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Cdc6 protein causes premature entry into S phase in a mammalian cell-free system

Kai Stoeber[1,2], Anthony D.Mills[1,2], Yumiko Kubota[1,2], Torsten Krude[1,2], Piotr Romanowski[1,2], Kathrin Marheineke[1,2], Ronald A.Laskey[1,2] and Gareth H.Williams[1,3,4]

[1]Wellcome/CRC Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, [2]Department of Zoology and [3]Departments of Pathology and Oncology, University of Cambridge, Cambridge, UK

[4]Corresponding author
e-mail: ks247@hermes.cam.ac.uk

We exploit an improved mammalian cell-free DNA replication system to analyse quiescence and Cdc6 function. Quiescent 3T3 nuclei cannot initiate replication in S phase cytosol from HeLa or 3T3 cells. Following release from quiescence, nuclei become competent to initiate semiconservative DNA replication in S phase cytosol, but not in G_0 phase cytosol. Immunoblots show that quiescent cells lack Cdc6 and that minichromosome maintenance (MCM) proteins are not associated with chromatin. Competence of G_1 phase nuclei to replicate *in vitro* coincides with maximum Cdc6 accumulation and MCM protein binding to chromatin *in vivo*. Addition of recombinant Cdc6 to permeabilized, but not intact, G_1 nuclei causes up to 82% of the nuclei to initiate and accelerates G_1 progression, making nuclei competent to replicate prematurely.

Keywords: Cdc6 protein/CDKs/cell cycle/*in vitro* replication/quiescence

Introduction

A network of signal transduction and cell-cycle progression pathways controls the regulated passage of eukaryotic cells through the cell division cycle (reviewed by Stillman, 1996; Michell, 1997). Key decision points lie at the entry to and the exit from the quiescent G_0 phase. Here we investigate the molecular basis of quiescence by assaying nuclei from quiescent 3T3 cells, or cells that have been released from quiescence, as templates for initiation of DNA replication in S phase cytosolic extracts from HeLa cells *in vitro*.

One of the major mechanisms by which DNA replication is controlled involves the regulated assembly of pre-replicative complexes (pre-RCs) or 'replication licences' at origins of replication during G_1 (Diffley *et al*., 1994; reviewed by Donovan and Diffley, 1996). The pre-RC includes two heteromeric protein complexes, the minichromosome maintenance complex (MCM) and the origin recognition complex (ORC), together with the monomeric Cdc6 protein (reviewed by Romanowski and Madine, 1996, 1997; Dutta and Bell, 1997; Newlon, 1997). The six-subunit ORC binds specifically to *Saccharomyces cerevisiae* autonomously replicating sequences (ARS) throughout the cell cycle (Bell and Stillman, 1992; Diffley and Cocker, 1992; Aparicio *et al*., 1997; Liang and Stillman, 1997; Tanaka *et al*., 1997). Although origins of replication have been difficult to define in higher eukaryotes, homologues of the yeast ORC proteins are required for initiation of replication and may therefore have similar functions (Gavin *et al*., 1995; Carpenter *et al*., 1996; Coleman *et al*., 1996; Romanowski *et al*., 1996a; Rowles *et al*., 1996).

The monomeric Cdc6 protein is essential for the initiation of DNA replication in yeast, *Xenopus* and human cells and is required for the assembly and maintenance of the pre-RC (Kelly *et al*., 1993; Liang *et al*., 1995; Nishitani and Nurse, 1995; Piatti *et al*., 1995; Cocker *et al*., 1996; Coleman *et al*., 1996; Muzi-Falconi *et al*., 1996; Detweiler and Li, 1997, 1998; Yan *et al*., 1998). The six members of the MCM protein family (Mcm2–7) are also components of the pre-RC, and association of these proteins with chromatin is required for initiation of DNA replication (Chong *et al*., 1995; Dalton and Whitbread, 1995; Kubota *et al*., 1995; Madine *et al*., 1995a). During replication, the MCM proteins become phosphorylated and displaced from chromatin (Kimura *et al*., 1994; Chong *et al*., 1995; Kubota *et al*., 1995; Madine *et al*., 1995a,b; Todorov *et al*., 1995; Coué *et al*., 1996; Hendrickson *et al*., 1996; Krude *et al*., 1996). Assembly of the pre-RC is sequential, with ORC recruiting Cdc6, which in turn promotes loading of MCM proteins onto chromatin (Coleman *et al*., 1996; Romanowski *et al*., 1996a; Rowles *et al*., 1996; Donovan *et al*., 1997; Tanaka *et al*., 1997). The regulated assembly and disassembly of the pre-RC during replication serves as a ratchet to prevent re-initiation within a single cycle (reviewed by Botchan, 1996; Laskey *et al*., 1996; Romanowski and Madine, 1996, 1997; Stillman, 1996).

Here we report a mammalian cell-free DNA replication system which we exploit to analyse the roles of Cdc6 and Mcm5 in establishing a replication-competent state in mammalian nuclei following release from quiescence. This system achieves efficient initiation of replication *in vitro* and, furthermore, gives significantly improved signal-to-background noise ratios over the published human cell-free system (Krude *et al*., 1997). Nuclei from murine 3T3 cells are synchronized in G_1 phase by release from quiescence and incubated together with cytosol from S phase human HeLa cells. We show that quiescent cells lack Cdc6 and that MCM proteins are not associated with chromatin. Competence to initiate DNA replication *in vitro* arises suddenly following release from quiescence coinciding with maximum Cdc6 accumulation and binding of the MCM protein complex to chromatin. The addition of recombinant *Xenopus* Cdc6 protein to G_1 phase nuclei accelerates G_1 progression, causing premature entry into

S phase. The ability of Cdc6 protein to cause premature competence to replicate is dependent on a permeable nuclear envelope which then becomes resealed in the S phase extract, revealing striking parallels to the *Xenopus* egg replication system (reviewed by Romanowski and Madine, 1996, 1997).

Results

G_1 but not G_0 phase 3T3 nuclei initiate DNA replication in HeLa S phase cytosol

The capacity of nuclei from quiescent (G_0) murine 3T3 cells or from G_1 phase 3T3 cells released from quiescence to replicate in HeLa S phase cytosolic extract was tested for two reasons. First, we wished to know if G_0 nuclei are competent to replicate *in vitro* and second we sought to improve the ratio of signal-to-background noise experienced with our published human cell-free replication system (Krude *et al*., 1997).

Untransformed 3T3 cells were synchronized in G_0 by contact inhibition and subsequently released from quiescence by subculturing the confluent cells at lower density. Cells begin to enter S phase ~21 h after the release as determined by flow cytometry (Figure 1B). Nuclei were isolated from cell cultures at various times after release from G_0 and incubated in S phase cytosol from chemically synchronized HeLa cells supplemented with ribonucleoside and deoxyribonucleoside triphosphates (NTPs and dNTPs) and an energy regeneration system to allow initiation of DNA replication *in vitro* (Krude *et al*., 1997). Replication reactions also contained biotin-16-dUTP as a marker to allow detection of DNA synthesized during the *in vitro* incubation by confocal fluorescence microscopy. The proportion of nuclei replicating was determined from printed images of nuclei stained for the presence of DNA by propidium iodide and for synthesis of DNA *in vitro* by fluorescein-linked streptavidin.

Figure 1A shows that G_1 phase 3T3 nuclei prepared 18 h after release from quiescence initiate DNA replication when incubated in HeLa S phase cytosol. Note that 3T3 nuclei released from the natural synchrony of quiescence give greatly improved signal-to-background noise ratios compared with the published system using chemically synchronized HeLa cells (Krude *et al*., 1997). Similar results were obtained with S phase cytosol derived from 3T3 cells, although the efficiency of initiation was two-thirds of that obtained with HeLa S phase cytosol (data not shown). Thus HeLa S phase cytosol was used in standard replication reactions, because it is both more efficient and easier to prepare in large amounts as HeLa cells grow to high density. In contrast to nuclei from G_1 phase cells, nuclei prepared from quiescent (G_0) 3T3 cells fail to replicate in S phase cytosol (Figure 1A). Control incubations were performed to exclude the possibility that the DNA replication signal observed in the G_1 phase nuclei represents elongation in nuclei that have already entered S phase and therefore initiated *in vivo* (S phase contaminants). These consisted of parallel incubations of G_0, G_1 or S phase nuclei in 3T3 G_0 cytosol or in buffer A supplemented with NTPs and dNTPs and bovine serum albumin (BSA). No signal (<1%) was detected for G_0 phase nuclei in control incubations. A low level of

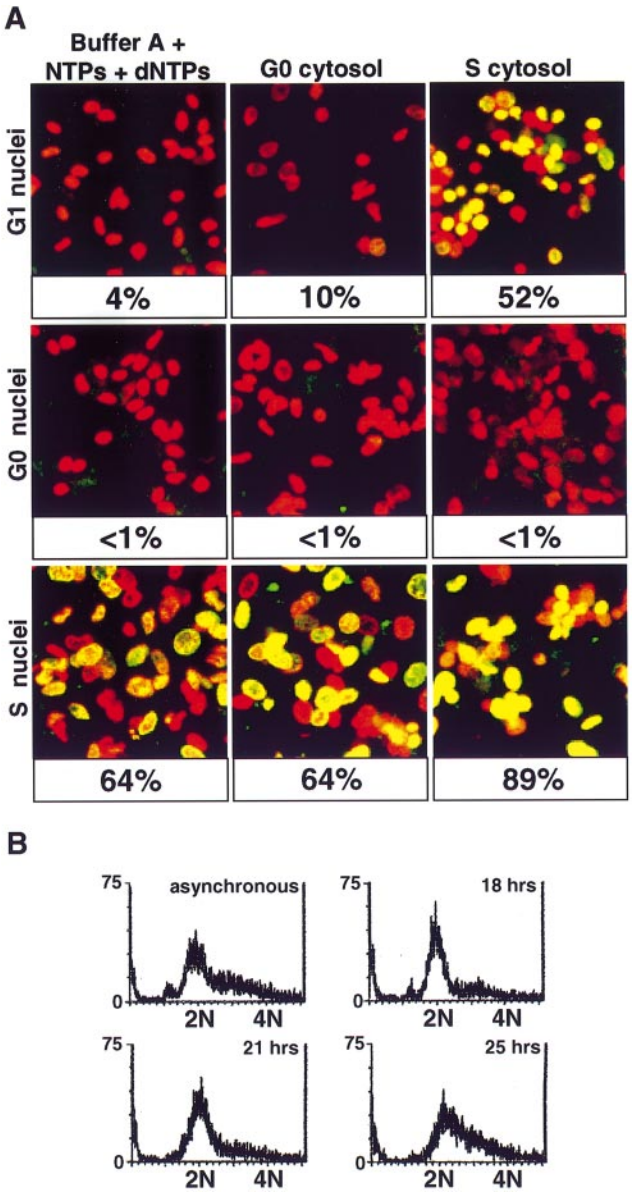

Fig. 1. Initiation of DNA synthesis in G_1 phase 3T3 nuclei incubated in HeLa S phase cytosolic extract. (**A**) Synchronized murine 3T3 G_1 phase nuclei prepared 18 h after release from quiescence were incubated with G_0 or S phase HeLa cytosol or buffer A/BSA, each containing NTPs (0.1 mM each), dNTPs (0.1 mM each) and an energy regeneration mix. After incubation for 3 h with biotin-16-dUTP, nuclei were washed, fixed in paraformaldehyde and stained with propidium iodide to reveal DNA (red) and with fluorescein–streptavidin (green) to detect biotin incorporation resulting from DNA synthesis. G_1 phase nuclei initiate only in S phase cytosol whereas G_0 nuclei fail to respond in any of the three incubations including S phase cytosol. The control reactions using S phase 3T3 nuclei show that buffer A/BSA, G_0 cytosol and S phase cytosol are all efficient in supporting elongation of DNA replication even though G_0 cytosol and buffer A/BSA do not support initiation. (**B**) FACS analysis of asynchronous or G_1 and S phase 3T3 nuclei. G_1 and S phase nuclei were obtained by releasing confluent quiescent 3T3 cells through subculturing at lower density. Analysis of the cell cycle position of these nuclei shows that at 18 h 3T3 cells are in G_1 phase and they begin to enter S phase ~21 h after release from quiescence. The majority of cells are in S phase by 25 h.

S phase contaminants (4–10%) was confirmed for G_1 phase nuclei firstly by a low DNA replication signal in G_0 cytosol (Figure 1A), and secondly by bromodeoxyuridine (BrdU) *in vivo* labelling (<5%, data not shown). In contrast, when

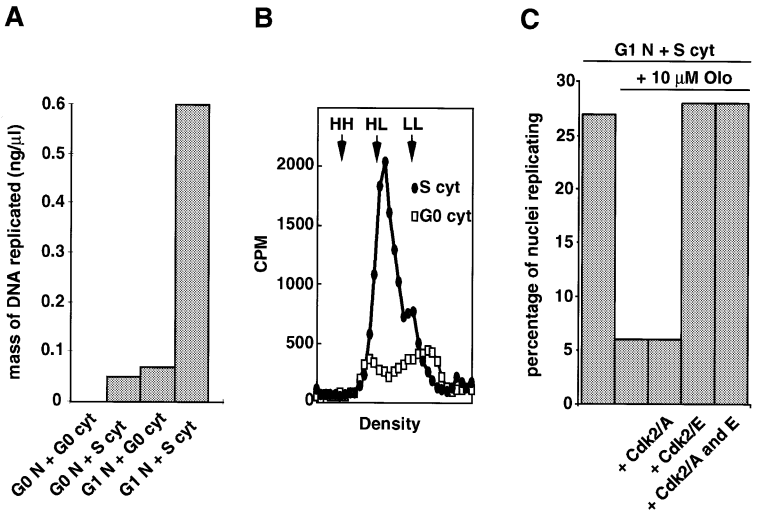

Fig. 2. G_1 phase 3T3 nuclei initiate a single round of semiconservative DNA replication *in vitro* which is cyclin–Cdk2 dependent. (**A**) Incorporation of [α-^{32}P]dATP by G_0 or G_1 phase nuclei in G_0 or S phase cytosol. A >7-fold increase in DNA synthesis was detected when G_1 phase nuclei where incubated in S phase cytosol (0.6 ng DNA/µl) compared with G_0 phase cytosol controls (0.07 ng DNA/µl). (**B**) G_1 phase nuclei initiate and elongate a single round of semiconservative DNA replication in S phase cytosol. Caesium chloride buoyant density gradient centrifugation was performed on DNA synthesized by G_1 nuclei in either S phase cytosol (o) or G_0 cytosol (□) together with BrdUTP and [α-^{32}P]dATP. LL shows the position of unsubstituted parental DNA, HL of DNA fully substituted with BrdU in only one strand after one round of semiconservative replication, and HH of DNA fully substituted in both strands after two rounds of replication. (**C**) The CDK inhibitor olomoucine (final concentration 10 µM) abolishes initiation of DNA replication by G_1 replication-competent nuclei (16.25 h release) incubated in S phase cytosol. The same concentration of olomoucine has no effect on the percentages of replicating nuclei when added to control reactions in which S phase 3T3 nuclei are incubated in S phase cytosol (data not shown). Inhibition is relieved fully by addition of recombinant cyclin E–Cdk2, but not by an equal amount of H1 kinase activity of cyclin A–Cdk2. The combination of both complexes rescues to the same extent as cyclin E–Cdk2 alone.

S phase 3T3 nuclei were incubated in buffer A with NTPs, dNTPs and BSA, or in G_0 cytosol or S phase cytosol, all efficiently supported elongation of DNA replication. These control incubations confirm that the majority of G_1 phase nuclei that are labelled in S phase cytosol represent initiation of DNA replication *in vitro*. Elongation by nuclei that had entered S phase *in vivo* is excluded by the fact that these nuclei would have elongated in G_0 cytosol or buffer A with NTPs, dNTPs and BSA (Figure 1A).

To confirm the initiation of DNA replication biochemically, *in vitro* incubations were performed in the presence of [α-^{32}P]dATP. Figure 2A shows a comparison of the incorporation obtained with G_0 phase and G_1 phase 3T3 nuclei (16.25 h release). G_0 phase nuclei failed to show significant amounts of DNA synthesis when incubated in S phase cytosol (0.05 ng DNA/µl; 0.4% of the genome). In contrast, G_1 phase nuclei synthesized 0.6 ng DNA/µl (5% of the genome) when incubated in HeLa S phase cytosol, whereas they synthesized 0.07 ng DNA/µl (0.6% of the genome) in G_0 phase cytosol controls (Figure 2A). The 2 h incubations are only one quarter of the S phase *in vivo*, and 40% of the nuclei initiate. Therefore, 5% of the genome indicates that DNA is synthesized *in vitro* at half the synthesis rate *in vivo*.

To confirm that DNA synthesized after initiation *in vitro* is due to semiconservative DNA replication, we used BrdU substitution and buoyant density gradient centrifugation (Blow and Laskey, 1986; Leno *et al*., 1992). G_1 phase 3T3 nuclei were incubated in replication reactions containing BrdUTP, [α-^{32}P]dATP and S phase cytosol from HeLa cells. After a 3 h incubation, genomic DNA was analysed by buoyant density gradient centrifugation. Figure 2B shows that a low level of DNA was synthesized by G_1 nuclei in G_0 cytosol with an intermediate density between unsubstituted (LL) and hemisubstituted DNA (HL), consistent with repair or partial DNA replication. However, S phase cytosol stimulated DNA synthesis producing predominantly hemisubstituted DNA (HL), indicating a single round of semiconservative replication. There was no evidence of synthesis of fully substituted DNA (HH) characteristic of multiple rounds of replication. These data demonstrate that G_1 phase 3T3 nuclei initiate and efficiently elongate a single round of semiconservative DNA replication in cytosolic extracts from S phase HeLa cells, but not in extracts from G_0 phase 3T3 cells.

Further evidence that DNA synthesis in G_1 nuclei is due to true initiation of replication is shown in Figure 2C. Initiation in G_1 nuclei (16.25 h release) is inhibited by the cyclin-dependent kinase (CDK) inhibitors olomoucine (final concentration 10 µM, IC_{50} for Cdk2 is 7 µM; Figure 2C) or roscovitine (data not shown). Olomoucine and roscovitine selectively inhibit both cyclin A–Cdk2 and cyclin E–Cdk2 at their IC_{50} concentrations (Vesely *et al*., 1994; Meijer, 1996). The IC_{50} for Cdk2 is several fold lower than for other kinases such as Cdk4 and MAP kinases. The same concentration of olomoucine has no effect on the percentages of replicating nuclei when added to control reactions in which S phase 3T3 nuclei are incubated in S phase cytosol (data not shown). Addition of active recombinant cyclin A–Cdk2 complex did not restore initiation. In contrast, addition of the same number of activity units of recombinant cyclin E–Cdk2 completely restores the replication signal. The combination of both complexes rescues initiation of replication to the same extent as cyclin E–Cdk2 alone (Figure 2C). Taken together, these results indicate a requirement for active cyclin E–Cdk2, but not for cyclin A–Cdk2, to initiate DNA replication *in vitro*.

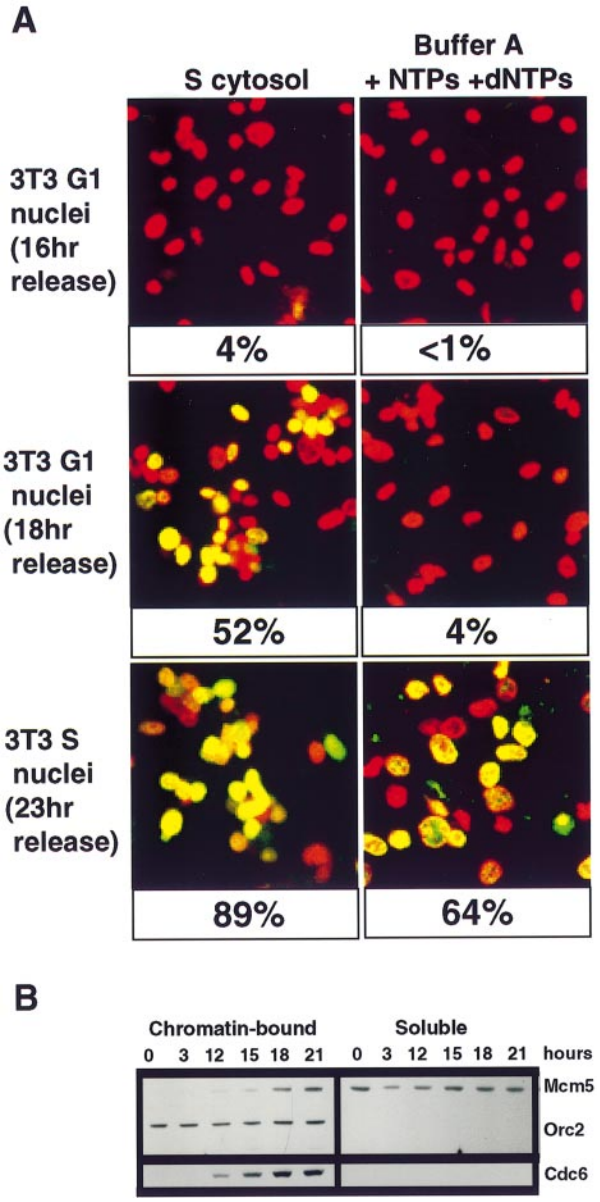

Fig. 3. G_1 phase nuclei become competent to replicate *in vitro* at a discrete point in G_1 coinciding with maximum Cdc6 accumulation and MCM binding to chromatin *in vivo*. (**A**) Nuclei were prepared from G_1 phase cells 16 or 18 h after release from quiescence. Nuclei prepared 18 h, but not 16 h, after quiescence initiate replication in HeLa S phase cytosol. S phase nuclei were prepared 23 h after the release and, unlike G_1 phase nuclei, they synthesize DNA in either S phase cytosol or buffer A/BSA containing NTPs and dNTPs. (**B**) Immunoblots of Mcm5, Orc2 and Cdc6 in chromatin-bound and soluble fractions of 3T3 cells during quiescence (0 h) or the indicated times after release from quiescence (3–21 h). The soluble fraction contains cytoplasmic and soluble nuclear proteins.

Competence of G_1 phase nuclei to replicate in vitro coincides with maximum Cdc6 accumulation and chromatin binding of MCM proteins in vivo

G_1 phase 3T3 nuclei were prepared at various timepoints after release from quiescence and incubated in HeLa S phase cytosol. Nuclei prepared from cells within 16 h after release from quiescence failed to respond to S phase cytosol (Figure 3A). However, between 16 and 18 h after release from quiescence, G_1 phase nuclei suddenly become competent to replicate *in vitro*, at least 3 h before they would enter S phase *in vivo* (Figure 3A). G_1 phase nuclei prepared from cells beyond 18 h after release from quiescence contained higher levels of S phase contaminants, hence lowering the signal-to-background noise ratio of the assay. Note that the exact time in G_1 phase at which cells become competent to replicate DNA *in vitro* in S phase cytosol varies slightly with growth conditions.

To investigate why G_0 nuclei or G_1 phase nuclei within 16 h of release from quiescence fail to initiate DNA replication *in vitro* and to identify any relevant changes that follow the release from quiescence, we have investigated the presence and distribution of proteins of the pre-RC, specifically Orc2, Mcm5 and Cdc6. Immunoblot studies were performed to look for changes in the abundance of these proteins as nuclei progress from an initiation-incompetent G_0 to a competent G_1 state. Homogenates were prepared from 3T3 cells in the G_0 state and after release from quiescence at various timepoints through G_1 phase until entry into S phase. These homogenates were fractionated into soluble and chromatin-bound fractions and immunoblotted with antibodies against human Orc2, Cdc6 and Mcm5. Note that the soluble fraction contains cytoplasmic and soluble nuclear proteins. As shown in Figure 3B, Orc2 is present exclusively in the chromatin-bound fraction and its levels do not vary from G_0 to S phase. In contrast, Cdc6 is completely absent from quiescent cells but becomes detectable on chromatin 12 h after release from quiescence. The levels of soluble Mcm5 protein do not vary significantly during the release. However, Mcm5 is absent from the chromatin-bound fraction in quiescent cells and binds to chromatin 15–18 h after the release. Chromatin-associated Mcm5 and Orc2 can be solubilized by DNase treatment (data not shown). The appearance of Mcm5 on chromatin follows that of Cdc6, and the levels of both proteins on chromatin reach saturation at ~18 h after release from quiescence. Interestingly, G_1 phase 3T3 nuclei become competent to initiate DNA replication in the cell-free system at the time of maximum Cdc6 accumulation and subsequent chromatin binding of Mcm5.

Together, these results suggest that the inability of G_0 phase nuclei to replicate in the mammalian cell-free system might be due to the lack of Cdc6 and that competence to replicate *in vitro* following release from quiescence corresponds to the assembly of pre-RCs from pre-existing ORC, newly synthesized Cdc6 and chromatin binding of MCM proteins *in vivo*.

Addition of recombinant Cdc6 increases initiation and promotes premature initiation of DNA replication in G_1 phase nuclei

If the synthesis of Cdc6 following release from quiescence is the reason why nuclei become competent to replicate, then addition of exogenous Cdc6 should make early G_1 phase 3T3 nuclei competent to replicate prematurely *in vitro*. To test this, incubations were performed similarly to those in Figure 1 but in the presence of a baculovirus-expressed six histidine-tagged version of *Xenopus* Cdc6 (His6-XCdc6; final concentration 0.65 μM). We tested XCdc6 initially because it was available in the laboratory (Coleman *et al.*, 1996). We intended to test human Cdc6 if the *Xenopus* protein failed to stimulate replication. No

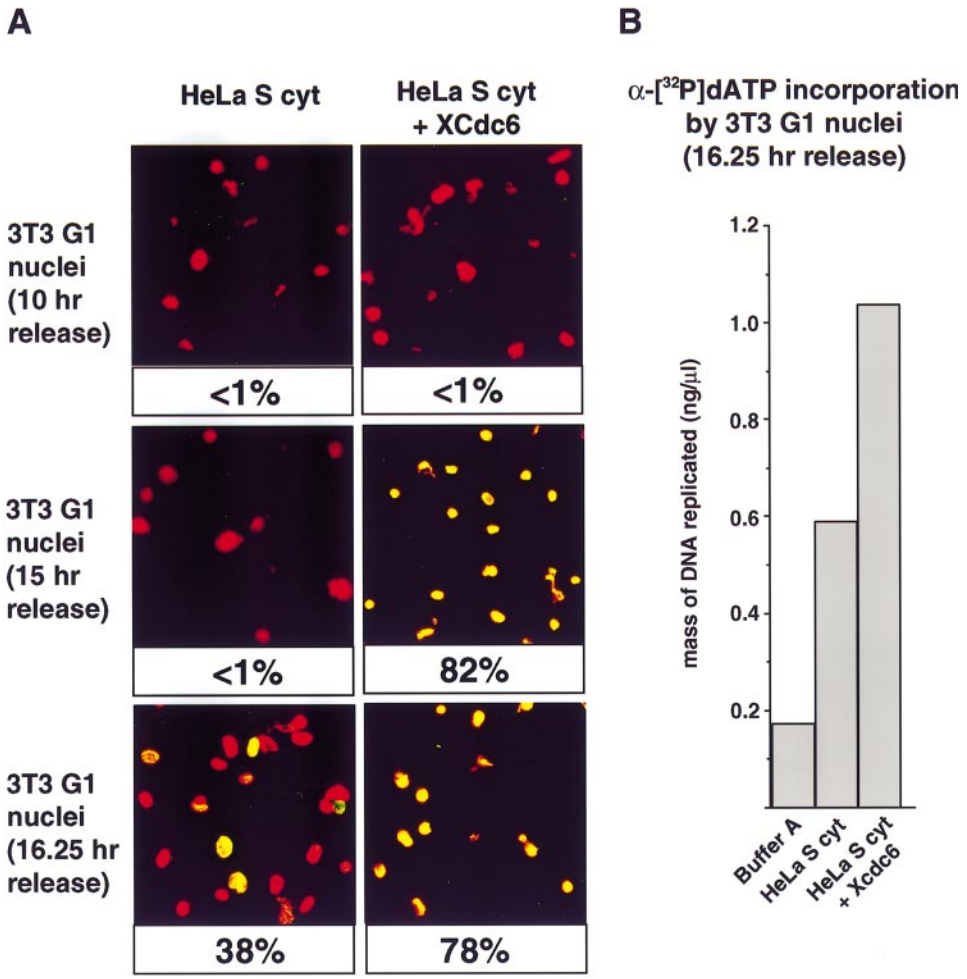

Fig. 4. Cdc6 addition promotes premature initiation of DNA replication in G_1 phase nuclei. (**A**) The addition of recombinant *Xenopus* Cdc6 protein to incubations of G_1 nuclei in S phase cytosol induces the premature entry of G_1 phase nuclei into S phase. Increased entry into S phase was detected for G_1 nuclei prepared between 15 and 18 h after release from quiescence. G_1 phase nuclei prepared at earlier timepoints failed to respond to exogenous recombinant Cdc6. (**B**) [α-^{32}P]dATP incorporation shows a 6-fold increase in DNA synthesis over the buffer A control (0.17 ng DNA/μl) when replication reactions are performed in the presence of recombinant XCdc6 and S phase cytosol (1.04 ng DNA/μl) compared with a 3-fold increase in the presence of S phase cytosol alone (0.59 ng DNA/μl). Note that nuclei for (B) were prepared only 16.25 h after release from quiescence compared with 18 h in Figure 2A.

replication (<1%) was detected for G_1 nuclei prepared 10 h after release from quiescence when incubated in HeLa S cytosol in the presence or absence of XCdc6 (Figure 4A). Similarly, G_1 phase nuclei prepared 15 h after release from G_0 did not initiate replication in HeLa S cytosol (<1%). However, addition of XCdc6 to the reaction resulted in 82% of the nuclei replicating (Figure 4A). G_1 phase nuclei taken 16.25 h after release resulted in 38% of the nuclei replicating in HeLa S cytosol without exogenous XCdc6. An additional 40% replicated in response to XCdc6, making a total of 78% (Figure 4A).

To determine the amount of DNA synthesized in G_1 phase nuclei in the presence or absence of XCdc6, incorporation of [α-^{32}P]dATP into nuclear DNA was measured using nuclei prepared 16.25 h after release from G_0 (Figure 4B). A low background (0.17 ng DNA/μl, 1.44% of the genome) of DNA synthesis in these nuclei was observed when incubated in buffer A with NTPs, dNTPs and BSA, representing a low level of S phase contaminants in the nuclear preparation (see Figure 2A). The same nuclei synthesized 0.59 ng DNA/μl (4.9% of the genome, similar to Figure 2A) when incubated in HeLa S cytosol. However, XCdc6 stimulated DNA synthesis ~2-fold more than S phase cytosol alone (1.04 ng DNA/μl, 8.64% of the genome). An interesting possibility is that the observed increase of DNA synthesis in the presence of XCdc6 might be due to re-initiation of DNA replication. To examine whether addition of XCdc6 causes DNA re-replication, we used BrdU substitution and buoyant density gradient centrifugation (Blow and Laskey, 1986; Leno *et al*., 1992). DNA synthesized in the presence of XCdc6 was predominantly hemisubstituted (HL) with BrdU, indicating a single round of semiconservative replication. No DNA re-replication which would result in fully substituted DNA (HH) was detectable (data not shown). These results demonstrate that Cdc6 accelerates G_1 progression of 3T3 nuclei *in vitro*, resulting in premature entry into S phase.

Cdc6-induced premature entry into S phase requires permeabilization of the nuclear envelope

Nuclear preparation by Dounce homogenization of hypotonically swollen cells (Heintz and Stillman, 1988; Krude *et al*., 1997) produced 3T3 nuclei with variable nuclear

envelope permeability (0–60%) as shown by entry of fluorescein-linked dextran molecules (70 kDa) by diffusion (data not shown). On addition to HeLa S phase cytosol, a variable proportion of 3T3 nuclei were observed to be transiently permeable, but essentially all the nuclear membranes repaired within 15 min. The ability of exogenous XCdc6 to promote premature initiation of DNA replication was also found to vary between different batches of nuclei prepared by Dounce homogenization. To test for a correlation between nuclear membrane permeability and response to exogenous XCdc6, a preparation of G_1 phase nuclei (18 h release) was chosen that was impermeable to fluorescein-linked dextran molecules. One aliquot of these nuclei was then fully permeabilized by lysophosphatidylcholine (lysolecithin) treatment (Leno and Munshi, 1994). Resealing of the nuclear membranes of these fully permeabilized nuclei occurred within 30 min upon incubation in extracts. The response of these permeabilized and impermeable nuclear preparations to exogenous XCdc6 (final concentration 0.65 μM) was compared in the replication reactions. Figure 5 shows that although impermeable G_1 nuclei respond to S phase cytosol they fail to respond further to exogenous XCdc6 (Figure 5b and c). In contrast, 65% of the lysolecithin-treated (permeabilized) G_1 nuclei were observed to replicate in the presence of XCdc6 compared with 40% in its absence (Figure 5d and e). The DNA replication signal in G_1 nuclei incubated in HeLa S phase cytosol alone was not influenced by the permeabilization of the nuclear envelope with lysolecithin (Figure 5b and d), excluding the possibility that the increased signal is due to permeabilization alone.

Nuclear envelope permeabilization promotes binding of exogenous Cdc6 to chromatin

To confirm that permeabilizing the nuclear envelope allows binding of exogenous XCdc6 to chromatin, immunolocalization of His6-XCdc6 was determined for both permeabilized and impermeable G_1 phase nuclei (18 h release) using a monoclonal anti-His6 antibody (Qiagen) following incubation in HeLa S phase cytosol with XCdc6. Figure 6A shows the immunolocalization of His6-XCdc6 for nuclei analysed at the end of the *in vitro* replication reactions (3 h). Chromatin-bound exogenous XCdc6 was detected in 92.1% of the lysolecithin-treated (permeabilized) nuclei. In contrast, only a minority of the impermeable nuclei (6.5%) stained positively for XCdc6. Measurement of the intensity of immunostaining for XCdc6 confirmed the differences between permeabilized and impermeable nuclei. Figure 6B shows the average intensity of fluorescent labelling of XCdc6 performed on 80 nuclei after incubation for 3 h in HeLa S phase cytosol. Permeabilized nuclei show an 11-fold increase in the intensity of XCdc6 fluorescent staining compared with impermeable nuclei.

Our data do not distinguish between failure of exogenous Cdc6 to cross the nuclear envelope or failure to bind to chromatin followed by rapid release from nuclei after entry. Nevertheless, they show clearly that nuclear envelope permeabilization stimulates Cdc6 binding and that Cdc6 stimulates DNA replication only when the nuclear envelope is permeabilized.

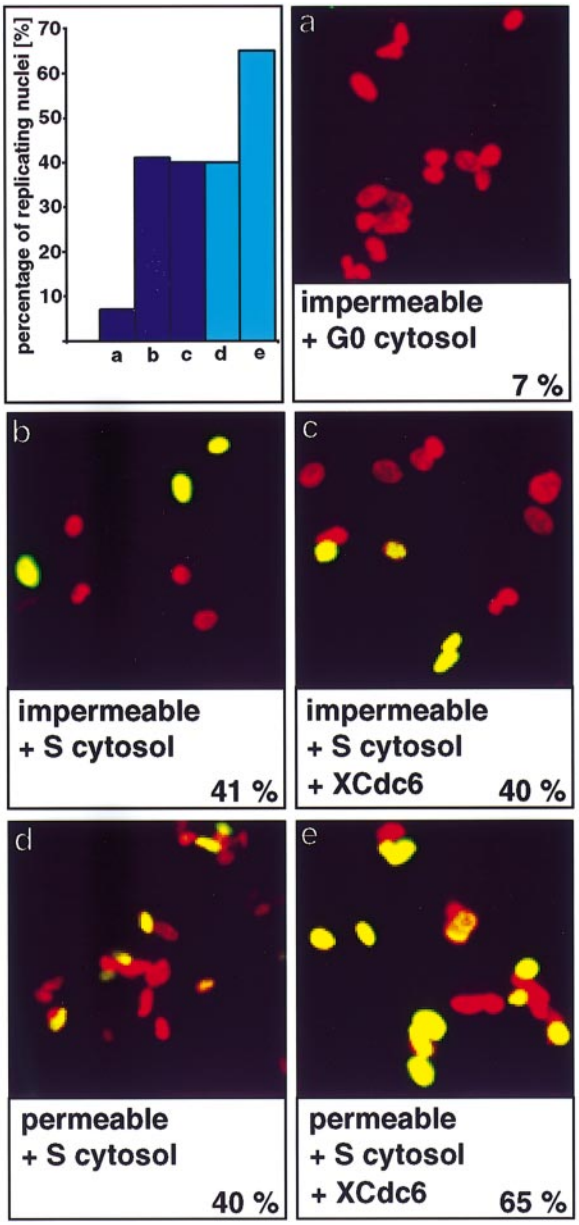

Fig. 5. Cdc6-induced premature initiation of DNA replication requires nuclear membrane permeabilization. G_1 phase nuclei prepared by gentle Dounce homogenization were observed to have impermeable nuclear envelopes by exclusion of fluorescein-linked dextran (data not shown). An aliquot was taken and the impermeable nuclei were subsequently permeabilized by lysolecithin treatment. The response of these permeabilized and impermeable G_1 nuclei to exogenous XCdc6 (final concentration 0.65 μM) was then compared in the *in vitro* replication system. A low DNA replication signal (7%) was observed for impermeable G_1 nuclei in G_0 cytosol [S phase contaminants; (**a**)]. Impermeable G_1 nuclei do not respond to exogenously added XCdc6 (**b** and **c**). In contrast, an increased replication signal (25%) was observed in the presence of XCdc6 in permeabilized G_1 phase nuclei compared with incubation in HeLa S phase cytosol alone (**d** and **e**). The DNA replication signal in G_1 nuclei incubated in S phase cytosol alone was not influenced by permeabilization of the nuclear envelope with lysolecithin (b and d). Note that the histogram summarizes quantitative data for (a–e) and that impermeable and permeable nuclei are represented by dark-blue and light-blue columns respectively.

Discussion

We describe an improved mammalian cell-free system for analysing the initiation of DNA replication. In this

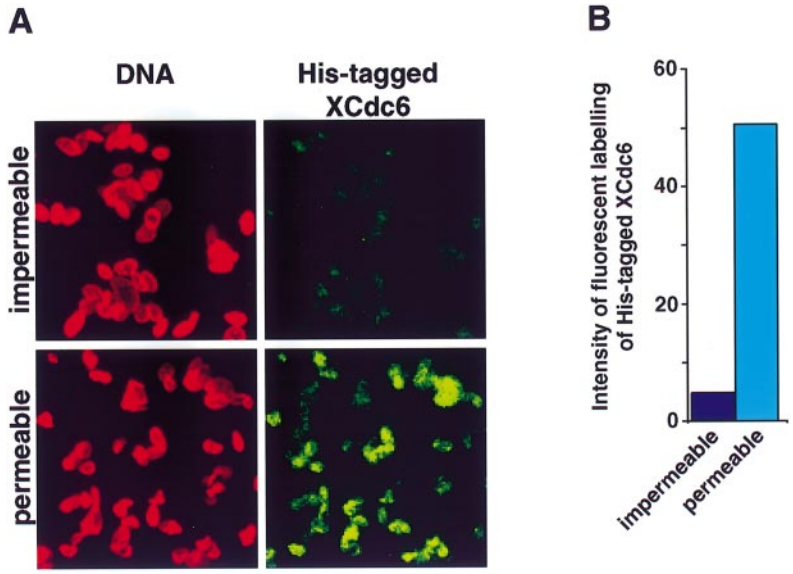

Fig. 6. Increased binding of recombinant Cdc6 to chromatin following permeabilization of G_1 phase nuclei. Monoclonal anti-His6 antibody (Qiagen) was used to detect specifically exogenous XCdc6 in impermeable and lysolecithin-treated (permeabilized) G_1 phase nuclei (18 h release) following 3 h incubations in S phase cytosol. The average intensity of fluorescent labelling of XCdc6 was determined from 80 nuclei using the LASERSHARP processing programme (Bio-Rad). (**A**) Chromatin-bound exogenous XCdc6 was detected in the majority of permeabilized G_1 nuclei (92.1%). In contrast, only a minority of the Dounced nuclei (6.5%) were found to be labelled for Xcdc6. (**B**) Permeabilized nuclei showed an 11-fold increase in the intensity of fluorescent staining of His6-XCdc6 compared with Dounced nuclei.

system, the natural synchrony of quiescence is exploited to produce an assay with a high ratio of signal-to-background noise, overcoming the main source of variability in the published human cell-free system which requires optimal synchrony procedures to obtain satisfactory signal-to-noise ratios (Krude *et al*., 1997). Somatic cell cycle control is retained in this version. G_1 phase 3T3 nuclei prepared by release from quiescence fail to initiate DNA replication when incubated in G_0 cytosolic extracts. Similarly, G_0 nuclei fail to initiate in S phase cytosolic extracts. In contrast, nuclei that have already entered S phase are easily distinguished by their ability to synthesize DNA in G_0 phase cytosol or even in buffer A supplemented with NTPs, dNTPs and BSA (Figure 1A). Initiation of replication in this system is inhibited by Cdk2 inhibitors and rescued by Cdk2–cyclin E (Figure 2C), consistent with previous studies showing a role for this cyclin–CDK complex in the G_1/S transition (Resnitzky *et al*., 1994; Ohtsubo *et al*., 1995; Resnitzky and Reed, 1995; Krude *et al*., 1997; Connell-Crowley *et al*., 1998). The [α-^{32}P]dATP incorporation and density substitution data (Figure 2A and B) demonstrate that DNA synthesis initiated in each G_1 phase nucleus *in vitro* proceeds at half the rate *in vivo* and is due to a single round of semiconservative DNA replication, not DNA repair.

Using this cell-free system, we have analysed the molecular mechanisms that establish a replication-competent state in mammalian nuclei following release from quiescence. The assembly of pre-RCs is essential for the initiation of DNA replication (reviewed by Botchan, 1996; Stillman, 1996; Romanowski and Madine, 1996, 1997; Dutta and Bell, 1997; Newlon, 1997). In yeast, it has been shown that Cdc6/Cdc18 has a critical regulatory role in the initiation of DNA replication (Kelly *et al*., 1993; Nishitani and Nurse, 1995; Piatti *et al*., 1995; Cocker *et al*., 1996, Muzi-Falconi *et al*., 1996; Detweiler and Li, 1997, 1998), but its function in mammalian cells is less well characterized (Williams *et al*., 1997; Yan *et al*., 1998). In yeast and *Xenopus*, the sequential binding of the ORC followed by the Cdc6 protein results in recruitment of MCM proteins and formation of pre-RCs which define a replication-competent state (Coleman *et al*., 1996; Romanowski *et al*., 1996a; Rowles *et al*., 1996; Donovan *et al*., 1997; Tanaka *et al*., 1997). The immunoblot data of extracts prepared from G_0 and G_1 phase 3T3 cells demonstrate that the replication-incompetent quiescent state in mammalian cells is characterized by the presence of chromatin-bound ORC but absence of Cdc6 (Figure 3B). Although MCM proteins are present, they are not associated with chromatin (Figure 3B). Consistent with this result from murine 3T3 cells, we have also found down-regulation of Cdc6 protein in quiescent human newborn fibroblasts and in the human bladder carcinoma cell line EJ30 arrested by serum starvation (data not shown). Furthermore, the absence of Cdc6 has been shown in quiescent human Wi38 and T24 cells (Williams *et al*., 1997; Yan *et al*., 1998). Thus absence of Cdc6 and displacement of MCM proteins from chromatin appear to be characteristic of quiescence in mammalian cells.

At 12 h after release from quiescence, Cdc6 becomes detectable in 3T3 cell extracts by immunoblotting. It is entirely chromatin-bound, and protein levels increase between 12 and 18 h after the release (Figure 3B). Although MCM proteins are present in G_0, their binding to chromatin follows the kinetics of Cdc6 accumulation as predicted for the formation of pre-RCs (Figure 3B). Interestingly, nuclei become competent to initiate DNA replication in the cell-free system at the time of maximum Cdc6 accumulation and MCM binding during G_1 phase progression *in vivo* (Figure 3A and B). These data suggest that Cdc6 may play a key regulatory role in determining the quiescent state and the competence of nuclei to initiate replication during progression through G_1 phase. The absence of Cdc6 in quiescence, in contrast to its presence

in proliferating cells, suggests that Cdc6 may be used as a novel and powerful proliferation marker. Antibodies against human Cdc6 and Mcm5 have already been exploited to identify pre-malignant cells and thus to improve the sensitivity of the standard Papanicolaou (Pap) cervical smear test (Williams *et al*., 1998).

If the synthesis of Cdc6 following release from quiescence is rate-limiting for nuclei to enter a replication-competent state, then addition of exogenous Cdc6 should make early G_1 phase 3T3 nuclei competent to replicate prematurely *in vitro*. To test this hypothesis, exogenous recombinant XCdc6 was added to the cell-free system. XCdc6 (final concentration 0.65 μM) was found to cause premature entry of G_1 nuclei into S phase (Figure 4A). These data demonstrate that Cdc6 does indeed play a critical regulatory role in determining progression through G_1 phase prior to the onset of S phase. Interestingly, a window of response between 15 and 18 h after release from G_0 was identified in which G_1 progression was accelerated, causing premature entry into S phase. Nuclei prepared earlier than 15 h after release from quiescence failed to respond to exogenous Cdc6 (Figure 4A). It will be interesting to determine the additional differences that prevent nuclei from initiating in response to exogenous Cdc6 when they are prepared within 15 h of quiescence. Figure 4A shows that addition of exogenous Cdc6 protein increases the proportion of nuclei replicating when nuclei are prepared between 16 and 18 h after release from quiescence. However, Cdc6 also causes nuclei taken before 16 h to initiate prematurely. Thus addition of exogenous Cdc6 protein can increase further the signal-to-background noise ratio in the system, e.g. from <1 to 82% in Figure 4A for nuclei taken 15 h after quiescence. It is important to note that this dependence on Cdc6 and its ability to induce replication in nuclei that are even further from the G_1/S border reinforces our conclusion that the cell-free system is inducing initiation of DNA replication and not just elongation of existing S phase contaminants.

In contrast to other proteins which are thought to be components of the pre-RC, the abundance of Cdc6 is tightly controlled throughout the cell cycle (Kelly *et al*., 1993; Zwerschke *et al*., 1994; Piatti *et al*., 1995; Coleman *et al*., 1996) and the protein is targeted for rapid degradation at the G_1/S boundary at least in yeast (Nishitani and Nurse, 1995; Muzi-Falconi *et al*., 1996; Piatti *et al*., 1996). In *Schizosaccharomyces pombe*, overexpression of Cdc18 has been shown to result in multiple rounds of DNA replication without intervening mitosis (Nishitani and Nurse, 1995; Muzi-Falconi *et al*., 1996). The increased DNA synthesis observed in the mammalian cell-free system following addition of exogenous Cdc6 (Figure 4B) was predominantly hemisubstituted with BrdU, indicating a single round of semiconservative DNA replication. In contrast to *S.pombe*, there was no evidence for re-replication in the presence of increased levels of Cdc6. Thus additional control mechanisms for the prevention of re-replication must be active in mammalian cells as has been observed for *Saccharomyces cerevisiae* in which neither stable derivatives of Cdc6 nor overexpression of the protein induce re-replication (Bueno and Russell, 1992; Piatti *et al*., 1996; Drury *et al*., 1997). Such additional levels of control may include the ability of high levels of S phase-promoting and mitotic cyclin–CDK complexes to inhibit re-assembly of pre-RCs (Hayles *et al*., 1994; Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Dahmann *et al*., 1995; Labib *et al*., 1995; Jallepalli and Kelly, 1996; Piatti *et al*., 1996; Hua *et al*., 1997; Liang and Stillman, 1997; Coverley *et al*., 1998; Walter *et al*., 1998).

In vertebrate cells, the nuclear membrane plays a crucial role in restricting DNA replication to once per cell cycle (reviewed by Laskey *et al*., 1996). When nuclei that have replicated once in *Xenopus* egg extract are isolated, permeabilized and added to fresh extract, these nuclei re-replicate (Blow and Laskey, 1988). In contrast, intact nuclei fail to re-replicate. Similarly, permeabilized somatic G_2 HeLa nuclei, but not intact G_2 nuclei, re-replicate in *Xenopus* egg extract (Leno *et al*., 1992). These and earlier observations (Laskey *et al*., 1981) led to the proposal that a chromatin-binding factor is essential for replication and that the compartmental distribution of the factor between nucleus and cytosol serves to restrict DNA replication to a single round per cell cycle (Blow and Laskey, 1988; Blow, 1993; Kubota and Takisawa, 1993). Further studies in *Xenopus* extracts demonstrated that the nuclear envelope prevents re-initiation of replication by regulating the binding of MCM proteins to chromatin (Chong *et al*., 1995; Kubota *et al*., 1995; Madine *et al*., 1995a,b). Evidence exists that the nuclear envelope may impede both access of positive factors (Coverley *et al*., 1993; Chong *et al*., 1995; Kubota *et al*., 1995; Madine *et al*., 1995b) and exit of negative factors (Hua *et al*., 1997; Walter *et al*., 1998). It will be interesting to see how these phenomena relate to the behaviour of Cdc6 described here.

It has been shown that recombinant XCdc6 cannot rescue the replication defect of intact nuclei that have been assembled previously in XCdc6-depleted egg extracts (Coleman *et al*., 1996). Consistent with this data, we show that nuclear membrane permeabilization is a prerequisite for exogenously added recombinant XCdc6 to promote initiation of DNA replication in the mammalian system we describe here (Figures 5 and 6). Further studies of the mammalian system should help to resolve the precise roles of the nuclear envelope in regulating DNA replication and in restricting it to once per cell cycle.

In summary, we have established a mammalian cell-free system that initiates DNA replication efficiently *in vitro* under somatic cell cycle control. With this system, we have demonstrated that the Cdc6 protein plays a key regulatory role in determining quiescence and G_1 progression in mammalian cells.

Materials and methods

Cell culture and synchronization

NIH-3T3 and HeLa S3 tissue culture cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 10 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma). To prepare nuclei and cytosolic extracts from G_0 phase cells, 3T3 cells were driven into quiescence by contact inhibition leaving them to accumulate in G_0 for 3 days. G_1 phase nuclei and cytosolic extracts were obtained by releasing confluent quiescent cells through subculturing and replating 1 in 4. Progress through G_1 into S phase was monitored by BrdU incorporation and fluorescence-activated cell sorting (FACS) analysis (Krude *et al.*, 1997). The majority of 3T3 cells (65–70%) synchronized and released in this way begin to enter S phase ~21 h after replating. To prepare S phase HeLa cytosolic extracts, HeLa cells were synchronized in S phase by a single block in culture medium containing 2.5 mM thymidine (Sigma) for 25 h (Rao

and Johnson, 1970), followed by release into culture medium for 2 h. Cell synchronization was determined by FACS analysis of isolated nuclei (Krude *et al.*, 1997).

Preparation of nuclei and cytosolic extracts

Cytosols and nuclear preparations were produced by hypotonically swelling, scrape-harvesting and Douncing essentially as described (Heintz and Stillman, 1988; Krude *et al.*, 1997) with the following minor modifications. Cytosolic supernatant was taken from the first spin (4000 r.p.m. in an Eppendorf 5415C centrifuge for 3 min) and respun at 14 000 r.p.m. for 20 min. Supernatant fractions were then aliquoted, snap-frozen and stored in liquid nitrogen. Nuclear pellets from the first separating spin were resuspended in 1 ml of ice-cold SuNaSp/BSA (250 mM sucrose, 75 mM NaCl, 0.5 mM spermine trihydrochloride, 0.15 mM spermidine tetrahydrochloride, 3% BSA) and respun (4000 r.p.m. in an Eppendorf 5415C centrifuge for 2 min). The supernatant was discarded and the packed nuclear pellet was resuspended in an equal volume of SuNaSp/BSA, frozen, and stored at –80°C.

Permeabilization of 3T3 nuclei with lysophosphatidylcholine (lysolecithin)

For permeabilization, frozen 3T3 nuclei were resuspended in 500 μl of SuNaSp, supplemented with 10 μg/ml leupeptin, pepstatin A and aprotinin (Sigma), and spun down (2000 r.p.m. in an Eppendorf 5415C centrifuge for 2 min at 4°C). Nuclei were washed three times and resuspended in 500 μl of SuNaSp. For permeabilization, 50 μl aliquots of lysolecithin (2 mg/ml; Sigma) were added sequentially, incubations were mixed, and the permeability of the nuclear membrane was assessed by monitoring the extent of inclusion of fluorescein-linked dextran molecules (Molecular Probes) in nuclei stained with Hoechst 33258 (Sigma), (Coverley *et al.*, 1993). Note that fluorescein-linked dextran (70 kDa) lacks a nuclear localization signal and is large enough to be retarded by an intact nuclear envelope. Determination of the extent of nuclear membrane permeability was made immediately by fluorescence confocal microscopy. The permeabilization reaction was stopped by addition of 500 μl of 3% BSA/SuNaSp. Nuclei were washed three times and resuspended in an equal volume of 3% BSA/SuNaSp.

Expression and purification of human cyclin–Cdk complexes

Recombinant cyclin A–Cdk2 and cyclin E–Cdk2 complexes were expressed and purified as previously described (Krude *et al.*, 1997). Purification resulted in an enrichment of cyclin–Cdk activity of ~50 000 for each cyclin–Cdk from infected insect cell lysates, with specific histone H1 kinase activities of 6.2 nmol/min/mg for cyclin A–Cdk2 (25 μg/ml) and 12.5 nmol/min/mg for cyclin E–Cdk2 (25 μg/ml).

Expression and purification of His6-XCdc6

Sf9 cells were grown in 400 ml of Grace medium (Gibco–BRL) containing 5% FCS and 5 μg/ml gentamycin (Gibco-BRL) in spinners at 27°C up to a density of 1.5×10^6/ml. Cells were pelleted, resuspended in 50 ml of medium containing recombinant baculovirus and incubated for 2 h with gentle mixing. The culture was diluted to 400 ml with fresh medium and incubated for a further 48 h. Insect cell culture (400 ml) was harvested 48 h after infection. Cell were washed gently with ice-cold phosphate-buffered saline (PBS) and resuspended in 10 ml of hypotonic buffer [10 mM HEPES pH 7.4, 10 mM NaCl, 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml of each: leupeptin, aprotinin, pepstatin and chymostatin]. Cells were allowed to swell for 10 min and lysed by passing through a tight-fitting Dounce homogenizer (Wheaton). Immediately after lysis, the NaCl concentration was restored to 150 mM. The lysate was spun at 5000 *g* for 10 min. The supernatant was transferred to a clean tube and spun again at 100 000 *g* for 1 h in a Beckman Ti55 rotor. The supernatant was transferred to a clean tube and bound to 1 ml of Ni^{2+}-NTA–agarose (Qiagen) in a batch mode at 4°C for 2 h. A wash (2 vols) and three elution steps (5 ml each) were performed in a Sigma 3794 liquid chromatography column at 4°C with 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF and 10 μg/ml of each: leupeptin, aprotinin, pepstatin and chymostatin, containing 10, 20, 50 and 100 mM imidazole, respectively. Protein-containing fractions were pooled and dialysed against 20 mM HEPES pH 7.5; 150 mM NaCl, 5% glycerol, 1 mM dithiothreitol (DTT) at 4°C overnight. The biochemical activity of nickel-affinity-purified His6-XCdc6 was tested by rescuing replication of exogenously added sperm chromatin in Cdc6-depleted *Xenopus* egg extract as described by Coleman *et al*. (1996).

Assaying for DNA replication

DNA replication reactions containing 30 μl of cytosolic HeLa S phase extract (250–300 μg of protein), a buffered mix of NTPs, dNTPs, an energy regeneration system and 1×10^5 3T3 nuclei were performed as previously described (Krude *et al.*, 1997). For detection of DNA replication by immunofluorescence, the nucleotide mix was supplemented with a final concentration of 0.25 μM biotin-16-dUTP (Boehringer Mannheim). For quantitation of nuclear DNA synthesis, reactions also contained 1–2 μCi of [α-^{32}P]dATP with substitution of biotin-16-dUTP by 0.1 mM dTTP, or for density substitutions, by 0.1 mM BrdUTP (Sigma). His6-XCdc6 in 20 mM HEPES pH 7.5; 150 mM NaCl, 5% glycerol, 1 mM DTT was added to *in vitro* replication reactions at a final concentration of 0.65 μM where indicated. An equal volume of XCdc6 buffer was added to control replication reactions.

Quantitation of DNA synthesis

In vitro DNA replication reactions were incubated at 37°C in the presence of [α-^{32}P]dATP for 2 h. Two volumes of stop mix (1% SDS, 5 mM DTT, 1 mM EDTA, 50 mM Tris pH 6.8) were added after the incubations and aliquots were pipetted onto GFC filters in quadruplicate. Two filters were dried and used to measure total counts, whilst the other two were trichloroacetic acid (TCA)-precipitated (10% TCA containing 2% NaPPi). By using the ratio of the incorporated radiolabel to the total radiolabel available in the incubation and relating this to the concentration of the dATP pool, the mass of DNA synthesized was calculated using the formula [(TCA c.p.m./total c.p.m.)×0.101 = ng/μl DNA synthesized].

Density gradients

Caesium chloride density gradient centrifugation of the purified DNA product of the replication reactions was performed as described previously (Blow and Laskey, 1986; Leno *et al.*, 1992).

Polyclonal antisera production

Antibodies to human Orc2 and Mcm5 proteins were raised and affinity purified as previously described (Romanowski *et al*., 1996a,b). Antibodies were also raised against the human Cdc6 protein. Several human expressed sequence tags (ESTs) encoding a putative human homologue of Cdc6 were identified on the basis of their homology to yeast Cdc6/Cdc18 and human Orc1. Corresponding cDNA clones (110966, 204214 and 294716; Image Consortium, Research Genetics Inc., USA) were sequenced by primer walking. Fragments corresponding to amino acids 145–360 and 364–547 were cloned into pET23a expression vector (Novagen) and expressed in *Escherichia coli* CL41 strain (Miroux and Walker, 1996). The expressed protein fragments were purified by nickel affinity chromatography and used to immunize rabbits. The immunization protocol and affinity purification of antibodies were performed as described previously (Romanowski *et al*., 1996a,b).

Immunoblotting

For immunoblot analysis, 3T3 cells were synchronized following the protocol described above. Cells were taken at 0 h (G_0) and at 3, 12, 18 (G_1) and 21 h (S) following release from quiescence and Dounced in 10% Triton. Total extracts were fractionated into soluble and chromatin-bound fractions (Heintz and Stillman, 1988), separated on SDS–12% polyacrylamide gels and immunoblotted with antibodies against hOrc2, hCdc6 and hMcm5. Note that the soluble fraction contains cytoplasmic and soluble nuclear proteins. Blocking, antibody incubation, and washing steps were performed in Tris-buffered saline (TBS)/1% Tween-20/10% milk. Immunoreactive bands were visualized on a pre-flashed Kodak XLS1 Scientific Imaging film by enhanced chemiluminescence (ECL, Amersham).

Fluorescence confocal microscopy

Nuclei from *in vitro* DNA replication assays were resuspended in PBS (0.5 ml) and fixed for 5 min by adding an equal volume of 8% paraformaldehyde. After fixation, nuclei were spun through 30% sucrose/PBS onto polylysine-coated coverslips. All subsequent washing and staining steps were carried out in PBS/0.2% Triton X-100/0.04% SDS. Coverslips were washed, stained for incorporated biotin-16-dUTP with fluorescein-linked streptavidin (1:100 dilution, Amersham) and for DNA with propidium iodide/RNase A (both at 50 ng/ml, Sigma), washed again and mounted in 1 mg/ml phenylenediamine (Sigma) in 90% glycerol/10% PBS. Confocal fluorescence microscopy of random fields of nuclei was performed on a Bio-Rad 1024 confocal microscope. Images were collected, and merged pictures of the propidium iodide channel (red) and fluorescein channel (green) were obtained using

Adobe Photoshop with standardized brightness- and contrast-enhanced operations for all samples. Images were printed, and the number of nuclei incorporating biotin-16-dUTP *in vitro* (yellow) and non-replicating nuclei (red) were counted. Routinely, 800–1000 nuclei were scored for each replication reaction and quantitated as percentages of the total number of nuclei which synthesized DNA *in vitro*.

Immunolocalization of chromatin-bound His6-XCdc6 was performed using monoclonal anti-His6 antibody (Qiagen) to detect specifically exogenous XCdc6 in G_1 phase 3T3 nuclei following 3 h incubations in the *in vitro* replication reaction mix. Replication reactions were stopped by addition of equal volumes (500 µl) of 0.5% Triton/PBS and 8% paraformaldehyde (500 µl). Nuclei were spun through 30% sucrose/PBS onto coverslips and washed three times with PBS. Coverslips were incubated with monoclonal anti-His6 antibody (1:500 dilution) overnight. Following incubation overnight, coverslips were washed three times with PBS and incubated with Cy2-anti-mouse antibody (1:1000 dilution; Amersham). Unbound secondary antibody was washed away twice with PBS, and DNA was stained with TOTO-3 (1:10000 dilution; Molecular Probes) for 30 min. Coverslips were washed twice with PBS and mounted in 1 mg/ml phenylenediamine (Sigma) in 90% glycerol/10% PBS. Nuclear localization of XCdc6 was analysed by confocal fluorescence microscopy (Bio-Rad 1024 confocal microscope), and the intensity of fluorescent labelling was measured using the LASERSHARP processing programme (Bio-Rad).

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