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Both cdc2 and cdk2 promote S phase initiation in *Xenopus* egg extracts

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SUMMARY

***Xenopus* egg extracts induce S phase DNA replication in added sperm pronuclei in a highly regulated manner, similar to events in vivo. Removal of cyclin-dependant kinases (cdks) or cdk2 from these extracts using affinity matrices severely inhibits initiation of S phase. We have used p13^{suc1} beads to remove both cdk2 and cdc2 proteins from egg extracts and developed a method to replace either protein alone to assess their capacity to initiate DNA replication. Re-addition of either cdk2 or cdc2 proteins to depleted extracts, through translation of their respective mRNAs, restimulated replication, judged by both total synthesis and labelling index. An ATP-binding-site mutant cdk2 mRNA (cdk2.R33) failed to stimulate replication and inhibited S phase initiation in mock-depleted extracts. Both human and *Xenopus* cdc2 mRNAs rescued replication in this system. Human mutant mRNAs have been used to show that the stimulation induced requires cdc2 catalytic activity, though not its mitotically active form. Rescue of replication by p34^{cdc2} is also observed in extracts depleted of cdks with a cdk2 antibody, which still retain much of their endogenous cdc2 protein. We conclude that newly synthesised p34^{cdc2}, but not the inherited 'old' form, can induce S phase and in this form may overlap in function with p33^{cdk2}.**

Key words: cdc2, cdk2, DNA replication, *Xenopus* extract

INTRODUCTION

The period of DNA duplication is tightly regulated during the cell cycle of eukaryotes. Once S phase is initiated, DNA replication continues until the DNA is precisely doubled, before mitosis can occur. Indeed, if replication is disrupted, mitosis is delayed (Dasso, 1993; Sheldrick and Carr, 1993). The behaviour of *Xenopus* egg extracts appears to reflect these controls in that each nucleus initiates and terminates replication once in any interphase, and must undergo mitotic events before re-initiation can take place (Blow and Laskey, 1986; Blow and Watson, 1987; Hutchison et al., 1987, 1988). In addition, at appropriate nuclear concentrations mitosis is delayed when replication is blocked (Dasso, 1993). Initiation of S phase only occurs after the template has been assembled into nuclei and does not occur in intact G_2 nuclei (Blow and Watson, 1987; Hutchison et al., 1988; Newport, 1987; Coverley et al., 1993). Two unusual features of cell cycles in early *Xenopus* embryos are also observed in these extracts. Neither transcription nor translation is necessary for S phase and thus an analogue of the restriction point of somatic cells is not present (reviewed by Norbury and Nurse, 1992). Secondly, S phases are short, achieved through synchronous initiation at multiple replicons (Callan, 1972; Mills et al., 1989). Thus, this model system provides a special opportunity for biochemical dissection of the regulation of S phase.

The cell division cycle kinase, p34^{cdc2}, is required twice in the yeast cell cycle, at START before S-phase and in G_2 for mitosis (Nurse and Bisset, 1980; Piggott et al., 1982; Nurse, 1990). While cdc2 kinase is universally required for entry into mitosis, the requirements for initiation of S phase are not well understood. The situation is potentially complicated in multicellular eukaryotes by the identification of a family of cdc2-related cyclin-dependent kinases (cdks; Paris et al., 1991; Meyerson et al., 1992). Expression studies in several human cell lines, using wild-type and dominant negative mutant cdks, have implicated cdk2 and cdk3 (but not cdk4, 5 or 6) in the progression from G_1 to S phase while expression of mutant cdc2 caused cells to accumulate only in G_2 (van den Heuvel and Harlow, 1993). cdc2 mutant mouse cells grown at the restrictive temperature were also arrested in the cell cycle only in G_2 (Hamaguchi et al., 1992). However, a dominant negative allele of cdc2 in *Schizosaccharomyces pombe* also only arrested the cell cycle in G_2, indicating that the G_1 requirement for p34^{cdc2} was not exposed (Fleig et al., 1992). In addition, timing correlations and inhibition studies have suggested that both cdc2 and cdk2 are involved in the G_1/S phase transition in different mammalian cells (Pagano et al., 1993; Tsai et al., 1993; Marraccino et al., 1992).

The strategy of selective inhibition or removal of components from egg extracts by antibodies or affinity matrices has identified several proteins required for DNA replication,

including lamin B3 (Meier et al., 1991; Newport et al., 1990; Jenkins et al., 1993), annexin II (Vishwanatha and Kumble, 1993) and replication factor-A (RPA) (Fang and Newport, 1993; Adachi and Laemmli, 1994). RPA binds to decondensing chromatin in punctate pre-replication centres, but nuclear membrane formation is required for subsequent DNA unwinding and replication (Adachi and Laemmli, 1992, 1994). Preferential removal of cdk2 and cdc2 using $p13^{suc1}$-beads does not prevent the localisation of RPA but inhibits initiation of S phase (Blow and Nurse, 1990; Fang and Newport, 1991, 1993; Adachi and Laemmli, 1994). In this situation, replication could be restimulated by addition of the protein fraction recovered from suc1-beads enriched in cdc2 and cdk2 (Blow and Nurse, 1990; Fang and Newport, 1991). Both Blow and Nurse (1990) and Fang and Newport (1991) also used extracts immunodepleted with cdc2 antibodies, but with apparently different effects on the initiation of DNA replication. To investigate the roles of cdks in the induction of S phase we have used suc1-bead depletion to inhibit DNA replication in egg extracts and have replaced either cdk2 or cdc2 proteins individually through translation of synthetic mRNA. Each protein stimulates S phase initiation. Both *Xenopus* and human cdc2 mRNA stimulated initiation while mutant forms failed to restore this activity. In the case of mutant cdk2, replication was inhibited in a dominant negative manner. The results demonstrate that newly synthesised cdc2 can regulate S phase initiation and may overlap in function with $p33^{cdk2}$.

MATERIALS AND METHODS

Oocyte preparation, oligonucleotide injection and in vitro maturation

Manually isolated oocytes were incubated in Barth X (Gurdon and Wickens, 1983) at 19°C after addition of 5 μg/ml progesterone (Sigma), and groups of 15 cells were taken at the indicated times for electrophoresis. Percentage GVBD was estimated hourly by external inspection. Samples for electrophoresis were homogenised in NP-40 lysis buffer (Harlow and Lane, 1988) with 8.3 μg/ml aprotinin, mixed with SDS sample buffer after centrifugation, and analysed by western blotting using a rabbit polyclonal antibody to cdk2 diluted 1/500 (serum P5, a gift from Dr T. Hunt). For Fig. 1B oocytes were injected with 25 nl of water, or antisense oligodeoxynucleotide to cdk2 (nucleotides 5 mg/ml) and incubated for 4 hours at 21°C before reinjecting some with cdk2 mRNA (1 mg/ml). Progesterone was then added and incubation was continued overnight at 19°C. Samples were prepared from eggs and uninjected or injected oocytes, and analysed by western blotting using 0.1 μg/ml anti-PSTAIRE monoclonal antibody (a gift from Dr M. Yamashita; Yamashita et al., 1991). First antibodies were detected using peroxidase-coupled second antibodies.

Egg extract preparation and incubation

Egg extracts were prepared as previously described (Hutchison et al., 1988) and frozen with 3% glycerol in liquid nitrogen. Extracts which synthesised at least 30% of the input DNA and showed strong translation were chosen. Thawed samples were supplemented with 3 mM phosphocreatine and 7.5 μg/ml creatine phosphokinase before mixing with sucl- or BSA-beads (50 μl beads/100 μl extract) for 1 hour at 4°C. Supernatants were removed after brief centrifugation and made 5% in rabbit reticulocyte S100 (Mathews and Colman, 1991). Aliquots (40 μl) of supernatant were incubated at 21°C for 30 minutes with 2 μl of mRNA or water and [^{35}S]methionine before taking 20 μl samples, adding 1 μl containing 2×10^4 demembranated sperm together with either 1 μCi of [^{32}P]dCTP or biotin-11-dUTP (Hutchison et al., 1988), and incubating for a further 150 minutes.

Depletion procedure using suc1 protein or cdk2 antibody

suc1 protein was produced using pRK172 (a gift from J. Hayles and P. Nurse) and purified by heat denaturation and chromatography on Superose 12 and Mono Q. Equal volumes of $p13^{suc1}$ or BSA (each at 10 mg/ml) and CNBr-activated Sepharose beads (Pharmacia) were coupled using the manufacturer's instructions. Beads were washed twice in bead wash buffer (BWB, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 4.5 mM NaF, 100 μM benzamidine, 0.1% NP-40 and 10 μg/ml of leupeptin, aprotinin and trypsin inhibitor) and three times in SuNaSp (Hutchison et al., 1988) before using with extracts. After depletion, pellets were similarly washed in BWB before adding SDS sample buffer. Protein A-Sepharose 6MB (Sigma) was mixed with serum P5 for 2 hours at 4°C, pelleted, washed and stored in BWB containing 0.2% sodium azide. Prewashed cdk2 antibody Protein A-Sepharose beads and extract were mixed at a ratio of 50 μl beads to 80 μl extract, for 1 hour at 21°C. Supernatants were taken after brief centrifugation (2× 4 seconds) in a microfuge and supplemented with 1.5 mM phosphocreatine, 3.75 μg/ml creatine phosphokinase (final concentrations) before adding S100 and mRNAs as above.

In vitro mRNA preparation

Capped transcripts were prepared (Mathews and Colman, 1991) and dissolved in DEPC-treated water (0.25-2.0 mg/ml depending on the translation level observed). *Xenopus* cdc2 cDNA was a gift from J. Newport (Milarski et al., 1991), and was transferred into pSP64T (Melton et al., 1984) by J. Shuttleworth. The Hscdc2 clones (wt, D161, A14F15, in pSP64T) were gifts from C. Norbury (Norbury et al., 1991). Xlcdk2.wt and Xlcdk2.R33 both in pEPEX, were gifts from J. Gautier and J. Paris (Paris et al., 1994), respectively. pGEMXl-cycB1 was a gift from T. Hunt.

Quantification of DNA synthesis

At the end of the incubations ^{32}P-labelled samples were analysed on 0.9% agarose gels as described (Hutchison et al., 1988). The equivalent of 7.5 μl of original cytoplasm was loaded in each track. For quantification each track from the dried gel was cut out and counted in liquid scintillant.

Protein analysis

Samples of supernatant and washed pellet were taken immediately following depletion into SDS sample buffer for comparison with undepleted extracts. Following incubation, [^{35}S]methionine-labelled samples were also taken. All samples (1.25 μl equivalent cytoplasm per track) were analysed on 12% polyacrylamide gels and autoradiographed following electrotransfer. cdks were revealed as described for oocyte samples.

Cytological analysis

Samples containing biotin-11-dUTP were fixed and processed onto coverslips (Hutchison et al., 1988) and at least 100 nuclei were scored blind for each coverslip. Where nuclei had entered mitosis, condensed DAPI-stained foci were scored as nuclei. Nuclei were photographed at ×400 on a Zeiss Axiophot microscope using constant exposure times for each filter setting.

RESULTS

cdk2 accumulates late in maturation

Paris et al. (1991) suggested that cdk2 might be involved in entry into S phase, on the basis of its sequence similarity to cdc2, its failure to complement cdc2 mutants, and the

polyadenylation changes of its mRNA, which implied that the protein would be synthesised during oocyte maturation. In *Xenopus*, the capacity to initiate DNA replication on nuclear templates in vivo appears during oocyte maturation at the time of germinal vesicle breakdown (GVBD; Gurdon, 1967; Furuno et al., 1994). Therefore, we investigated when the cdk2 protein accumulated during oocyte maturation by blotting with polyclonal cdk2 antibody (Fig. 1A) and cdk2 C-terminal peptide antibody (data not shown). cdk2 protein is just detectable in oocytes and increases substantially in amount during maturation, particularly after seven hours, when the first oocytes have undergone GVBD (Fig. 1A).

The increase in cdk2 during maturation was also detected in immunoblots where cdks were revealed using a PSTAIRE peptide monoclonal antibody (Yamashita et al., 1991). In Fig. 1B, only the upper bands observed with this antibody, designated cdc2, reacted with a C-terminal cdc2 antibody (data not shown). Scanning densitometry indicated that, while total cdc2 changed little, the lower band increased some 3- to 4-fold between oocyte and egg (Fig. 1B, Ooc, Egg). This increase was prevented by injection of cdk2 antisense oligonucleotide and could be replaced by injection of cdk2 mRNA four hours after the antisense injection, prior to maturation (Fig. 1B, AS, AS+cdk2). These observations confirm that the lower band detected with PSTAIRE peptide antibody is cdk2. We noted in these experiments that eggs produced after cdk2 antisense injection were activated on pricking and subsequently showed abortive cleavage furrows. However, we did not determine the nuclear status of these activatable eggs. The major accumulation of cdk2 during maturation is consistent with our observation that cdk2 mRNA is polyadenylated only during this period (Paris et al., 1991). Moreover, its translation is not essential for production of activatable eggs. We conclude that most cdc2 protein is inherited from the oocyte while the majority of the cdk2 present in an egg is accumulated late during maturation.

cdk2 or cdc2 can be efficiently removed and specifically replaced in egg extracts

Xenopus egg extracts normally initiate S phase replication efficiently on added sperm pronuclei, but this can be inhibited by prior depletion with $p13^{suc1}$ beads (Blow and Nurse, 1990; Fang and Newport, 1991). We have used $p13^{suc1}$ beads to remove both cdk2 and cdc2 proteins from egg extracts and developed a method to replace either protein alone, to assess their capacity to initiate DNA replication. In comparison with an untreated extract (Fig. 2A, Untreated) the supernatant remaining after removal of the suc1 beads retained 20% of the starting cdc2 and less than 10% of the cdk2, judged by densitometry (Fig. 2A, Suc1 snt). All of these proteins remained in the supernatant after treatment with BSA beads (Fig. 2A, BSA snt, BSA pellet). After the bead treatment, supernatants were supplemented with 5% reticulocyte S100 to stimulate total translation. Different mRNAs or water were then added to supernatant aliquots and incubated for 30 minutes before adding demembranated sperm heads and incubating for a further 150 minutes. At the end of the incubation, immunoblots were autoradiographed to reveal total protein synthesis (Fig. 2A, lower panel) and cdks were detected on the same blot with PSTAIRE peptide antibody (Fig. 2A, middle panel). Nuclear labelling indices in the same samples were estimated cytologically through biotin-11-dUTP incorporation, while DNA replication was quantified in parallel incubations containing [^{32}P]dCTP (Fig. 2B).

Addition of cdk2 mRNA (wt or R33) generated clear bands, detected immunologically, which are not detected following endogenous translation alone (Fig. 2A, + water, middle panel). Translation of added *Xenopus* cdc2 mRNA (with or without cyclin B) substantially increases the intensity of the bands detected by blotting, compared to the residual cdc2 present in the water control. This cdc2 translation generates a strong radioactive band that is detected autoradiographically. No radioactive band comigrating with *Xenopus* cdc2 is observed in the absence of added mRNA (Fig. 2A, lower panel). Autoradiography of the endogenous translation products revealed a

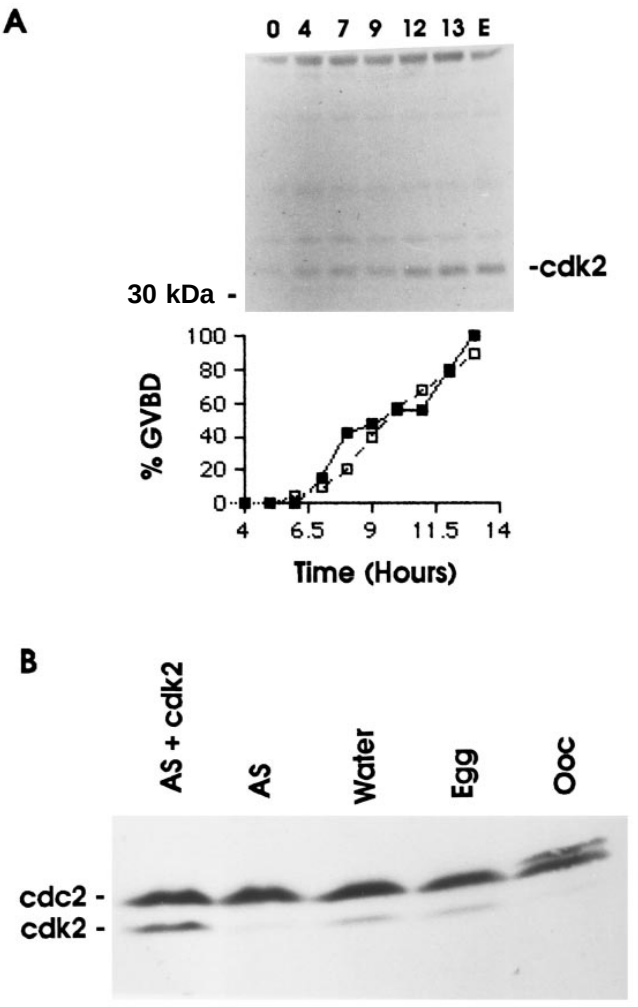
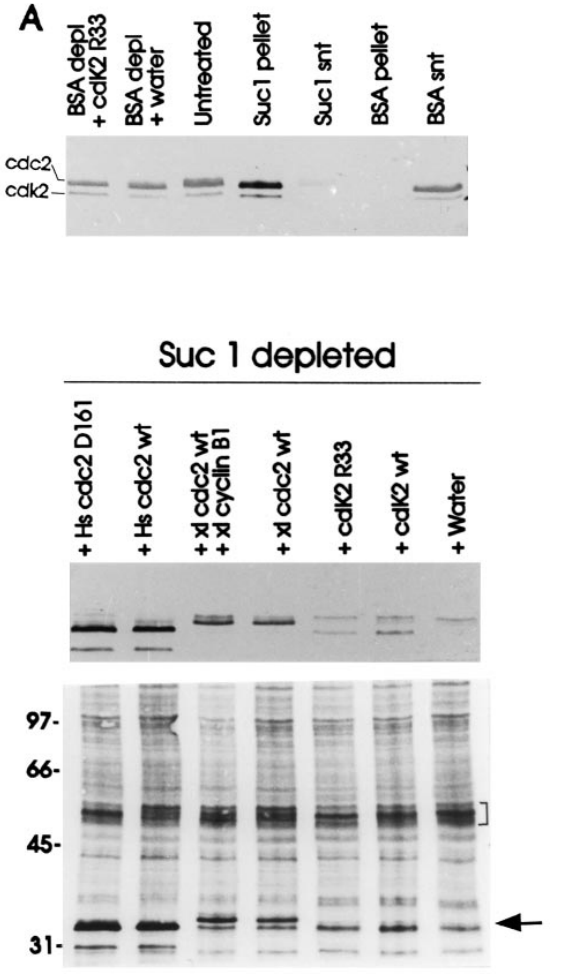


Fig. 1. cdk2 protein increases substantially late in oocyte maturation. (A) Samples were taken at the times indicated in hours after progesterone addition (E, unfertilised eggs shed by another female). Volumes equivalent to 1.25 oocytes were loaded in each lane of a 12% polyacrylamide gel. Proteins were electro-transferred onto a nitrocellulose filter, which was probed with polyclonal antibody to cdk2. Below is the time course of germinal vesicle breakdown (% GVBD) of the population of oocytes (□) from which small groups of oocytes were taken for electrophoresis (■). (B) PSTAIRE antibody detection of cdc2 and cdk2 proteins in oocytes (Ooc), eggs obtained following progesterone maturation (Egg), and eggs matured after injection with water, antisense oligonucleotide to cdk2 (AS) or antisense oligonucleotide followed by cdk2 mRNA (AS + cdk2).

band comigrating with cdk2 (Fig. 2A, arrow, lower panel) but it is not detected by western blotting with PSTAIRE antibody (Fig. 2A, +Water, middle panel). Together these results indicate that no detectable cdc2 or cdk2 proteins are synthesised without added mRNA (Fig. 2A, Suc1 depleted+water) although considerable translation of endogenous mRNAs



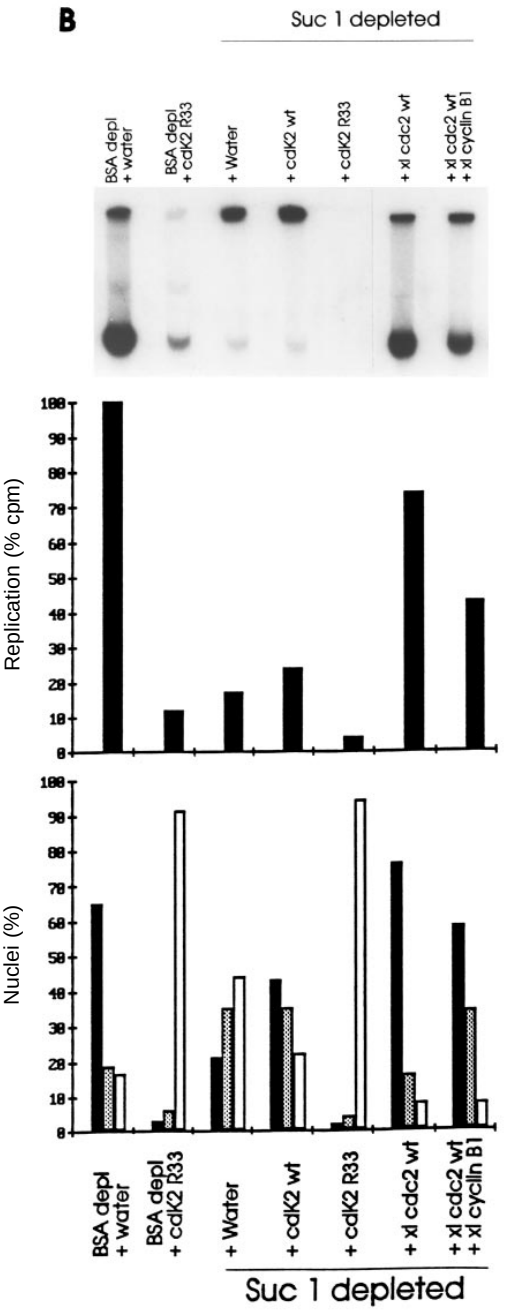


Fig. 2. Replication, inhibited by sucl depletion, is rescued by cdk 2 or cdc2 wild-type proteins and inhibited by cdk2.R33. Following depletion with suc1- or BSA-beads, extract supernatants were separated from bead pellets, supplemented with 5% reticulocyte S100, and aliquots were incubated with mRNA or water as indicated. After 30 minutes at 21°C, demembranated sperm heads were added (10^3/μl final concentration) and incubation was continued for a further 150 minutes. For each mRNA condition, parallel aliquots contained either [^{35}S]methionine and biotin-11-dUTP for blot analysis, autoradiography and labelling index measurement or [^{32}P]dCTP for quantification of DNA synthesis. (A) Detection of cdks with anti-PSTAIRE antibody following western blotting and protein synthesis by autoradiography. Upper panel (right): cdks in supernatant (snt) and pellet fractions taken immediately after depletion compared with control (Untreated) extract. Upper panel (left): cdks in BSA-bead depleted (BSA depl) supernatant after incubation with water or cdk2.R33 mRNA. Middle panel: cdks in sucl depleted supernatant after incubation with mRNA or water as indicated. Lower panel: protein synthesis was revealed by autoradiography of the blot used to detect cdks in the middle panel. As in Fig. 1B, the PSTAIRE antibody detects two *Xenopus* cdc2 bands above a single cdk2 band, though human cdc2 protein migrates at essentially the same position as *Xenopus* cdk2. The migration position of B cyclins is marked by a bracket. The arrow marks an endogenous radiolabelled band which comigrates with p33^{cdk2} but is not detected by PSTAIRE blotting. (B) Analysis of DNA replication. Upper panel: autoradiograph of agarose gels of [^{32}P]dCTP incorporation in parallel samples to those shown in A. DNA either remains at the pocket or migrates almost to the 23 kb marker. Middle panel: quantification of the gel autoradiographed in the upper panel. Each track from the dried gel was cut out and counted in liquid scintillant. The undepleted control synthesised 39% of the input sperm DNA during the incubation. In this experiment the BSA depleted control synthesised 25% of the input DNA. Lower panel: nuclear labelling indices of the samples shown in A. Biotin-11-dUTP incorporation was detected with Texas Red/streptavidin and for each sample nuclei were scored as strongly labelled (black bar), weakly labelled (stippled bar) or unlabelled (open bar).

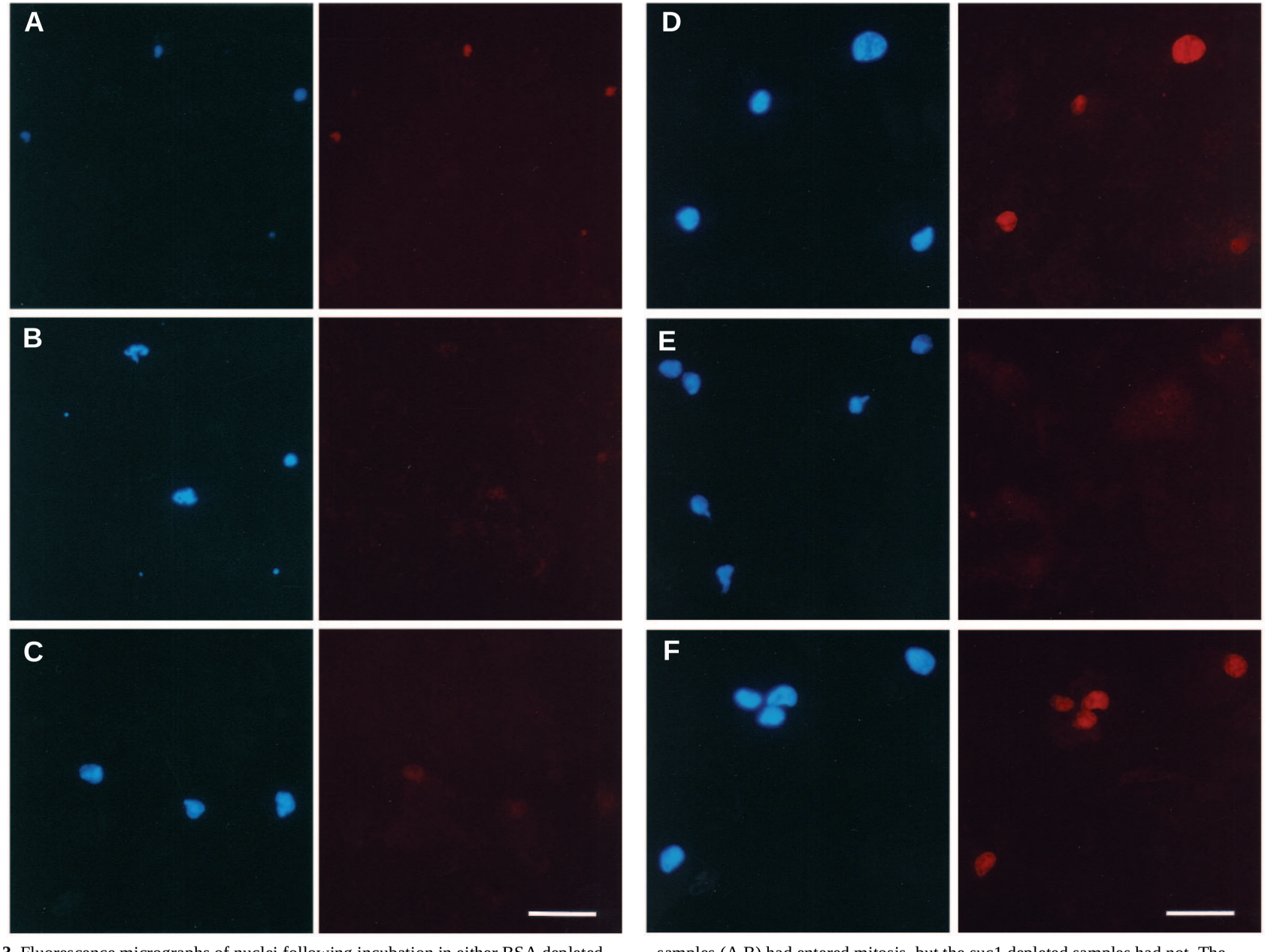


Fig. 3. Fluorescence micrographs of nuclei following incubation in either BSA depleted extract with: A, water (control); B, cdk2.R33 mRNA; or in sucl depleted extract with: C, water (control); D, cdk2.wt mRNA; E, cdk2.R33 mRNA, and F, Xlcdc2 mRNA and XlcycB1 mRNA. For each pair of photographs DAPI staining of DNA is shown to the left of the biotin-streptavidin image of the same field. In this experiment BSA depleted samples (A,B) had entered mitosis, but the suc1 depleted samples had not. The streptavidin image in C. (suc1 depleted control) shows one weakly labelled and two unlabelled nuclei. The image in D. shows two strongly labelled and two weakly labelled nuclei. In E all of the nuclei are unlabelled, while in F all in this field are strongly labelled. Bar, 10 μm.

occurred, including B cyclins (Fig. 2A, bracket, lower panel). The majority of cdc2 remaining in the extract shifts to a more slowly migrating form, presumably reflecting tyrosine phosphorylation triggered by association with newly synthesised cyclin B during the incubation. Densitometric comparison of the amount of cdk2 or cdc2 protein accumulated on addition of its mRNA with the untreated cytoplasm indicates that each is close to the level observed before depletion (Fig. 2A, top and middle panels). The addition of human cdc2 mRNAs generates major products that migrate essentially at the position of *Xenopus* cdk2, with a smaller component at 31 kDa, which is probably a degradation product (Fig. 2A; and Norbury et al., 1991). In all cases the range and level of endogenous proteins synthesised were the same (Fig. 2A, lower panel). Thus, under these conditions the majority of endogenous PSTAIRE-reactive proteins can be removed, and either cdk2 or cdc2 proteins replaced at physiological levels only through translation of their respective mRNAs.

cdk2 or cdc2 alone rescue replication in suc1 depleted egg extracts

DNA replication has been followed in parallel aliquots of depleted extracts using [^{32}P]dCTP incorporation to quantify DNA replication and biotin-11-dUTP to measure nuclear labelling indices (Fig. 2B). As anticipated from previous work (Blow and Nurse, 1990; Fang and Newport, 1991) depletion with p13^{suc1} beads severely inhibits replication compared to either mock-depleted or non-depleted samples (Fig. 2B, upper panel). Quantification of DNA synthesis indicates that p13^{suc1} depletion inhibits replication by about 84% compared to the BSA depleted control (Fig. 2B, middle panel). However, this varied from 50 to 90% in different experiments even though the level of depletion of PSTAIRE-reactive proteins appeared similar, as observed previously (Blow and Nurse, 1990; Fang and Newport, 1991). When wild-type cdk2 mRNA is preincubated in suc1 depleted extract, subsequent replication is stimulated to above the level found in the control preincubated with water. While, quantitatively, this stimulation was only 40%, it was correlated with a doubling in the percentage of strongly labelled nuclei (Fig. 2B, middle and lower panels). In scoring slides for labelling indices, biotin-11-dUTP incorporation detected through Texas Red/streptavidin fluorescence was categorised as strong, weak or absent. Weakly labelled nuclei presumably reflect those nuclei in which replication has only recently been initiated, since individual nuclei initiate replication autonomously and asynchronously in frozen egg extracts (Blow and Watson, 1987). In BSA depleted control extracts the majority of nuclei were strongly labelled (Fig. 2B, lower panel; Fig. 3.1). However, almost 50% of nuclei incubated in suc1 depleted extracts without added mRNA, remained unlabelled (Fig. 2B, Fig. 3C). The drop in labelling index reflects a reduction in the capacity to initiate S phase following suc1 depletion. Reintroduction of cdk2 protein, through translation of its mRNA, doubles the proportion of strongly labelled nuclei, indicating that the frequency of S phase initiation has increased (Fig. 2B, Fig. 3D).

Both cdk2 and cdc2 proteins are removed from extracts during suc1 bead treatment. We therefore added cdc2 protein to depleted extracts through translation of its mRNA. S phase replication is strongly stimulated by this treatment, judged both by a quantitative increase in [^{32}P]dCTP incorporation and an increased labelling index. Indeed, less than 10% of nuclei remain unlabelled under this condition (Fig. 2B, lower panel) and total incorporation was stimulated fivefold to 75% of the mock depleted level (Fig. 2B, middle panel). Taken together, these results indicate that either cdk2 or cdc2 proteins, added singly through mRNA translation, can increase S phase initiation in suc1 depleted extracts.

Mutant cdk2 does not rescue replication and acts in a dominant negative manner

In view of the observation that replication in *Xenopus* egg extracts required p33^{cdk2} but not endogenous p34^{cdc2} (Fang and Newport, 1991), we were surprised that, under the conditions used here, p34^{cdc2} stimulated replication and did so more effectively than p33^{cdk2}. However, in other experiments where the level of p33^{cdk2} translation was increased, replication was stimulated to levels close to those observed with p34^{cdc2} (Fig. 4). In addition, if sperm heads were added to suc1 depleted extracts at the same time as cdk2 mRNA, so that nuclear formation occurred before significant levels of p33^{cdk2} had been synthesised, no stimulation of replication was detected (data not shown). To assess whether the cdk2 protein synthesised on addition of its mRNA was responsible for the stimulation of replication observed, we replaced wild-type cdk2 with an inactive mutant cdk2 mRNA in which lysine 33, a conserved amino acid in the ATP-binding site, had been changed to arginine (cdk2.R33; Paris et al., 1994). Translation of this mutant mRNA failed to stimulate replication in suc1 depleted extracts and, indeed, reduced replication further (Fig. 2B, top, middle). In this situation, analysis of labelling indices revealed that very few nuclei were biotin labelled (either strongly or weakly), indicating that the reduced rate of initiation in suc1 depleted extracts had been decreased still further (Fig. 2B, lower panel; Fig. 3E). More strikingly, expression of cdk2.R33 in mock depleted extracts severely inhibited the number of nuclei initiating S phase and consequently the total incorporation observed (Fig. 2B). Thus, even in the presence of endogenous cdk2, synthesis of cdk2.R33 reduced the total labelling index from 83% to 9% and total incorporation by 88%. In this experiment, the BSA depleted control had entered mitosis by the end of the incubation (Fig. 3A). Similarly, in the presence of cdk2.R33 the extract had also entered mitosis, but essentially no replication had occurred (Fig. 3B). Thus, in this situation, cdk2.R33 is inhibiting S phase entry but not preventing cdc2 kinase activation for mitosis. These results indicate that the cdk2.R33 mutant is selectively inhibiting replication even in the presence of wild-type p33^{cdk2}. This dominant negative effect implies that cdk2 kinase is normally required for S phase initiation in *Xenopus* extracts.

Newly synthesised p34^{cdc2} but not the endogenous protein can induce S phase

Replication in suc1 depleted extracts was stimulated by new synthesis of cdc2 or cdk2 (Figs 2, 4). This could be explained if cdk2 and cdc2 either overlap in function or are both required for initiating S phase in egg extracts. However, Fang and Newport (1991) have stated that, in activated egg extracts, removing endogenous p34^{cdc2} did not inhibit S phase, while removing p33^{cdk2} did. In the light of our results this suggests that endogenous and newly synthesised p34^{cdc2} have different properties. To address this directly, antibody to cdk2 protein was used to immunodeplete egg extracts. Fig. 4A shows that

this treatment removes the cdk2 protein and about an equal amount of cdc2, leaving some 75% of endogenous cdc2 protein in the supernatant. mRNA additions were made, as before, to reintroduce $p33^{cdk2}$ or to supplement the endogenous $p34^{cdc2}$ with newly synthesised cdc2 protein. The nuclear labelling index was substantially inhibited following depletion, with only 26% of nuclei being strongly labelled and almost 50% unlabelled (Fig. 4B). However, addition of either cdk2 or cdc2 mRNAs stimulated initiation, with the pattern of nuclear labelling returning close to that seen with untreated extract. Notably, newly translated $p34^{cdc2}$ increased the labelling index even though most of the endogenous cdc2 protein had not been removed. In these experiments added mRNA stimulated more translation of cdks than in suc1 depletion experiments (compare Figs 2 and 4). For $p33^{cdk2}$, increased expression correlates with greater rescue, but for $p34^{cdc2}$ this is not the case. Since, in suc1 depletion experiments, the level of new cdc2 protein synthesis approaches the endogenous levels present before depletion, rescue of replication does not reflect an anomolous effect of overexpression. These results indicate that, not only can replication be restimulated by either cdk2 or cdc2 proteins, but in the case of cdc2, it can be achieved despite the presence of the majority of the endogenous 'old' $p34^{cdc2}$. It appears that newly synthesised $p34^{cdc2}$ has a capacity to induce nuclear replication that is not shared with the pre-existing pool.

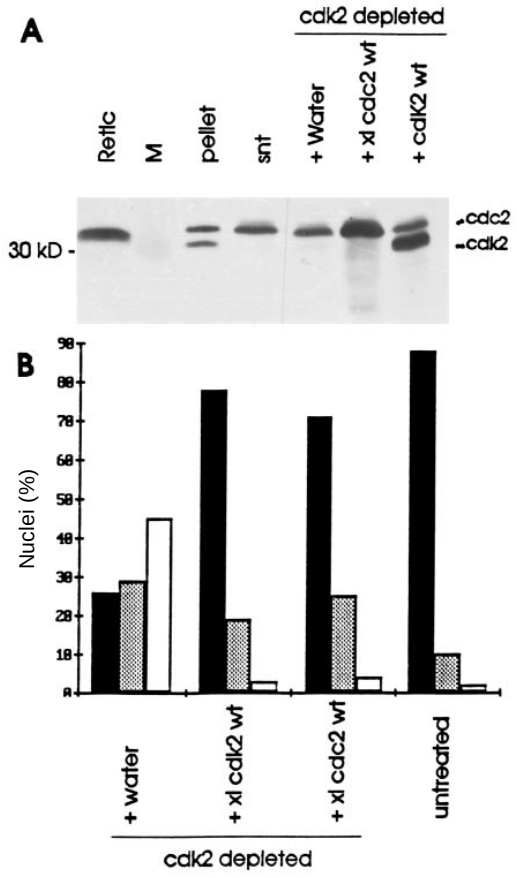


Fig. 4. Endogenous cdc2, in the absence of cdk2, cannot initiate S phase but newly synthesised cdc2 can. Following depletion with cdk2 antibody-Protein A-Sepharose, the extract was preincubated with water or mRNA for thirty minutes before adding sperm heads and biotin-11-dUTP (as in Fig. 2) and incubating for a further two hours. (A) Western blot of extract samples using anti-PSTAIRE monoclonal antibody. Lanes contain, from left to right, cdc2 mRNA translated in reticulocyte lysate (Retic); molecular mass markers (M); pellet and supernatant (snt) immediately after depletion; depleted supernatant incubated with sperm heads, biotin-11-dUTP and water, *Xenopus* cdc2 or cdk2 mRNA (+ Water, + Xlcdc2wt and + cdK2wt, respectively). The level of translation of added mRNA was higher than in Fig. 2. (B) Biotin-11-dUTP incorporation into nuclei was detected and scored as in Fig. 2 for cdk2 depleted extract samples incubated alone (+ water) or with wild-type cdk2 or cdc2 mRNA. The labelling index of an untreated extract is shown as control. Both mRNAs stimulated replication to similar extents.

The cdc2 kinase activity that induces S phase is not the mitotic form

The possibility that the newly synthesised cdc2 was present in a different form to old inherited cdc2 and consequently capable of inducing replication was tested in two ways using suc1 depleted extracts. The first was to cotranslate cyclin B with cdc2 to promote formation of cdc2/cyclin B G_2 complexes (pre-MPF) and compete out formation of cdc2/G_1 cyclin complexes. In practice, this did not lead to substantial overexpression of cyclin B but did reduce the level of stimulation compared to that with cdc2 alone (Fig. 2) even though mitosis was not induced (Fig. 3F).

The second approach was to use the human cdc2 double mutant (ala14, phe15, A14F15), which develops H1 kinase activity directly, as cyclin B is synthesised in *Xenopus* egg extracts (Norbury et al., 1991). Initially we established that translation of wild-type human cdc2 mRNA stimulates replication in suc1 depleted extracts (Figs 5, 6.1). The degree of stimulation was similar to that observed with *Xenopus* cdc2, judged either by labelling index or by [^{32}P]dCTP incorporation (compare Xlcdc2.wt and Hscdc2.wt, Fig. 5). That this stimulation was dependant on kinase activity of cdc2 was tested using a D161 mutant mRNA, whose product lacks T161, binds little B cyclin and does not show H1 kinase activity (Norbury et al., 1991). This mutant failed to stimulate replication in suc1 depleted extracts despite clear synthesis of the protein (Fig. 5). As expected, mitosis did not occur (Fig. 6B). In comparison, translation of cdc2.A14F15 mRNA also failed to stimulate replication, but the added sperm heads were advanced into mitosis (Figs 5, 6C). Indeed, when the double mutant mRNA was added to mock depleted extract, replication was severely inhibited (Fig. 5). These results indicate that the kinase activity of cdc2 can substantially rescue DNA replication in suc1-depleted extracts and that this activity is distinguishable from the cdc2/cyclin B kinase that is active in mitosis.

DISCUSSION

The results presented here demonstrate that either $p33^{cdk2}$ or $p34^{cdc2}$ can promote S phase entry in extracts depleted of both proteins and in extracts preferentially depleted of $p33^{cdk2}$. It is surprising that any single protein can relieve the inhibition of replication produced by suc1 depletion, since a range of proteins in addition to cdks are removed by this procedure (Blow and Nurse, 1990). These include any cyclin partners bound to cdks at the time of depletion, including cyclin E (data not shown). Normally, new protein synthesis is not required for first S phase in complete extracts (Hutchison et al., 1988). However, the

depleted extracts used here were active in synthesising proteins from endogenous mRNA, including cyclin B (Fig. 2A). In the absence of added mRNA this synthesis was not sufficient to rescue replication and no endogenous synthesis of $p34^{cdc2}$ was detected by either western blotting or [^{35}S]methionine incorporation. An endogenous radioactive band was observed at the position of $p33^{cdk2}$, but it was undetectable by western blotting. The failure of the human cdc2.D161 mutant mRNA to rescue replication implies that the stimulation observed with wild-type mRNAs is not a fortuitous consequence of addition of high concentrations of single mRNA species. In particular, it indicates that rescue is not achieved by altering endogenous translation in some undetected way. Thus we conclude that the rescue of replication that is observed is dependent on the translation of the added wild-type mRNAs. Since cdks require cyclins to become active we consider that the relevant cyclin partners remain in the extract after depletion or are made from endogenous templates during the incubation.

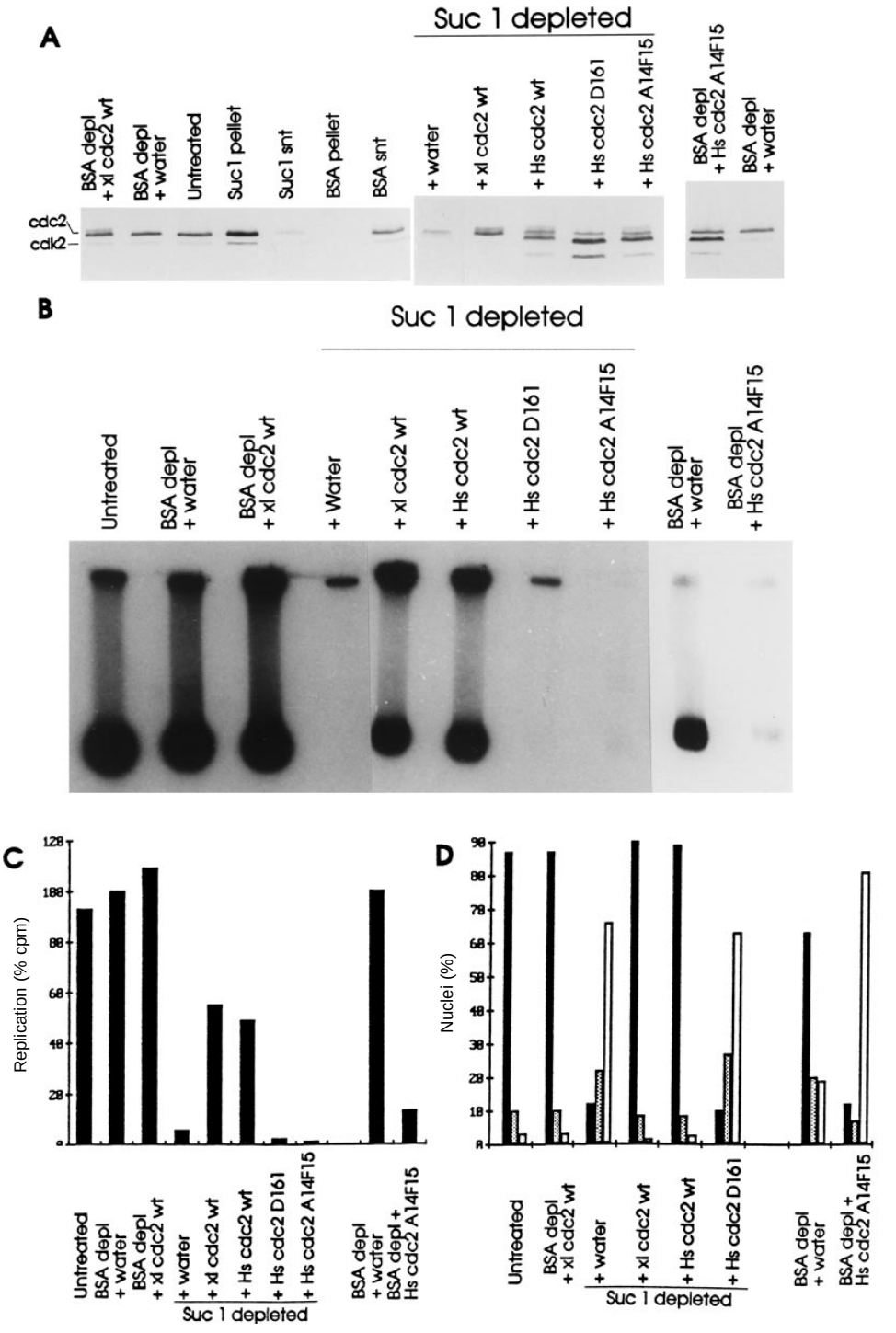


Fig. 5. Human wild-type cdc2 rescues replication but phosphorylation site mutants do not. (A) Detection of cdks on western blots using PSTAIRE antibody. An extract was treated with either suc1-beads or BSA-beads and samples of pellets and supernatant (snt) taken immediately for electrophoresis or incubated as in Fig. 2 (+water or different mRNAs as indicated) before electrophoresis. A different extract, treated with BSA-beads (BSA depl), was incubated with either water or Hscdc2.A14F15 mRNA. (B) Autoradiograph of agarose gels of [^{32}P]dCTP incorporation in parallel samples to those shown in A. (C) Quantification of the agarose gel of [^{32}P]dCTP incorporation into DNA in sperm heads added to the incubations as in A, 30 minutes after mRNA addition. (D) Nuclear labelling indices of the samples shown in A. Where samples had entered mitosis by the end of the incubation (BSA depl + Hscdc2.A14F15) mitotic clumps of DNA were scored as single nuclei. The biotin positive scores in this case were in interphase, judged by morphology and the presence of lamina.

The mutant cdk2.R33 behaves in a dominant negative manner in egg extracts, preventing replication in the presence of wild-type protein. This is consistent with results with human cells, using the equivalent human mutant (van den Heuvel and Harlow, 1993). The inhibition of replication may reflect effective competition for a cyclin or non-productive interaction in larger complexes such as sites of replication, since cdk2 has been found colocalised with replication foci (Cardoso et al., 1993). Removal of $p33^{cdk2}$ without removing cdc2 also inhibited replication (Fang and Newport, 1991; this study). In our experiments the inhibition induced by depletion can be rescued by addition of cdk2 mRNA. However, the degree of rescue appears to depend on the amount of $p33^{cdk2}$ present, particularly at early times, since no rescue is observed when mRNA and sperm heads are added at the same time (data not shown). suc1 depletion 15 minutes after addition of sperm heads fails to inhibit replication (Blow and Nurse, 1990), suggesting that the components normally removed by this treatment operate early in nuclear formation, or are rapidly sequestered in a compartment (perhaps the nucleus) that is inaccessible to suc1 beads. In the depleted extracts used here, nuclear labelling indices are reduced, while events of elongation still occur (Blow and Nurse, 1990), both of which are consistent with a lesion at or prior to initiation of S phase. RPA, a trimeric component necessary for origin unwinding in SV40 replication in vitro, is necessary for replication in *Xenopus* extracts, but its nuclear form is not phosphorylated in cdk2 depleted extracts (Brill and Stillman, 1989; Fang and Newport, 1993). RPA localises to pre-replication centres in suc1 depleted mitotic extracts, a process which is prevented by

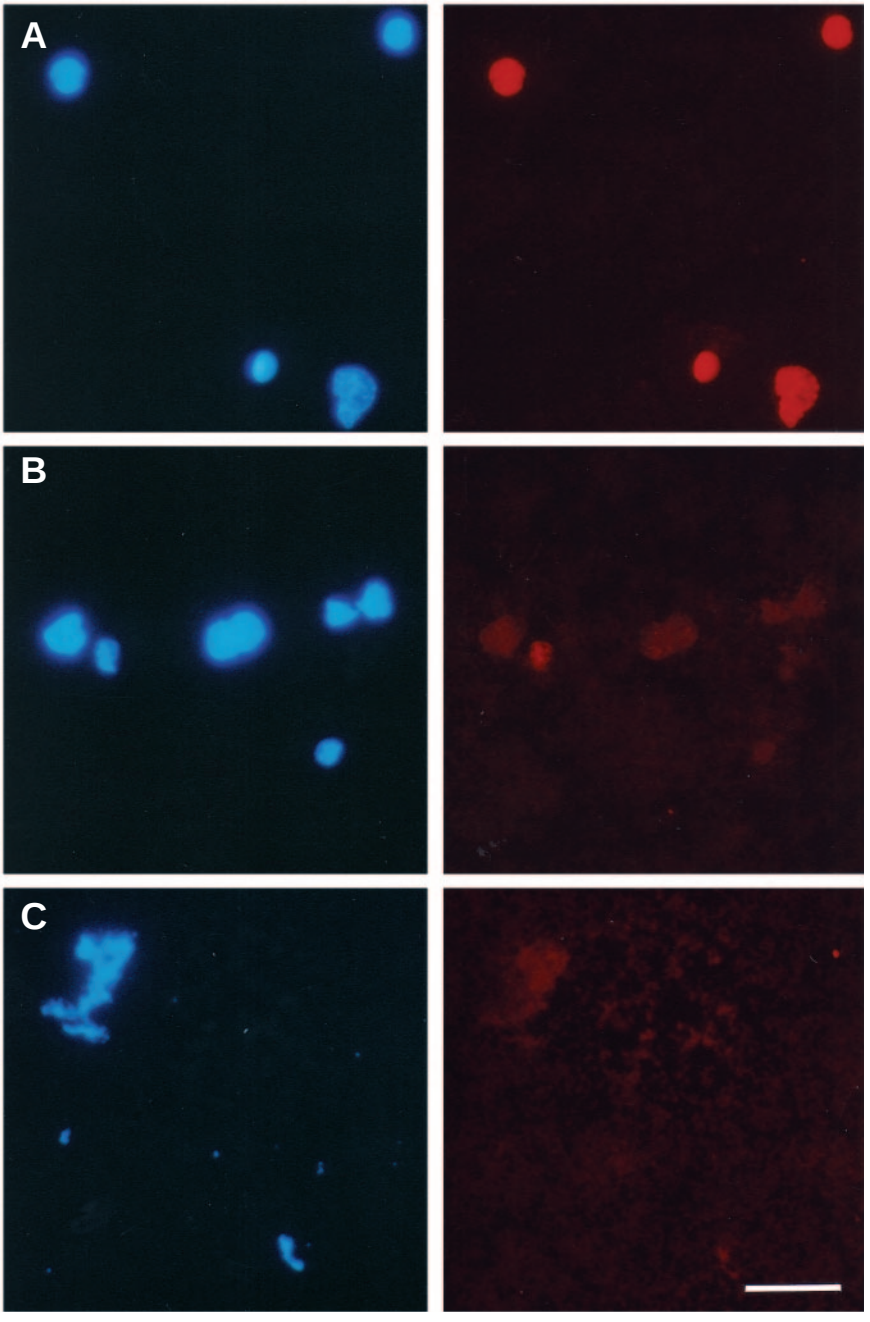


Fig. 6. Fluorescence micrographs of nuclei incubated in suc1 depleted extract with: A, Hscdc2.wt mRNA; B, Hscdc2.D161 mRNA; C, Hscdc2.A14F15 mRNA. In A, all nuclei are strongly labelled. In B, one nucleus is weakly labelled, the remaining nuclei being unlabelled. In C, unlabelled mitotic fragments are seen. DNA (DAPI staining) is shown to the left of the biotin/streptavidin image of the same field. Experimental details were as for Figs 2 and 3. Bar, 10 μm.

active cdc2-cyclin B complexes but not by cdk2-cyclin A (Adachi and Laemmli, 1994). These results point to a role for $p33^{cdk2}$ from an early stage during chromatin decondensation in providing the necessary modifications that allow assembly and/or activity at replication foci.

Newly synthesised $p34^{cdc2}$ (human or *Xenopus*) also rescued replication in depleted extracts. The human mutant cdc2.D161 failed either to stimulate replication or to induce mitosis in suc1 depleted extracts. Presumably, since this form does not bind detectable cyclin B or generate H1 kinase activity in *Xenopus* extracts (Norbury et al., 1991), it also fails to bind a partner for S phase entry. In contrast, the mutant cdc2.A14F15, which forms active H1 kinase as B cyclins are synthesised (Norbury et al., 1991), causes mitosis without replication in both suc1 and mock depleted extracts. The simplest interpretation of this is that kinase activity develops sufficiently rapidly so that nuclear envelopes never form round the sperm heads, thus precluding S phase initiation. These results indicate that a cdc2 kinase activity can induce replication in depleted extracts but it is not the mitotic form.

The presence of endogenous $p34^{cdc2}$ is not sufficient to sustain replication after depletion of $p33^{cdk2}$, but despite this S phase initiation is restimulated by newly synthesised $p34^{cdc2}$ (Fig. 4). From these data it appears that $p34^{cdc2}$, when newly translated, can bind productively with cyclin partner(s) to induce S phase initiation, while inherited (old) cdc2 protein cannot. In this newly synthesised form $p34^{cdc2}$ may usurp the role of $p33^{cdk2}$. Alternatively, since, in these experiments, depletion does not eliminate replication entirely, the new $p34^{cdc2}$ may be increasing the efficiency of the residual $p33^{cdk2}$. At present, this latter possibility seems less likely, as we have not detected an additive effect of cotranslation of cdk2 and cdc2 mRNAs (data not shown).

In human cells, cdc2 gene transcription and translation start in each cell cycle at the G_1/S transition (McGowan et al., 1990; Welch and Wang, 1992). By the end of interphase 80% of the old $p34^{cdc2}$ is replaced by newly synthesised protein. In T-lymphocytes inhibition of $p34^{cdc2}$ synthesis by injection of antisense oligonucleotides blocked S phase entry (Furukawa et al., 1990). cdc2/cyclin A kinase is active at G_1/S, though injection of cdc2 antibodies fails to block replication (Pagano et al., 1993). However, the newly synthesised $p34^{cdc2}$ may not be accessible to antibodies at this time. The failure of dominant negative mutants of cdc2 to block in G_1 (van den Heuvel and Harlow, 1993) does not exclude a role for cdc2 in G_1/S. In contrast, antibodies to cdk2 and dominant negative mutants of cdk2 both block initiation of S phase (Pagano et al., 1993; van den Heuvel and Harlow, 1993; this work). The functional assay we have used demonstrates that newly translated $p34^{cdc2}$, distinct from the mitotically active kinase, induces S phase entry. We conclude that, by using mRNA translation, we have uncovered a form of $p34^{cdc2}$ that regulates S phase initiation.

Blow and Nurse (1990) showed that cdc2 or a closely related protein was required for replication in *Xenopus* egg extracts. These experiments included depletions using a yeast cdc2 antibody, but distinctions between *Xenopus* cdk2 and cdc2 were not clearly made, as cdk2 had not been characterised at that time. Fang and Newport (1991) stated that removal of cdc2 from activated egg extracts did not inhibit replication. Our data indicate that a form of cdc2 protein distinct from the G_2/M form stimulates S phase initiation. We suspect that such a form exists in somatic cells at G_1/S when new synthesis of $p34^{cdc2}$ starts, but disappears as cyclin B accumulates, since cotranslation of cdc2 and cyclin B mRNAs reduces the level of stimulation (Fig. 2). These observations are very reminiscent of experiments with *Schizosaccharomyces pombe* cdc2 and cdc13 mutants, which have been interpreted in terms of different forms of cdc2, with cdc2-cyclin B identifying the cell as in G_2 (Broek et al., 1991; Hayles et al., 1994). In early embryos and egg extracts S phase initiation rapidly follows chromatin decondensation and nuclear formation. Suppression of protein kinases in M phase (CSF) extracts pushes them into interphase, but S phase does not occurr and the nuclei formed have a G_2/prophase morphology (Blow, 1993). At present, we cannot exclude the possibility that a fraction of $p34^{cdc2}$ inherited in eggs exists in a G_1/S form transiently as cells exit mitosis. Selective depletion of $p34^{cdc2}$ from CSF extracts is being used to test this.

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