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Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts

Ulrich P. Strausfeld[1,*], Mike Howell[1,†], Patrick Descombes[1], Stephane Chevalier[1], Rachel E. Rempel[2], Jörg Adamczewski[1,‡], James L. Maller[2], Tim Hunt[1] and J. Julian Blow[1,§]

[1]Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Herts EN6 3LD, England
[2]Howard Hughes Medical Institute Research Laboratories, University of Colorado School of Medicine, Denver, Colorado 80262, USA

*Present address: Fakultät Biologie, Universität Konstanz, 78434 Konstanz, Germany
†Present address: Ludwig Institute for Cancer Research, UCL School of Medicine Branch, London W1P 8BT, UK
‡Present address: Institut de Genetique et de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Sante et de la Recherche Medicale, Illkirch, France
§Author for correspondence

SUMMARY

Extracts of activated *Xenopus* eggs in which protein synthesis has been inhibited support a single round of chromosomal DNA replication. Affinity-depletion of cyclin dependent kinases (Cdks) from these extracts blocks the initiation of DNA replication. We define 'S-phase promoting factor' (SPF) as the Cdk activity required for DNA replication in these Cdk-depleted extracts. Recombinant cyclins A and E, but not cyclin B, showed significant SPF activity. High concentrations of cyclin A promoted entry into mitosis, which inhibited DNA replication. In contrast, high concentrations of cyclin E1 promoted neither nuclear envelope disassembly nor full chromosome condensation. In the early embryo cyclin E1 complexes exclusively with Cdk2 and cyclin A is complexed predominantly with Cdc2; only later in development does cyclin A associate with Cdk2. We show that baculovirus-produced complexes of cyclin A-Cdc2, cyclin A-Cdk2 and cyclin E-Cdk2 could each provide SPF activity. These results suggest that although in the early *Xenopus* embryo cyclin E1-Cdk2 is sufficient to support entry into S-phase, cyclin A-Cdc2 provides a significant additional quantity of SPF as its levels rise during S phase.

Key words: Cyclin A, Cyclin E, Cdk, DNA replication, SPF

INTRODUCTION

Cyclin dependent protein kinases (Cdks) play crucial roles in cell cycle regulation. They consist of a small kinase subunit which is inactive until complexed to a cyclin protein. In metazoans, the Cdks are grouped into seven families (Cdk1-8) and the cyclins are grouped into families A-H (Nigg, 1995). B-type cyclins activate Cdc2 (Cdk1) on passage into mitosis to form the mitotic inducer maturation promoting factor (MPF). Other cyclins and Cdks are involved in progression through G_1 and S-phases of the cell cycle. Expression of dominant negative mutants of Cdk2 (van den Heuvel and Harlow, 1993) and microinjection of anti-Cdk2 antibodies (Pagano et al., 1993) blocked S-phase progression in mammalian cells. Cyclins A and E, both of which can associate with Cdk2 (Pines and Hunter, 1990, 1991; Pagano et al., 1992), also appear to regulate S-phase progression in mammalian cells (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Ohtsubo and Roberts, 1993). In *Drosophila* embryos, cyclin E genes are necessary to undergo S-phase (Knoblich et al., 1994), though no similar S-phase role for cyclin A has been detected (Lehner and O'Farrell, 1990; Knoblich and Lehner, 1993).

To study the role of cyclins and Cdks in S-phase control, we have used *Xenopus* egg extracts that support chromosomal DNA replication (Blow and Laskey, 1986). DNA added to this system is assembled into interphase nuclei and then undergoes a single round of DNA replication (Lohka and Masui, 1983; Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987; Blow and Sleeman, 1990). Complete DNA replication still occurs even when protein synthesis is blocked (Harland and Laskey, 1980; Blow and Laskey, 1988), meaning that all proteins essential for DNA replication are present in the *Xenopus* egg, and all replication control is by post-translational modification.

Affinity-depletion of Cdks from *Xenopus* extracts using $p13^{suc1}$-coupled Sepharose blocks subsequent DNA replication (Blow and Nurse, 1990; Fang and Newport, 1991; Chevalier et al., 1995; Jackson et al., 1995). Suc1 depletion can block DNA replication in at least two distinct ways. Depletion or inhibition of Cdc2 during metaphase blocks activation of replication licensing factor (RLF) (Blow and Laskey, 1988; Blow and Nurse, 1990; Blow, 1993; Kubota and Takisawa, 1993; Vesely et al., 1994). RLF is normally activated during anaphase and is then required for replication of DNA in the

subsequent cell cycle (Blow, 1993; Chong et al., 1996). DNA replication can be blocked in a different way by inhibition of Cdks in interphase extracts, which already contain active RLF (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld et al., 1994; Chen et al., 1995; Jackson et al., 1995). We call this second Cdk activity, 'S-phase promoting factor' (SPF), by analogy to the mitotic inducer MPF, as it appears to function close to the start of S phase (Strausfeld et al., 1994).

In interphase *Xenopus* extracts treated with protein synthesis inhibitors, SPF activity appears to depend on Cdk2, as DNA replication is blocked by immunodepletion of Cdk2 (Fang and Newport, 1991, 1993; Chevalier et al., 1995) or by the Cdk inhibitor $p21^{Cip1}$ (Adachi and Laemmli, 1994; Strausfeld et al., 1994; Shivji et al., 1994; Chen et al., 1995; Jackson et al., 1995). Most Cdk2 is complexed with cyclin E in the early *Xenopus* embryo (Rempel et al., 1995), and immunodepletion of cyclin E also blocks replication (Jackson et al., 1995). However, Cdc2 can also supply SPF activity (Chevalier et al., 1995): when translation is permitted to continue in the cell-free system, addition of Cdc2 mRNA can efficiently rescue DNA replication in $p13^{suc1}$-depleted extracts. This suggests that SPF activity can also be supplied by Cdc2 complexed to a cyclin normally translated in the early embryo.

In this paper, we assess the ability of different *Xenopus* cyclins and Cdks to provide SPF activity and restore DNA synthesis to Suc1-depleted extracts. Our results suggest that the cyclin A-Cdc2 complex provides a significant quantity of SPF activity in addition to that provided by cyclin E-Cdk2. The sequential activation of different Cdk-cyclin complexes provides a consistent model for the way that DNA replication is controlled in the cell cycle.

MATERIALS AND METHODS

Preparation of *Xenopus* extracts

Interphase and metaphase-arrested *Xenopus* egg extracts were prepared as described (Blow, 1993) and frozen in liquid nitrogen. After thawing, extracts were supplemented with an ATP-regenerating system (25 mM phosphocreatine and 5 μg/ml creatine phosphokinase) and 250 μg/ml cycloheximide. Metaphase extracts were released into interphase by addition of 0.4 mM $CaCl_2$. Except for Fig. 7B, 'Suc1-depleted' extracts were prepared by mixing 2 volumes of freshly prepared interphase extract containing 250 μg/ml cycloheximide with 1 volume of $p13^{suc1}$-Sepharose beads (10 mg $p13^{suc1}$ protein coupled per ml beads) for 10 minutes at 22°C. Beads were separated by centrifugation (15 seconds at 1,000 ***g)*** and by filtration through a 25 μm Nybolt membrane (Plastok Ltd, Merseyside). Suc1-depleted extracts were frozen and stored in liquid nitrogen. In the experiment shown in Fig. 7B, extract was Suc1-depleted at 4°C as described (Blow and Nurse, 1990). Extracts were immunodepleted of cyclin E as described (Jackson et al., 1995) using anti-*Xenopus* cyclin E affinity-purified rabbit polyclonal antibody.

Protein expression and purification of cyclins and Cdks

Xenopus cyclin A1 NΔ56 cloned in pET3a (Minshull et al., 1990) was expressed in *Escherichia coli* and purified from inclusion bodies. *Xenopus* cyclin E1 (Rempel et al., 1995) tagged with GST, 6His or 15His and *Xenopus* cyclin A2 tagged with GST were expressed in *E. coli* and purified on glutathione agarose or nickel agarose. GST-tagged human Cdk2 was prepared as described (Poon et al., 1993). *Xenopus* cyclin B1 tagged with maltose binding protein was constructed by M.-A. Félix (EMBL, Heidelberg) and provided by K. Yamashita (ICRF Clare Hall). Monoclonal antibodies against the Cdk-specific PSTAIRE-peptide were a gift from Y. Nagahama and M. Yamashita (Yamashita et al., 1991).

C-terminally His tagged *Xenopus* Cdk2, C-terminally His tagged *Xenopus* Cdc2, *Xenopus* cyclin E and *Xenopus* cyclin A1 were cloned into vector pVL1392 (Pharmingen) and *Xenopus* cyclin A2 was cloned into vector pVL1393 (Pharmingen). Recombinant baculoviruses were engineered using a BaculoGold kit following suppliers instructions (Pharmingen). Recombinant proteins were produced by coinfection into Sf9 cells according to the method of O'Reilly et al. (1992). Cells were lysed in lysis buffer: 20 mM Tris-HCl, pH 8.0, 10 mM β-glycerophosphate, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 1 μg/ml each pepstatin, leupeptin, chymostatin and aprotinin. NaCl was added to 20 mM, the extract cleared by centrifugation and glycerol added to 5%. Extract was loaded onto Q-Sepharose (Pharmacia) equilibrated in column buffer (lysis buffer plus 5% glycerol and 20 mM NaCl), and Cdk complexes were eluted with 200 mM NaCl (cyclin E1-Cdk2) or 300 mM NaCl (cyclin A1-Cdc2 and cyclin A2-Cdk2) in column buffer minus DTT. Active fractions were pooled, supplemented with 10 mM $CaCl_2$ and loaded onto Ni^{++}NTA Agarose (Qiagen) in column buffer containing 10 mM $CaCl_2$ and no DTT. Column buffer was changed to XX buffer: 50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 10 mM imidazole, 10% sucrose, 0.1 mM PMSF and 1 μg/ml each pepstatin, leupeptin, chymostatin and aprotinin. Proteins were eluted in XX buffer containing 80 mM imidazole.

Histone H1 kinase assay

Histone H1 kinase assays with samples of *Xenopus* egg extracts were prepared by diluting tenfold in buffer (Poon et al., 1993). After adding 35 μl kinase buffer (1 mg/ml histone H1 (Sigma), 200 μM ATP, 100-250 cpm/pmol [γ-^{32}P]ATP (Amersham), 10 mM $MgCl_2$, 0.5 mM EGTA, 20 mM Hepes, pH 7.5, 1 mM DTT 10% sucrose) and incubation at 22°C for 10 minutes the reaction was stopped on Whatman P81 paper. P81 paper was washed 3× 30 minutes in deionised water and incorporated ^{32}P counted by liquid scintillation. Alternatively, proteins were run on a 15% polyacrylamide gel and quantified by Phosphorimager.

DNA synthesis and microscopy

DNA synthesis was measured by adding 3-12 ng demembranated *Xenopus* sperm nuclei and 0.05 μCi [α-^{32}P]dATP (Amersham; 3,000 Ci/mmol)/μl *Xenopus* egg extract. Replication was assayed by TCA precipitation and BrdUTP density substitution as described (Blow and Laskey, 1986). Nuclear formation and breakdown were monitored under phase contrast optics using a Zeiss Axioskop microscope. In parallel DNA was stained with 10 μg/ml Hoechst 33258 and viewed under UV excitation.

RESULTS

Assay for SPF activity

Cell-free extracts were prepared from *Xenopus* eggs activated by calcium ionophore, which releases them from arrest in meiotic metaphase II and allows them to progress into interphase of the first (embryonic) cell cycle. Extracts contain approximately 600 nM Cdc2 and 60 nM Cdk2 (Kobayashi et al., 1991, 1994). Since cyclins A and B are degraded as cells exit from metaphase, their levels remain low in extracts treated with protein synthesis inhibitors. Cyclin E1, which is not degraded on exit from mitosis, remains relatively constant at 30-60 nM (Rempel et al., 1995).

Interphase extracts were supplemented with the protein

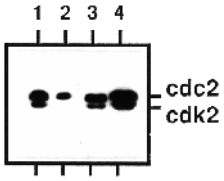

Fig. 1. Depletion of Cdc2 and Cdk2 by Suc1 Sepharose. Interphase *Xenopus* extracts before (lane 1) and after (lane 2) Suc1 depletion were western blotted with an anti-PSTAIRE antibody. Lanes 3 and 4, material eluted from the Suc1 beads. Markers show the migration of Cdc2 and Cdk2.

synthesis inhibitor cycloheximide and affinity-depleted of Cdks and associated proteins using $p13^{suc1}$ beads, leaving them unable to support the initiation of DNA replication (Blow and Nurse, 1990). Not all Cdc2 need be removed from the extracts for DNA replication to be blocked. Fig. 1 shows extracts before and after Suc1-depletion immunoblotted with an anti-'PSTAIRE' antibody which recognises a common motif in both Cdc2 (upper band) and Cdk2 (lower band). The depleted extract still contained 50-100 nM Cdc2 (10-20% of starting levels), although it supported very little DNA replication. Virtually no Cdk2 can be seen in the Suc1-depleted extract. Since in *Xenopus* extracts most Cdk2 is complexed with cyclin E1 whilst most Cdc2 is monomeric, one explanation for the proportionately greater depletion of Cdk2 compared to Cdc2 is that Suc1 binds Cdk-cyclin complexes with a higher affinity than it does monomeric Cdks.

DNA replication can be restored to these Suc1-depleted extracts (reaching 30-90% of levels in undepleted extract) by small quantities of undepleted *Xenopus* extract or by material eluted from the Suc1 beads (Blow and Nurse, 1990). We have used this assay system to identify proteins capable of restoring initiation competence to Suc1-depleted interphase *Xenopus* extracts, an activity we define as 'S-phase promoting factor' (SPF).

H1 kinase and MPF activity of cyclins A and E

Xenopus cyclins (A1, A2 and E1) and Cdk2 were expressed in bacteria, isolated, and purified. We first tested the ability of the cyclins to generate histone H1 kinase activity on addition to *Xenopus* extract (Fig. 2). Cyclin A1 induced significant H1 kinase in *Xenopus* extract, whether or not Cdk2 was also added (Fig. 2A). Similar results were obtained with bacterially-produced cyclin B (data not shown). With cyclin A, maximal kinase activity took 20-60 minutes to develop, presumably due to the time required for assembly into an active complex with Cdc2 (data not shown). Unlike cyclin A (which complexes with Cdc2 in the early embryo, see below), recombinant cyclin E1 (which complexes with Cdk2) did not induce high levels of H1 kinase unless the extract was also supplemented with Cdk2 (Fig. 2B). Cdk2 was therefore titrated into the extract to measure its effect on H1 kinase in the presence of 910 nM added cyclin A1 or 400 nM cyclin E1 (Fig. 2C). Although Cdk2 only slightly increased the cyclin A1-induced H1 kinase, Cdk2 levels of >100 nM strongly increased cyclin E1-induced H1 kinase. Similar results were obtained in normal and Suc1-depleted extracts (data not shown).

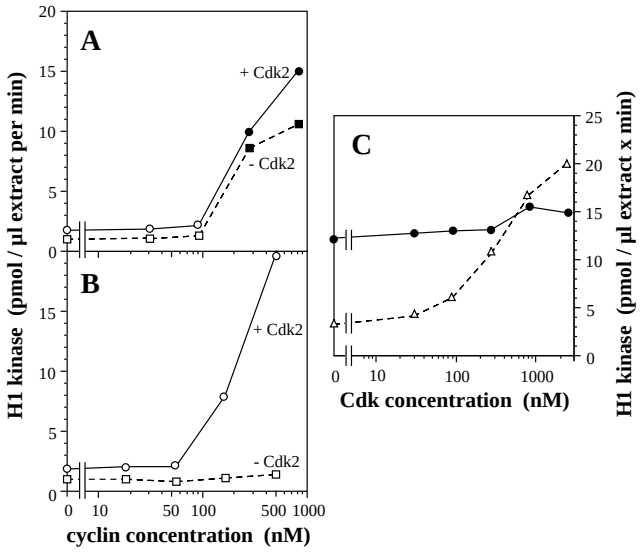

Fig. 2. Induction of H1 kinase activity in extracts supplemented with recombinant cyclins and Cdks. (A and B) Interphase extracts containing 15 ng sperm DNA/µl were supplemented with 2.5 µM GST-tagged human Cdk2 (circles) or buffer alone (squares) and incubated 90 minutes at 22°C. Extracts with assembled nuclei were then mixed with (A) *Xenopus* cyclin A1 NΔ56 protein and (B) *Xenopus* GST-tagged cyclin E1. After 2 hours samples were taken for histone H1 kinase activity assays and nuclear morphology was monitored by microscopy. Open symbols, intact interphase nuclei; filled symbols, nuclear envelope breakdown. (C) Interphase *Xenopus* extract was supplemented with 910 nM *Xenopus* cyclin A1 NΔ56 (circles) or 400 nM 15His-tagged *Xenopus* cyclin E1 (triangles) and mixed with various amounts of GST-tagged human Cdk2. After 45 minutes incubation at 21°C samples were taken for histone H1 kinase assays.

MPF activity of cyclins A and E

We next analysed the effect of added cyclins A1 and E1 on the morphology of nuclei assembled in *Xenopus* extract, which normally contain decondensed DNA (Fig. 3J) surrounded by phase-dense nuclear envelopes (Fig. 3I). Addition of high levels of cyclin A1 to interphase extract induced morphological changes associated with entry into mitosis, including disassembly of the nuclear envelope (Fig. 3A) and condensation of DNA into chromosome-like threads (Fig. 3B). Similar results were obtained with bacterially-produced cyclin B1 (data not shown). In contrast, whether or not recombinant Cdk2 was also added, bacterially produced cyclin E1 did not induce these mitotic changes (Fig. 3E-H) although high H1 kinase levels were induced when both proteins were added together. The nuclear envelope remained clearly intact (Fig. 3E,G) and condensed chromosomes were not observed (Fig. 3F,H). The ability of different concentrations of cyclins A1 and E1 to induce mitosis is indicated in Fig. 2A and B (filled versus open symbols).

Ability of cyclins A, B and E to generate SPF activity

We next measured the ability of cyclins A1 (Fig. 4A), B1 (Fig. 4B) and E1 (Fig. 4C) to provide SPF activity in the presence of 2.5 µM recombinant Cdk2. At concentrations between 10 and 100 nM, cyclin A1 induced significant DNA synthesis in Suc1-depleted extract (Fig. 4A, squares). At higher concentra-

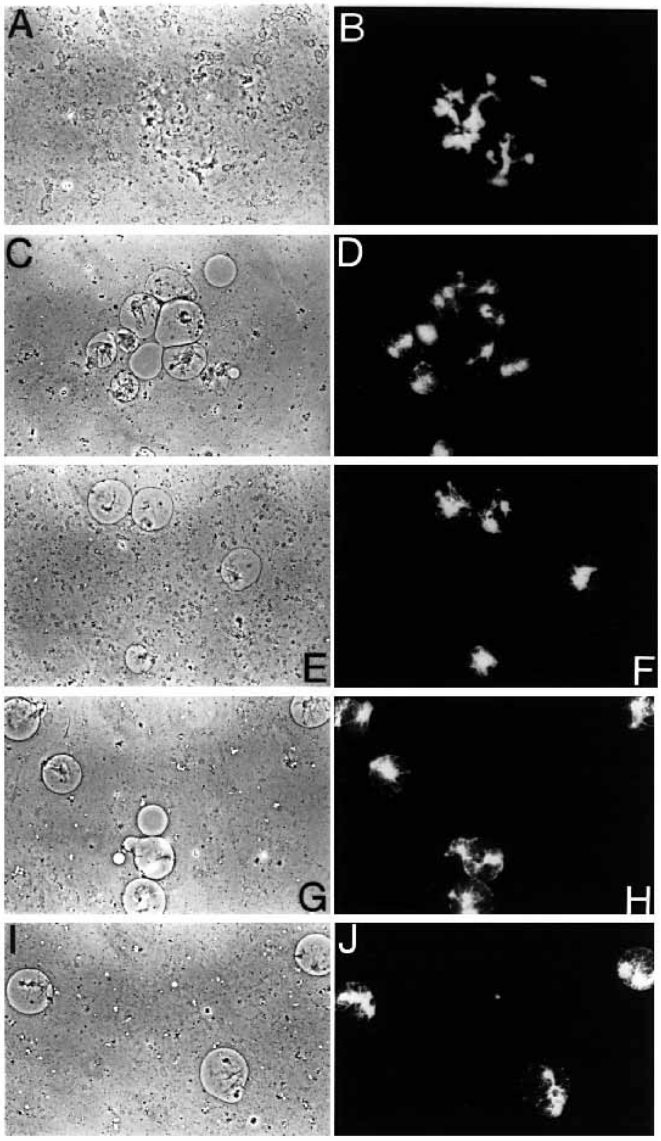

Fig. 3. Cyclin A but not cyclin E induces nuclear envelope breakdown. Photomicrographs of intact nuclei treated with different concentrations of recombinant cyclin A1 or cyclin E1 in the presence of 2.5 μM Cdk2. (A,C,E,G,I) phase contrast; (B,D,F,H,J) DNA stained with Hoechst 33258 and viewed under UV fluorescence. (A,B) 826 nM cyclin A1. (C,D) 31 nM cyclin A1. (E,F) 495 nM cyclin E1. (G,H) 18 nM cyclin E1. (I,J) buffer alone. Samples were from the same experiment as is shown in Fig. 2.

tions cyclin A1 induced high histone H1 kinase (Fig. 4A, circles) and drove the extract into mitosis (filled symbols), under which conditions the initiation of DNA replication does not occur (Blow and Sleeman, 1990). Similar results were obtained with a range of cyclin A constructs, including *Xenopus* cyclin A2 and bovine cyclin A (data not shown). BrdU density substitution demonstrated that the DNA synthesis induced by cyclin A1 represented a single complete round of semiconservative replication (data not shown). Although cyclin B1 was capable of inducing similar levels of H1 kinase, and could drive extracts into mitosis, induction of DNA synthesis was not seen (Fig. 4B). In contrast to cyclins A1 and B1, cyclin E1 was unable to induce either mitosis or significant amounts of DNA synthesis, despite being able to induce high H1 kinase levels (Fig. 4C).

The inability of bacterially-expressed cyclin E1 to induce DNA synthesis was somewhat surprising (Strausfeld et al., 1994; Jackson et al., 1995). A number of other bacterially-expressed cyclin E1 constructs were tested: although some SPF activity was observed, this was always significantly lower than that generated by cyclin A (data not shown). In order to provide highly active Cdk-cyclin complexes, cyclins A2 and E1 were co-expressed with Cdk2 in Sf9 insect cells using the baculovirus expression system. These cyclin-Cdk2 complexes were then assayed for their ability to provide SPF activity by restoring DNA replication to Suc1-depleted extract (Fig. 5). Consistent with our previous results, cyclin A2-Cdk2 provided good SPF activity, reaching 32% of the levels seen with control extract (Fig. 5A, circles). Cyclin E1-Cdk2 also provided significant SPF activity, though higher concentrations were needed than cyclin A2-Cdk2 (Fig. 5A, triangles).

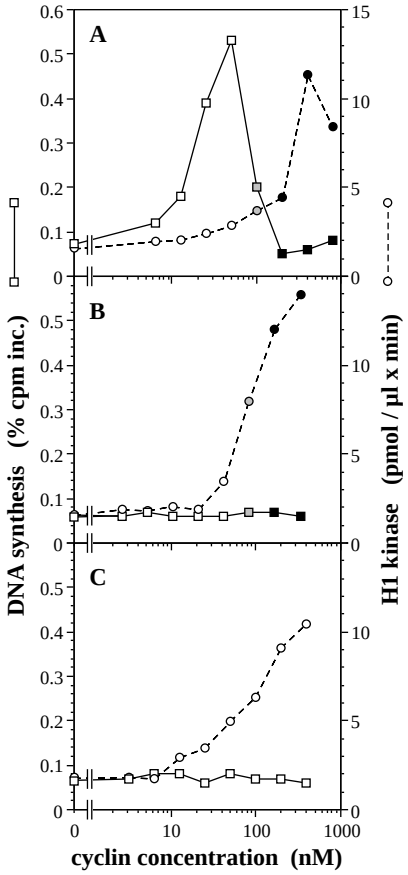

Fig. 4. SPF activity of recombinant cyclins A, B and E. Samples of a Suc1-depleted *Xenopus* interphase extract supplemented with 2.5 μM GST-tagged human Cdk2 protein were mixed with different quantities of (A) cyclin A1 NΔ56, (B) MBP-tagged *Xenopus* cyclin B1 or (C) GST-tagged *Xenopus* cyclin E1. After 45 minutes at 22°C, aliquots were taken for histone H1 kinase assays (circles). The remaining extract was supplemented with 2 ng/μl sperm DNA and [α-^{32}P]dATP to determine total DNA synthesis over a further 195 minutes (squares). Nuclear morphology was monitored by microscopy for condensed chromosomes (filled symbols) intact nuclei (open symbols), or partial nuclear disassembly (stippled symbols).

Fig. 5B shows the H1 kinase activity in the extract after addition of the cyclin-Cdk2 complexes. The specific H1 kinase activity of cyclin A2-Cdk2 induced in the extract was about 100 times that of cyclin E1-Cdk2, consistent with the specific activities of the partially-purified cyclin-Cdk complexes (0.9 nM cyclin A2-Cdk2 gave 0.45 pM/minute per μl, whilst 150 nM cyclin E1-Cdk2 gave 0.75 pM/minute per μl with histone H1 as a substrate). Fig. 5C shows a BrdU density substitution of DNA synthesis induced by cyclin A2-Cdk2 and cyclin E1-Cdk2. For both these complexes, >90% of the newly synthesised DNA banded at the density of heavy/light (HL) DNA, as expected of a single complete round of DNA replication. A small amount of heavy/heavy (HH) DNA was also observed, suggesting that some re-replication of DNA had occurred.

Cyclin A complexes with Cdc2 in early development

Cyclin E1 in *Xenopus* is exclusively complexed with Cdk2, as it is in other cell types (Rempel et al., 1995). Cyclin A can complex with either Cdc2 or Cdk2 under different circumstances (Minshull et al., 1990; Pines and Hunter, 1990; Kobayashi et al., 1992; Pagano et al., 1992; Rempel et al., 1995). Two different cyclin A proteins, A1 and A2, have been identified in *Xenopus* (Minshull et al., 1990; Howe et al., 1995). Cyclin A1 is found only in the early embryo, whilst cyclin A2 protein levels are relatively low in the early embryo and increase significantly at developmental stages 10-12 as cyclin A1 disappears (Howe et al., 1995). Fig. 6A compares levels of Cdc2 and Cdk2 protein during *Xenopus* development. Cdc2 levels stayed essentially constant throughout all developmental stages investigated. During early development Cdk2 protein levels were also constant at about 10% of Cdc2 levels, but between stages 23-26 it increased to levels similar to those of Cdc2, as is more typical of somatic cells. Antibodies against cyclins A1 and A2 were used to immunoprecipitate cyclin-Cdk complexes from *Xenopus* embryos, and the proteins were then blotted for Cdc2 and Cdk2 (Fig. 6B). In the early embryo, both cyclins A1 and A2 complexed exclusively to Cdc2, consistent with previous reports (Minshull et al., 1990; Rempel et al., 1995). After stages 12-14, cyclin A1 disappeared, and so no Cdks co-precipitated with cyclin A1 antibodies (Fig. 6B). However, between embryonic stages 14-19 a switch occurred, and cyclin A2 was found almost exclusively complexed with Cdk2 (Fig. 6B). This switch was unlikely to be due to differences in the quantities of Cdc2 and Cdk2, since their levels remained constant from the egg through to at least stage 23 (Fig. 6A).

In the SPF assays shown in Figs 4 and 5, recombinant Cdk2 was added to the assays, either added separately (Fig. 4) or as part of a cyclin-Cdk complex (Fig. 5). Fig. 7A shows that cyclin A can still efficiently rescue DNA replication in Suc1-depleted extracts without addition of further Cdk2. This suggests that this rescue is due to the formation of a cyclin A-Cdc2 complex, since the Cdk-depleted extracts used in these assays were not completely depleted of Cdc2 (Fig. 1). In order to prove that cyclin A-Cdc2 can provide SPF activity, we used preformed cyclin A1-Cdc2 and cyclin A2-Cdk2 produced in baculovirus. Both complexes could efficiently restore DNA replication to Suc1-depleted extracts (Fig. 7B). Further, both complexes could restore DNA replication to extracts immunodepleted of cyclin E (Fig. 7C).

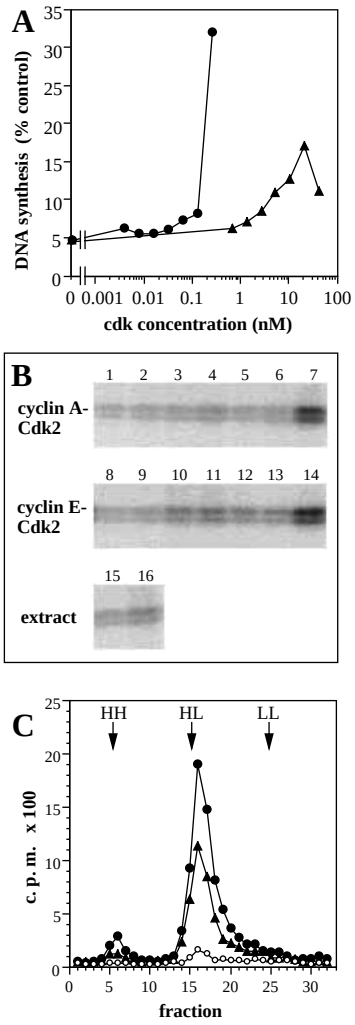

Fig. 5. SPF activity of baculovirus-expressed cyclin A2-Cdk2 and cyclin E1-Cdk2. Partially purified cyclin-Cdk2 complexes were titrated into Suc1-depleted extract, and assayed for their ability to induce DNA synthesis (A) or histone H1 kinase (B). (A) Total DNA synthesis over 150 minutes expressed as a percentage of that obtained by rescue with 10% (v/v) untreated interphase extract. Circles, cyclin A2-Cdk2; triangles, cyclin E1-Cdk2. (B) Autoradiograph of histone H1 labelled with [γ-^{32}P]ATP. Lanes 1-7, Suc1-depleted extract containing 4, 8, 16, 32, 64, 128, 256 pM cyclin A2-Cdk2. Lanes 8-14, Suc1-depleted extract containing 0.66, 1.3, 2.7, 5.4, 10.7, 21 and 42 nM cyclin E1-Cdk2. Lane 15, Suc1-depleted extract alone; lane 16, Suc1-depleted extract plus 10% (v/v) untreated interphase extract. (C) CsCl gradient of density substitution reactions with Suc1-depleted extract supplemented with 256 pM cyclin A2-Cdk2 (filled circles), 21 nM cyclin E1-Cdk2 (filled triangles) or Suc1-depleted extract alone (open circles). Arrows show the expected densities of heavy/heavy DNA (HH; 1.79 g/ml), heavy/light DNA (HL; 1.75 g/ml) and light/light DNA (LL; 1.71 g/ml).

In the early *Xenopus* embryo cyclin A is constitutively translated from maternal mRNA, and is degraded at the end of mitosis (Minshull et al., 1990). Cyclin A levels are therefore low at the start of interphase and build up later in the cell cycle. If the SPF activity of cyclin A is of physiological relevance it must be able to induce DNA replication in intact nuclei. Fig. 8 shows the ability of cyclin A1 to restore DNA synthesis to

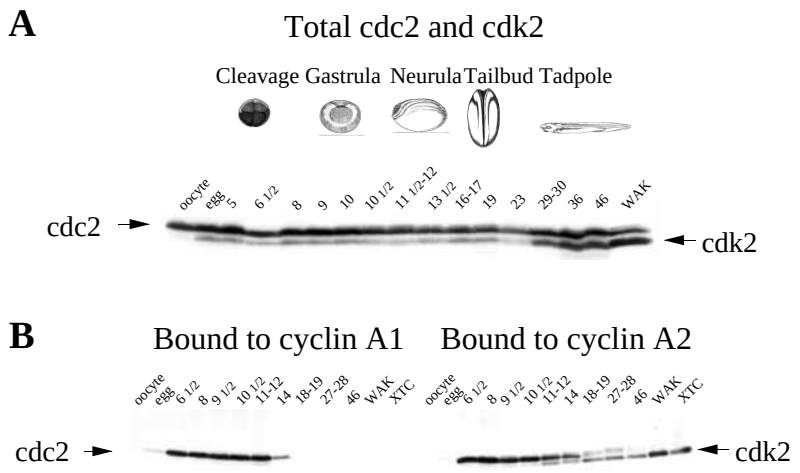

Fig. 6. Cdc2 and Cdk2 complexes present during *Xenopus* embryogenesis. (A) At the indicated stages of *Xenopus* development, embryos were western blotted with anti-PSTAIRE antibodies which recognise both Cdc2 and Cdk2 (arrowed). (B) Extracts were prepared from the indicated stages of development, and immunoprecipitates collected with antibodies to cyclins A1 and A2 were subjected to PAGE and western blotted with anti-PSTAIRE antibodies. XTC and WAK denote samples from two *Xenopus* cell lines.

Suc1-depleted extracts at various times after nuclear assembly. The open circles show a timecourse of DNA synthesis in a Suc1-depleted extract, with sperm chromatin (the DNA template) added at time zero. When cyclin A1 was also added to the extract at this time, efficient DNA synthesis was restored (Fig. 8, filled diamonds). Full nuclear assembly takes 0.5-1 hour in these extracts (Blow and Laskey, 1986; Blow and Watson, 1987; Blow, 1993). When cyclin A1 was added after this period, at either 1 hour (filled circles) or 2 hours (filled triangles), it could still efficiently rescue DNA synthesis. This demonstrates that the SPF function of cyclin A1 can be executed after nuclear assembly is complete.

DISCUSSION

Previous work has shown that Cdk activity is required for entry into and progress through S-phase of the cell cycle. We have used the *Xenopus* cell-free system to investigate in detail the role of different cyclin-Cdk complexes in this process. We show that cyclin A1-Cdc2, cyclin A2-Cdk2 and cyclin E1-Cdk2 can all provide SPF activity to interphase extracts of *Xenopus* eggs affinity-depleted of Cdks.

Cdk complexes found in the early *Xenopus* embryo

In *Xenopus* eggs and egg extracts Cdc2 is present at about 600 nM and Cdk2 at about 60 nM (Kobayashi et al., 1991, 1994). *Xenopus* cyclin E1 complexes exclusively with Cdk2, and levels remain at 30-60 nM in the early embryo (Kobayashi et al., 1991; Rempel et al., 1995). We show that recombinant cyclin E only provided high levels of histone H1 kinase activity when additional Cdk2 was also added. This suggests that in contrast to Cdc2, which is present in large molar excess over cyclins, there are only relatively low levels of free Cdk2 in egg extracts (Gabrielli et al., 1992).

In contrast to the stability of cyclin E1 at this early stage of development, cyclins A and B are degraded at the end of each mitosis, so that levels oscillate with the cell cycle. Cyclin A1 reaches peak levels in mitosis of at least 3 nM (Kobayashi et al., 1991). In early *Xenopus* development cyclin A1 and the smaller quantity of A2 both complex almost exclusively with endogenous Cdc2. The lack of detectable cyclin A-Cdk2 may be due to preferential association of Cdk2 with cyclin E, since if recombinant Cdk2 is also added, cyclin A-Cdk2 complexes are formed (Kobayashi et al., 1992). About developmental stage 10-12 a switch occurs, with levels of cyclin A1 and E1 falling and levels of A2 rising (Howe et al., 1995; Rempel et

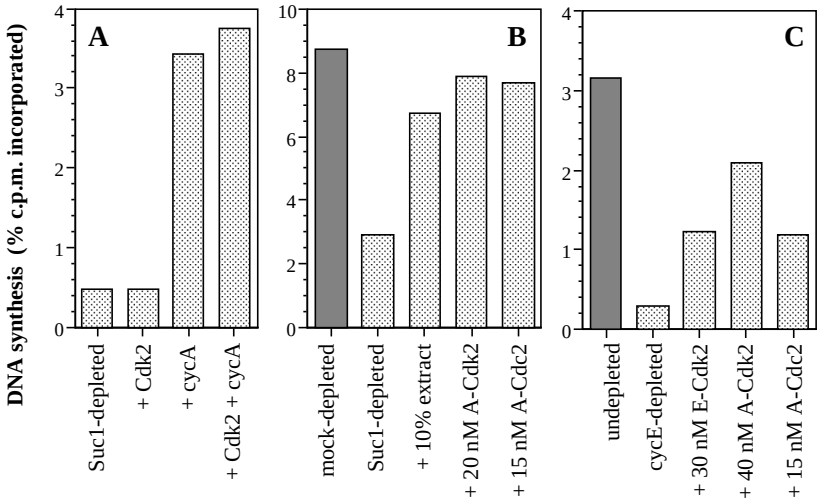

Fig. 7. Ability of different cyclin-Cdk complexes to provide SPF activity. (A) Suc1-depleted extract containing sperm chromatin (3 ng DNA/μl) with different combinations of 0.5 μM bacterially-produced Cdk2 and/or 100 nM bacterially-produced cyclin A1 NΔ56. (B) Suc1-depleted extract (light stippling) containing sperm chromatin (5 ng DNA/μl) was supplemented with 10% (v/v) undepleted extract or baculovirus-produced cyclin A2-Cdk2 or cyclin A1-Cdc2. Mock-depleted extract (heavy stippling) is also shown. (C) Extract immunodepleted of cyclin E (light stippling) containing sperm chromatin (3 ng DNA/μl) was supplemented with baculovirus-produced cyclin E1-Cdk2, cyclin A2-Cdk2 or cyclin A1-Cdc2. Undepleted extract (heavy stippling) is also shown. In all panels, resultant DNA synthesis is expressed as a percentage of total [α-^{32}P]dATP incorporated into DNA.

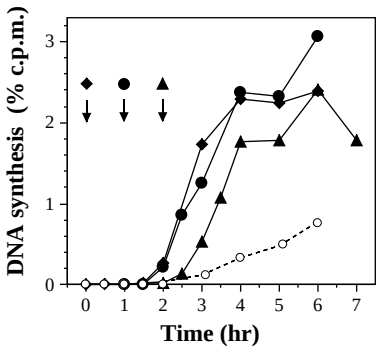

Fig. 8. Ability of cyclin A to rescue DNA synthesis at different times after nuclear assembly. Suc1-depleted *Xenopus* extract was supplemented with sperm chromatin (3 ng DNA/μl) and [α-^{32}P]dATP. After incubation at 22°C for 0 hour (filled diamonds), 1 hour (filled circles) or 2 hours (filled triangles), samples were supplemented with a final concentration of 80 nM *Xenopus* cyclin A1 NΔ56. Open circles, no cyclin addition. At the indicated times from the start of the incubation, samples were assayed for the extent of DNA synthesis.

al., 1995). Slightly later, between stages 14-19, cyclin A2 is predominantly complexed with Cdk2, as it is in somatic cells. These changes roughly correspond to the time when the short embryonic cell cycles of about 30 minutes lengthen to become more typical of somatic cells (Frederick and Andrews, 1994), and may have important functional consequences (Pagano et al., 1992).

MPF activity of cyclins A and B

MPF is an activity present in metaphase cells capable of inducing entry into mitosis. It consists of a heterodimer of Cdc2 and cyclin B, and probably represents the major histone H1 kinase activity in mitotic cells (Gautier et al., 1988; Labbé et al., 1989a,b). A-type cyclins can also generate MPF activity, however (Luca et al., 1991; Roy et al., 1991). There is a good correlation between H1 kinase and MPF activity induced by cyclins A and B, as similar levels of histone H1 kinase activity are required to induce mitosis in each case. We show here that this correlation does not hold for cyclin E1. When recombinant cyclin E1 and Cdk2 are added to *Xenopus* extracts, high levels of H1 kinase were induced but mitosis did not occur, even at kinase levels several-fold higher than is normally found in mitosis. This is consistent with studies in *Drosophila* and mammalian cells where deletion or overexpression of cyclin E had no observable mitotic effects (Ohtsubo and Roberts, 1993; Knoblich et al., 1994)

SPF activity of cyclins A and E

After exit from mitosis, *Xenopus* extracts enter interphase and support a single round of chromosome replication. Inhibition of Cdk2 function in interphase extracts, either by affinity-depletion with p13^{suc1} (Blow and Nurse, 1990; Fang and Newport, 1991; Chevalier et al., 1995; Jackson et al., 1995), immunodepletion (Fang and Newport, 1991; Chevalier et al., 1995) or by using the Cdk inhibitor p21^{cip1} (Strausfeld et al., 1994; Chen et al., 1995; Jackson et al., 1995) blocked the initiation of DNA replication. This defines an activity, called 'S-phase promoting factor' (SPF), required for the initiation of DNA replication. The ability of SPF to induce the initiation of DNA replication in the absence of further protein synthesis distinguishes it from certain Cdk complexes in other cell types (such as Cdk4-cyclin D or Cdc28-Cln) whose role in G_1 progression appears to be mediated by the induction of transcription.

SPF activity can be induced by recombinant cyclin A. In Suc1-depleted extracts, cyclin A induces maximal SPF activity at H1 kinase levels less than one third of those required to induce mitosis. At higher levels, the MPF activity induced by added cyclin A drives the extract into mitosis rather than S-phase, so initiation does not take place (Blow and Sleeman, 1990). DNA replication is therefore seen only within a narrow window of cyclin A kinase activity. These results are consistent with the ability of cyclin A to restore DNA replication to Cip1-treated extracts (Strausfeld et al., 1994). Although capable of inducing efficient DNA replication in Cip1-treated extracts (Strausfeld et al., 1994), bacterially-expressed cyclin E1 provided relatively low SPF levels in Suc1-depleted extracts. Similar results have been reported by Jackson et al. (1995). However, baculovirus complexes of cyclin A1-Cdc2, A2-Cdk2 and cyclin E1-Cdk2 could each provide SPF activity to Suc1-depleted extract. All three complexes could also rescue DNA replication in extracts immunodepleted of cyclin E. In contrast to our results with cyclins A and E, we could induce no SPF activity with bacterially-expressed cyclin B1, although it could drive extracts into mitosis. Cyclin B1 was similarly incapable of inducing DNA replication in Cip1-treated extracts (Strausfeld et al., 1994).

Functions of cyclins A, B and E in vivo

Fig. 9 summarises the roles of the three Cdk-cyclin complexes so far identified in the early *Xenopus* embryo: cyclin A-Cdc2, cyclin B-Cdc2 and cyclin E-Cdk2. Of these, only cyclin A-Cdc2 and cyclin E-Cdk2 provide SPF activity (Fig. 9, diagonal shading), whilst only cyclin A-Cdc2 and cyclin B-Cdc2 provide MPF activity (Fig. 9, grey shading). Once template DNA has been assembled into a nucleus in these extracts, DNA replication initiates almost immediately (Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987; Sheehan et al., 1988; Blow

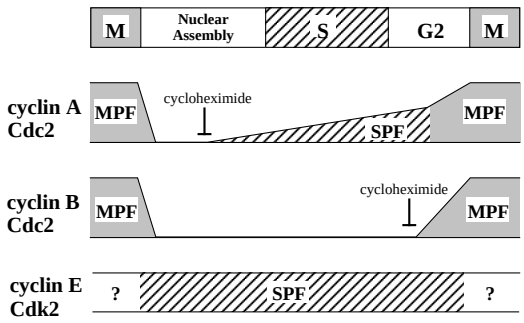

Fig. 9. Diagram showing the proposed roles of cyclins A, B and E in *Xenopus* eggs. The top panel shows the cell cycle events taking place in *Xenopus* eggs and egg extracts. MPF (stippling) and SPF (diagonal lines) activities of cyclin A-Cdc2, cyclin B-Cdc2 and cyclin E-Cdk2 at the different times are indicated. In the presence of cycloheximide, levels of cyclins A and B remain low, whilst levels of cyclin E are largely unaffected. See text for details.

and Sleeman, 1990). These extracts therefore lack a normal G_1 phase with nuclear assembly, rather than SPF, determining the timing of DNA replication (Fig. 9, 'nuclear assembly'). On exit from mitosis, cyclins A and B, but not cyclin E, are degraded (Minshull et al., 1990; Fang and Newport, 1991; Gabrielli et al., 1992; Rempel et al., 1995). If extracts are prepared in the presence of protein synthesis inhibitors such as cycloheximide, cyclin A is absent and all SPF activity is dependent on cyclin E1. Consistent with this, in the presence of cycloheximide DNA replication is inhibited by immunodepletion of either Cdk2 (Fang and Newport, 1991; Chevalier et al., 1995) or cyclin E1 (Jackson et al., 1995). Since cycloheximide does not affect progress through S-phase (Harland and Laskey, 1980; Blow and Laskey, 1988) this suggests that even in the absence of cyclin A, nuclear assembly rather than cyclin E-Cdk2 activity, still determines when DNA replication occurs.

During progress through a normal S-phase in *Xenopus* with ongoing protein synthesis, cyclin A-Cdc2 kinase is produced at levels of more than 3 nM (Minshull et al., 1990; Kobayashi et al., 1991), sufficient to provide a significant quantity of SPF. Since cyclin A can induce DNA synthesis even after nuclear assembly is complete, its role may be to induce initiation at any replicons that have not already fired. This would explain the observation that in translationally active Suc1-depleted extracts (which are likely to contain significantly more cyclin A than E), translation of Cdc2 stimulates more DNA synthesis than does translation of Cdk2 (Chevalier et al., 1995). Cyclin A is also required to delay mitosis if S-phase is incomplete (Walker and Maller, 1991), and so may function to mediate the progression from S-phase to mitosis. As cyclin A and cyclin B kinase levels build up later in the cell cycle, this becomes sufficient to generate MPF activity and induce entry into mitosis. Passage through mitosis is necessary for DNA to become relicensed for DNA replication in the next cell cycle, by causing nuclear envelope breakdown and RLF activation (Blow and Laskey, 1988; Blow, 1993; Kubota and Takisawa, 1993; Vesely et al., 1994; Chong et al., 1995, 1996). The co-ordination of these different cyclin dependent kinases is therefore capable of explaining the regulated replication of DNA during the cell cycle. Identification of the key substrates of these kinases is now of major importance.

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