity is switched from being growth dependent to growth independent.

To test if promoter sequences in the UNC region were sufficient to confer growth status-linked negative regulation in this assay, they were fused to an HSV tk-CAT reporter (pBL.CAT2) which is not down regulated in response to growth arrest (Figure 3C). Fusion of UNC sequences to the HSV-tk promoter (*cdc2*−TK) had no obvious effects on promoter activity in cycling cells but resulted in a 3.5-fold decrease in CAT activity in non-cycling cells.

It has been previously reported that $p34^{cdc2}$ and *cdc2* mRNA levels are very low in growth arrested baby rat kidney (BRK) cells compared with exponentially dividing

cells (Draetta *et al.*, 1988). This effect can be reversed by expressing the 12S.E1A gene product which induces cell proliferation and greatly enhances endogenous *cdc2* mRNA and $p34^{cdc2}$ levels (Draetta *et al.*, 1988; Wang *et al.*, 1991; S.Dalton, unpublished). To investigate growth control of the human *cdc2* promoter further, *cdc2*−CAT constructs were transfected into arrested BRK cells with or without 12S.E1A(pJF12) or 13S.E1A(pJN20) expression constructs (Schneider *et al.*, 1987). These experiments showed that the *cdc2* promoter had low activity in non-dividing BRK cells but was activated by 10- to 20-fold when co-transfected with 12S or 13S.E1A (see Figure 4). As a control, the adenovirus E4 promoter−CAT fusion gene (pE4.CAT) was shown in this system to have detectable basal activity in senescent BRK cells and was activated by 13S.E1A, in contrast to 12S.E1A which had no effect (see Moran and Matthews, 1987). These experiments again clearly demonstrate that the *cdc2* promoter is regulated in a growth dependent fashion.

The human cdc2 promoter is negatively regulated by the retinoblastoma gene product

As the *cdc2* promoter was shown to be repressed in stationary cells, the retinoblastoma (Rb) gene product was tested as a candidate negative regulator. This follows numerous reports that Rb acts as a negative regulator of cell growth (reviewed by Weinberg, 1990), possibly by targeting cell cycle regulatory genes and that it can be recruited to DNA via E2F sites found in several growth regulated promoters (see Discussion). These experiments were designed to answer two questions regarding possible transcriptional regulation of the *cdc2* promoter by Rb; (i) does Rb repress the *cdc2* promoter and if so, (ii) are E2F binding sites required? Co-transfection of an Rb expression plasmid (phu.Rb; Robbins *et al.*, 1990) with various *cdc2*−CAT fusion genes into cycling ICRF23 fibroblasts revealed that over-expression of Rb repressed the 2.5 kb *Bam*HI *cdc2* promoter by 10- to 20-fold (Figure 5). Similar changes were also seen when levels of endogenous transcripts were analysed (data not shown).

To identify promoter sequences required for Rb-mediated repression in more detail, a series of 5′ promoter deletions fused to a CAT reporter were co-transfected with phu.Rb. This analysis showed that sequences up to −245 were sufficient for high levels of basal promoter activity in cycling cells and that the −245 to −109 region was sufficient for responsiveness to Rb overexpression. Further truncation of the promoter to position −183 lowered the basal level of promoter activity by 3-fold and so further characterization of the promoter was restricted to the −245 endpoint. Co-transfection of phu.Rb with reporter genes driven by HSV tk, E4 (Figure 5A and B) and SV40 early promoters had no significant effect (data not shown) indicating that repression of the *cdc2* promoter was not just a general effect on transcription. Rb also down regulated basal level human c-*fos* promoter activity (Figure 5B) as previously reported by Robbins *et al.* (1990).

Analysis of the human *cdc2* promoter in the −245 to −109 region revealed a high affinity E2F binding site at position −125 (5′-TTTCGCGC-3′). Because a potential low affinity E2F site at −107 was also identified, this was mutagenized in combination with the −125 site so that it would not bind E2F. To see if these sites played a role in Rb-mediated repression, a construct with E2F site mutations in a −245 promoter background was tested for responsiveness to Rb overexpression. These experiments showed that Rb had only a minor effect on the mutant promoter (20% wild type repression) indicating that intact E2F sites were required for optimal repression (Figure 5C).

Discussion

Transcription of the human cdc2 gene is cell cycle regulated

Nuclear run-on assays show that *cdc2* transcription in HeLa cells is highest in S and G_2 phases of the cell cycle. This coincides with similar changes in *cdc2* mRNA and $p34^{cdc2}$ synthesis reported previously (McGowan *et al.*, 1990) indicating that transcriptional control is an important element in regulating new synthesis of $p34^{cdc2}$ (McGowan *et al.*, 1990). Although transcriptional control is important in determining levels of *cdc2* mRNA in the cell cycle, other levels of control such as regulation of mRNA stability may also be involved.

Since synthesis of $p34^{cdc2}$ itself shows similar cell cycle kinetics to transcription of *cdc2* (McGowan *et al.*, 1990; R.Marais and S.Dalton, unpublished), it is reasonable to assume that transcriptional control represents a rate-limiting step in $p34^{cdc2}$ synthesis. However, it is not clear how cell cycle-dependent transcription of *cdc2* and $p34^{cdc2}$ synthesis can be compatible with constant levels of $p34^{cdc2}$ in mammalian cells as judged by immunoblots (Draetta and Beach, 1988; McGowan *et al.*, 1990; R.Marais and S.Dalton, unpublished). One possibility is that newly synthesized $p34^{cdc2}$ forms only a small percentage of the overall steady-state pool and so will not greatly influence total levels in the cell. Alternatively, to balance constant levels of $p34^{cdc2}$ throughout the cell cycle (Draetta and Beach, 1988; McGowan *et al.*, 1990), $p34^{cdc2}$ could be degraded in a cell cycle specific fashion inversely to its rate of synthesis. This may involve a mechanism where an 'old' pool of $p34^{cdc2}$ made in the previous cell cycle is preferentially degraded at the same time as nascent $p34^{cdc2}$ synthesis in the following cell cycle (see discussion by McGowan *et al.*, 1990).

Differences in cdc2 transcriptional control between humans and yeast

In fission yeast there appears to be little or no cell cycle regulated transcriptional control of the $cdc2^+$ gene (Durkacz *et al.*, 1986), in contrast to its human homologue. Although post-translational control of $p34^{cdc2}$ kinase activity is fundamentally conserved between the two species (Draetta, 1990; Nurse, 1990), it is not clear why additional levels of control are involved in regulation of $p34^{cdc2}$ synthesis in cycling mammalian cells. However, it has been suggested that transcriptional control may be relevant to more long term processes such as cell ageing, senescence and differentiation (Lee *et al.*, 1988a). The role of *cdc2*-like kinases in yeast cell cycle control appears to be distinctly different from that in humans. For example, multiple *cdc2*-like kinases have been identified in human cells which are proposed to have either a G_1/S or a G_2/M execution point, but not both (Fang and Newport, 1991; Elledge and Spottswood, 1991; Tsai *et al.*, 1991). Thus, as different *cdc2*-like kinases appear to have different execution points in the cell cycle, it may be

necessary to control when they are expressed by regulating transcription.

Rb as a negative regulator of the cdc2 gene

Mapping studies indicate that promoter (UNC) sequences between −722 and −109 negatively regulate *cdc2* transcription in G_0 cells as deletion of these sequences resulted in growth-independent promoter activity. It therefore seemed likely that a repressor protein(s) interacting through the UNC region was involved in negative regulation. Rb was tested as a candidate repressor as its properties were judged to be consistent with it having some role in regulating transcription of the human *cdc2* gene. For instance, Rb can negatively regulate transcription of several growth regulated genes including c-*fos* (Robbins *et al.*, 1990) and IL-6 (Santhanam *et al.*, 1991); it functions as a negative regulator of the cell cycle (Horowitz *et al.*, 1990; Lee *et al.*, 1988b) and its activity is thought to be cell cycle regulated (Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989). This possibility was tested and data presented show that Rb negatively regulates the *cdc2* promoter and that E2F binding sites are required for optimal repression. Deletion of two putative E2F binding sites, however, did not completely abolish Rb-dependent repression indicating that other sites in the promoter may also be involved; one potential target site is the medium affinity E2F site (5′-GTTGGCGC-3′) at −173. These results show that Rb negatively regulates the *cdc2* promoter via E2F binding sites: data showing that Rb interacts with an E2F-like transcription factor *in vitro* (Bandara and La Thangue, 1991; Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991) suggest that Rb may act directly on the *cdc2* promoter.

E1A-mediated activation of the *cdc2* promoter shown in this report could occur either by direct transactivation or by antagonizing a negative regulator such as Rb. The second possibility is attractive since 12S.E1A has no direct transactivation function (see Moran and Matthews, 1987), it can form stable complexes with Rb (Whyte *et al.*, 1989) and can disrupt Rb−E2F complexes *in vitro* (Bandara and La Thangue, 1991; Mudryj *et al.*, 1991). Therefore it is possible that one consequence of expressing 12S.E1A is to activate a set of cellular genes, including *cdc2*, required for cell cycling by removing a transcriptional block generated by Rb complexing with DNA bound transcription factors. According to this model, E1A mutations that block E1A−Rb interaction should also block activation of the *cdc2* promoter.

Does Rb have a role in cell cycle control of cdc2 transcription?

The observation that Rb forms complexes with an E2F-like factor only in G_1 (Bagchi *et al.*, 1991; Bandara *et al.*, 1991; Shirodkar *et al.*, 1992) suggests a model for how Rb may regulate the *cdc2* promoter in the cell cycle. This could involve unphosphorylated Rb associating with E2F sites in G_1 thereby blocking transcription and dissociating late in G_1 when phosphorylated in order to permit activation of *cdc2* transcription. Modulating the interaction between E2F and Rb during the cell cycle and as cells enter G_0, could also be a central factor in negative transcriptional control of growth/cell cycle regulated genes besides *cdc2*. Therefore, cell cycle regulation of *cdc2* transcription may depend on a transient association between E2F and Rb, an association regulated by the cell cycle dependent phosphorylation of Rb protein (see Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989). The observation that low *cdc2* transcription coincides to the time in the cell cycle when Rb is hypophosphorylated and capable of forming a complex with E2F supports the model that Rb is involved in negatively regulating the *cdc2* promoter in G_1 phase.

In summary, transcription of the human *cdc2* gene was shown to be cell cycle regulated and negatively controlled in response to arrest of cell division. Data are presented showing that intact E2F binding sites are required for optimal repression by Rb. These observations have implications for the regulation of other cell cycle control genes besides *cdc2*. For instance, the profile of cyclin A transcription in the cell cycle (Pines and Hunter, 1989) is similar to that of *cdc2* and could be regulated in a similar way.

Materials and methods

Isolation of Hg.cdc2 sequences

Overnight cultures of LE392 cells were infected with a human genomic phage library (gift from Dr A.Robins) for 15 min at 37°C, added to LMM agarose and plated on L-plates for 10 h at 37°C. Plates were cooled at 4°C and plaques lifted in duplicate onto Plaque Screen Membranes (NEN) which were air dried and autoclaved to lyse phage. Filters were air dried, baked at 60°C for 2 h and pre-hybridized at 42°C in 1% SDS, 10% dextran sulphate, 1 M NaCl, 50 mM Tris−HCl pH 7.5, 5 × Denhardt's solution and 0.1 mg/ml sonicated denatured salmon sperm DNA. Two 60mer oligonucleotides (DS789 and 790) were used to screen the library (one million plaques): both complementary to the 5′ coding cDNA sequence (Lee *et al.*, 1987). DS789; 5′-CTTTAATAGAGAAATTTCCCGAATTGCAGTAC-TAGGAACCCCTTCCTCTTCACTTTCTAG-3′ (codons 3−22) DS790; 5′-TCTACCCTTATACACAACTCCATAGGTACCTTCTCCAATTT-TCTCTATTTTGGTATAATC-3′ (codons 37−56). DS789, 790 were 5′ end labelled with [γ^{32}P]ATP using polynucleotide kinase (PNK); gel purified and added directly to the hybridization mix as a gel slice and incubated at 42°C for 18 h. Duplicate filters were probed separately with DS789 or DS790 probe. Filters were washed at room temperature and then at 42°C in 6 × SSC, 0.1% SDS. Plaques hybridizing with both probes were purified (usually through three rounds of screening or until pure plaques could be isolated).

Plasmids and DNA constructions

pHg.cdc2-03 was constructed by subcloning a 17 kb *Sal*I phage insert into pUC18. This insert was dissected in two by restriction at a unique site with *Sac*I. A *Sal*I−*Sac*II fragment of 6.8 kb was subcloned into pBluescript. KS$^+$ (p5′Hg.cdc2) after which restriction mapping and DNA sequence analysis were performed to confirm the clone as being human *cdc2* sequences. A second construct containing the other half of the Hg.cdc2-03 insert was also subcloned into pBluescript. KS$^+$ for sequence and mapping analysis (p3′Hg.cdc2). Fusions of the human *cdc2* promoter to a CAT reporter construct pBL.CAT2 (Luckow and Schutz, 1987) minus existing promoter sequences were made by inserting a 6.5 kb *Bgl*II−*Sac*II *cdc2* fragment generating the construct p5′ΔBgl. A series of 5′ *cdc2* promoter truncations were made directly from p5′ΔBgl by recircularization following restriction enzyme digestion at an appropriate restriction site internal to the human *cdc2* promoter and at a site flanking the promoter in the remaining polylinker. The −245 deletion was made for a PCR product generated from the *cdc2* 30mer (see below) and an oligonucleotide priming from −245 to −220 using p5′ΔBgl as template. This PCR product was digested with *Sph*I and *Sac*II and ligated into *Sph*I−*Sac*II digested p5′ΔSph vector. *Hin*dIII and *Sal*I restriction sites were inserted in place of natural E2F sites in the −245 *cdc2* promoter at positions −125 (TTCAAGCTTGCTCT) and −107 (CACCCGTCGACGC) respectively by PCR, generating p245.ΔE2F.

Cell culture and synchronization

HeLa and BRK cells were cultured at 37°C in DMEM plus 10% FCS. ICRF23 primary human embryonic lung fibroblasts were grown under the same conditions on plates never exceeding 30 passages. HeLa cells were synchronized at the G_1/S boundary by the thymidine−aphidicolin (Sigma) double block protocol described by Heintz *et al.* (1983). Synchrony of cells was monitored by assessing the rate of DNA replication using [^{3}H]-thymidine pulse labelling (as described in Dalton *et al.*, 1986) and by flow

cytometry analysis of cellular DNA content (see Pines and Hunter, 1989) using a Becton Dickson FACScan.

Primer extension, RNAase protection and nuclear run-on assays

Poly(A)$^+$ RNA was purified by chromatography over oligo-dT cellulose (Aviv and Leder, 1972). Primer extension analysis of RNA was by the method of McKnight *et al.* (1981) using human *cdc2* and cyclin B oligonucleotide primers phosphorylated with polynucleotide kinase at their 5′ ends using [γ-^{32}P]ATP. Both primers anneal to the 5′ untranslated region of the respective mRNAs. Sequences for these primers were as follows: cdc2 30mer: 5′-dCGGCTTATTATTCCGCGGCGGCGCAG-CGGC-3′ cyclin B 30mer: 5′-dTCACCAGGCAGCAGCTCAGCGGG-GAGAAGC-3′. RNAase protection assays were performed as described by Treisman (1985). A 182 bp ^{32}P-labelled RNA probe used to map 5′ ends of human *cdc2* mRNA was generated from *Sma*I linearized p5′Hg.cdc2 (described previously) using T7 RNA polymerase (New England Biolabs). Nuclear run-on assays were performed as described previously (Dalton and Wells, 1988) using [^{32}P]-labelled RNA to probe filter-bound *cdc2* and β-actin single strand cDNA inserts. Quantification of transcription was determined by scanning autoradiograms with an LKB Bromma Ultroscan XL Laser Densitometer.

Transfection of cells and CAT assays

Transfection of BRK and ICRF23 fibroblasts was by the calcium phosphate precipitation method described previously (Treisman, 1985) except that precipitates were left on ICRF23 fibroblasts for only 8 h instead of 12–16 h. 4 h before addition of precipitates, ICRF23 cells were changed to fresh DMEM + 10% FCS or, DMEM + 0.5% FCS where appropriate. 0.5–5 μg of test plasmid along with 2 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) per 1×10^6 cells were used in transient transfection assays. In serum starvation experiments, arrest of cell proliferation was routinely assessed by pulse labelling mock transfected cells with [^{3}H]thymidine for 60 min (see below) 30 h after washing away precipitates. [^{3}H]thymidine incorporation in serum starved cells was generally 5–10% of that seen in cycling cells grown in 10% FCS (data not shown). Freshly prepared BRK cells were routinely transfected 48–60 h after plating. CAT activities were determined by the TLC method (method 1) of Gorman *et al.* (1982) using equal amounts of extract protein per assay, or by the non-chromatographic method (method 2) as described by Sleigh (1986). Protein concentrations were determined using the Biorad protein assay kit. β-galactosidase activities (Hermbomel *et al.*, 1984) generally varied no more than 2-fold between different extracts indicating that transfection efficiencies between plates were comparable. Quantification of CAT activities from TLC plates were performed using an Ambis Radioanalytic Imaging System and then standardized using β-galactosidase activities.

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