Journal of Cell Science 109, 1173-1184 (1996)
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JCS3346

Xenopus cyclin E, a nuclear phosphoprotein, accumulates when oocytes gain the ability to initiate DNA replication

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SUMMARY

The capacity to initiate DNA replication appears during oocyte maturation in *Xenopus*. Initiation of S phase is driven by several components which include active cyclin/cdk complexes. We have identified three *Xenopus* cyclin E clones showing 59% amino acid identity with human cyclin E. The recruitment of cyclin E mRNA, like cdk2 mRNA, into the polysomal fraction during oocyte maturation, results in the accumulation of the corresponding proteins in unfertilized eggs. Cyclin E mRNA remains polyadenylated during cleavage and anti-cyclin E antibodies detect Xlcyclin E in embryonic nuclei at this time. Cdk2 protein is necessary for the phosphorylation of radiolabelled cyclin E added to egg extracts. Radiolabelled Xlcyclin E enters interphase nuclei and, though stable through interphase and mitosis, is not associated with condensed mitotic chromatin. In egg extracts, endogenous Xlcyclin E rapidly associates with nuclei before S phase and remains nuclear throughout interphase, becoming nucleoplasmic in G_2/prophase. Under conditions where initiation of replication is limiting in extracts, Xlcyclin E associates only with those nuclei that undergo S phase. These features are entirely consistent with the view that Xlcyclin E is required for initiation of S phase.

Key words: Cyclin, Cyclin dependent kinase, Maturation, Replication, Egg extract

INTRODUCTION

The cyclins are a large family of proteins first identified in marine invertebrates (Evans et al., 1983), where their expression oscillates through the cell cycle (Pines, 1992; Lew and Reed, 1992). They associate as regulatory subunits with cyclin-dependent kinase (cdks). The first characterized complex was cdc2/cyclin B which is conserved throughout evolution. Its activation triggers entry into mitosis and cyclin B destruction is necessary for exit from mitosis (King et al., 1995). Genetic analyses in yeasts indicated a requirement for cdc2 kinase at both the G_2/M transition and progress through G_1 to S phase (Hartwell et al., 1974; Nurse and Bisset, 1981; Piggott et al., l982). In budding yeast, three G_1 cyclins, (CLN1-3) are involved with Cdc28 kinase in passing START during G_1 (Hartwell et al., 1974; Reed, 1980, 1992). CLNl and CLN2 require CLN3 to be activated. This activation is necessary for the expression of two other cyclin genes, CLB5 and CLB6, which are important for the control of DNA replication (Epstein and Cross, 1992; Nasmyth, 1993; Schwob and Nasmyth, 1993). Several cyclins of higher eukaryotes (C, D and E) appear to be good candidates for roles in G_1 and S phase because they have been cloned by their ability to rescue the CLNl-3 triple mutant (Koff et al., 1991; Lew et al., 1991).

Studies of mammalian tissue culture cells have identified a regulatory point in G_l, called the restriction point, thought to be the equivalent of START in yeast (Pardee, 1989). Cells that progress past this point are committed to enter S phase and progress through to the next G_l. In multicellular eukaryotes, a family of cdc2-related cyclin dependant kinases has been identified (Paris et al., 1991; Meyerson et al., 1992). Both cdk2 and cdk3 have been implicated in the control of S phase initiation in mammalian cell lines (Van den Heuvel and Harlow, 1993; Tsai et al., l993) while cdk4 and cdk6 in association with D cyclins may trigger progression through the restriction point in G_l (Pagano et al., 1994; Resnitzky and Reed, 1995). In mammalian cell lines, overexpression of cyclin E shortens G_1 phase, suggesting that cyclin E is rate limiting for G_l progression (Ohtsubo and Roberts, 1993; Resnitzky et al., l994; Wimmel et al., l994). Cyclin E is associated with cdk2, and shows maximal Hl kinase activity at the G_l/S transition in mammalian cells (Koff et al., l992; Dulic et al., l992). Cyclin E/cdk2 has been found coprecipitated with the transcription factor E2F and p107, a protein of the pRb family (Lees et al., l992; reviewed by Nevins, 1992). The complex formed with cyclin E/cdk2 and E2F is cell cycle regulated, such that it is present primarily during G_1 and decreases as cells enter S phase (Lees et al., 1992). It has also

been suggested that cyclin E/cdk2 mediates phosphorylation of pRb to inhibit its interaction with E2F, permitting entry into S phase (Hinds et al., 1992). Anti-cyclin E antibody injection in mammalian cells blocks progression into S phase (Ohtsubo et al., 1995) while *Drosophila* cyclin E is required for S phase in both mitotic and endoreduplication cycles (Knoblich et al., l994; Sauer et al., 1995). In addition, cyclin E/cdk2 complex activity is targeted by inhibitors of G_1 progression (Koff et al., 1993; Peter and Herskowitz, 1994; Elledge and Harper, 1994). Thus cdk2/cyclin E may be required in G_1 for progression through the restriction point, transcription of components for S phase and G_2, and/or directly in initiation of DNA replication.

Direct involvement of cdk/cyclin complexes in S phase initiation can be assessed in *Xenopus* egg extracts where transcription is not required for cell cycle progression. *Xenopus* egg extracts assemble complex subcellular structures like the nucleus and recapitulate major events of the cell cycle with impressive fidelity (Lohka and Maller, 1985; Blow and Laskey, 1986; Hutchison et al., 1987, 1988; Murray et al., 1989). In embryos, the first twelve cell cycles are a succession of S and M phases without intervening gap phases and are driven entirely by maternally derived products. Depletion of cdk2 and cdc2 proteins from egg extracts using suc1-beads, or depletion of cdk2 protein using antibodies, inhibits S phase initiation (Blow and Nurse, 1990; Fang and Newport, 1991). We have recently demonstrated that newly translated cdk2 or cdc2 can promote S-phase entry in egg extracts depleted of both proteins and in extracts preferentially depleted of cdk2 protein, suggesting that the two cdks overlap in function for replication (Chevalier et al., l995). Both cyclin A and cyclin E restimulate replication in egg extracts in which cdk activity is inhibited by the inhibitor $p21^{Cip1}$ (Strausfeld et al., 1994). Cyclin E depletion also inhibits replication in egg extracts and this effect can be rescued by addition of cdk2/cyclin E or by cyclin A (Jackson et al., 1995). However, cyclin A does not appear to be essential for replication in extracts where cyclin A synthesis is blocked (Walker and Maller, 1991). To further characterise the cdk/cyclin complex(es) that could control the initiation of S phase in *Xenopus* embryos, we used human cyclin E cDNA as a probe to screen a *Xenopus* cDNA library to identify *Xenopus* cyclin E homologues. The same strategy has been used recently to identify a *Xenopus* cyclin E1 cDNA (Rempel et al., 1995). These authors showed that cyclin E associates with cdk2 but not cdc2 and has H1 kinase activity which is twofold higher in mitosis than interphase in egg extracts. We report here the identification of three Xlcyclin E cDNAs, E1A (corresponding to the clone identified by Rempel et al., 1995), E1B, having the same coding sequence and which may be an alternatively spliced version, and E2, having a slightly different coding sequence. The major (2.4 kb) transcript is poly-adenylated during oocyte maturation and cyclin E protein is synthesised at this time. Xlcyclin E is a nuclear phosphoprotein which associates with chromatin before S-phase and redistributes to the nucleoplasm after S-phase. Phosphorylation of Xlcyclin E depends on the presence of cdk2 protein. These properties of Xlcyclin E are consistent with a role in the control of S phase initiation in early *Xenopus* embryos.

MATERIALS AND METHODS

Xenopus cyclin E cDNAs: cloning and constructions

An unfertilized egg cDNA library prepared in lambda gt10 (Paris et al., l988) was screened with a human cyclin E cDNA provided by J. Roberts (Hu4; Koff et al., 1991). The human cyclin E cDNA (1.7 kb) was ^{32}P-labelled using a random primer DNA labelling kit (Pharmacia). Hybridization was carried out overnight at 55°C in 6× SSC (l× SSC is 150 mM NaCl, 15 mM sodium citrate), 5× Denhart's solution, 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml denatured salmon sperm DNA. Nitrocellulose membranes were washed for 5 minutes at 25°C in 2× SSC, 0.5% SDS and twice for 15 minutes at 25°C in 2× SSC, 0.1% SDS. Under these conditions, eight clones were isolated from 24,000 plaque forming units. Inserted cDNAs were released from phages by an *Eco*RI digestion and subcloned in Bluescript KS for DNA sequencing.

The coding region of Xlcyclin E2 was amplified by polymerase chain reaction (PCR) using oligonucleotides 5′-GCCCTGCTAGCC-CTGTGATAG-3′ (position 35-55) and 5′-GAGAACTCGAGGTCT-GCTGGATC-3′ (position 1,255-1,277). The PCR product of 1,227 bp digested by *Nhe*I and *Xho*I was inserted in respect of the open reading frame in PET21a (Novagen). The fusion protein contains two extra amino acids (A-S) after the methionine and eight at the COOH end (LEHHHHHH). The Xlcyclin E2 was removed from Bluescript KS and inserted into the *Eco*RI site of expression vector PET3cp (Studier and Moffat, 1986). The NH_2-terminal region of Xlcyclin E2 was amplified by PCR using oligonucleotides 5′-AGACCATGGCTGT-GATAAGAA-3′ (position 38-58) and 5′-CAAAGAAATCTTGTGC-TAGG-3′ (position 540-559) to generate a 442 bp fragment after digestion with *Nco*I and *Xba*I and subcloned between these sites of Pepex vector kindly provided by Dr J. Gautier (Gautier et al., 1991). The fragment of Xlcyclin E2 between the two *Xba*I sites (Fig. 1) was added to this construct at the *Xba*I restriction site. PET-Xlcyclin A1, PGEM-Xlcyclin B1, PET-Xlcyclin B2 clones were kindly provided by Dr Tim Hunt (ICRF, South Mimms, Herts, UK).

Northern blot analysis

Total RNA was extracted from stages I+II and stage V oocytes, unfertilized eggs and embryos 5 hours after fertilization, according to the method of Auffray and Rougeon (1980). Poly(A)$^+$ and (A)$^-$ RNAs were separated by oligo(dT) chromatography. Samples were separated on agarose gels containing 20 mM MOPS, 6% formaldehyde and blotted onto nylon membrane (Hybond, Amersham). Nylon filters were hybridized overnight at 42°C with the full length Xlcyclin E1B cDNA ^{32}P-labelled with a random primer DNA labelling kit (Pharmacia) in 50% formamide, 1% SDS, 10× Denhardt's solution, 10% dextran sulfate, 1% PPi, 1 M NaCl, 50 mM Tris-HCl, pH 8. Filters were extensively washed with 2× SSC containing 0.5% SDS at 65°C and autoradiographed. The blot was immediately hybridized with the Eg5 cDNA probe as previously described (Le Guellec et al., 1991) and autoradiographed again to detect the signals from both probes (Fig. 2).

Production of recombinant Xlcyclin E and antibodies

Full length Xlcyclin E1B cDNA subcloned in PET3cp was induced in BL21-DE3 strain and the protein, purified on acrylamide gels according to the method of Sambrook et al. (1989), used to generate rabbit serum 1 (seven injections of 200 μg). Rabbit serum 2 was generated with a C-terminal 24 amino acid synthetic peptide specific to Xlcyclin E1 (five 500 μg injections) produced in Eric Karsenti's laboratory (EMBL, Heidelberg). Antibodies were affinity purified on membrane saturated with bacterially expressed Xlcyclin E2 as described by Harlow and Lane (1988). Xlcyclin E2 (His)6 was produced in BL21-DE3 grown in 2× YT medium to an A_{660} of 0.7 and induced with l mM of iso-prenyl-beta-D-thiogalactoside (IPTG) for 3 hours at 22°C (Novagen).

In vitro translation, immunoprecipitation and in vitro dephosphorylation

PET Xlcyclin A1, PGEM Xlcyclin B1, PET Xlcyclin B2 and Pepex-Xlcyclin E2 clones were transcribed and translated in a nuclease treated rabbit reticulocyte lysate system containing [^{35}S]methionine (Amersham) according to the manufacturer's instructions (Promega). Reticulocyte lysate (5 μl) and 8 μl of anti-cyclin E serum 1 were diluted in 300 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA and 0.5% Np40, mixed for 4 hours at 4°C on a rotating wheel and immune complexes precipitated with CNBr-activated Sepharose 4B (Pharmacia) for 2 hours at 4°C. Immunoprecipitations of cytoplasm equivalent to 10 mature oocytes were carried out similarly. In vitro translated proteins and products of immunoprecipitation were analysed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed following Coomassie blue staining.

For dephosphorylation, proteins previously bound to suc1 beads were resuspended in 1 volume of potato acid phosphatase buffer (PAP buffer: 20 mM 2-[*N*-morpholino] ethane sulfonic acid, pH 6.5, 100 mM NaCl, 1 mM $MgCl_2$) and dialysed overnight at 4°C against this buffer. Then 2% SDS and 100 mM DTT were added and samples boiled for 2 minutes (Osmani et al., 1994). Proteins from 10 μl of original suc1 beads precipitate were treated with buffer or 0.5 unit of PAP (Sigma) supplemented or not with 50 mM Na_3PO_4 and analysed by western blotting.

Oocyte preparation, mRNA injection and in vitro maturation

Xenopus oocytes were manually defolliculated after 45 minutes incubation in OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.6) containing 2% collagenase (Sigma). Oocytes were injected with 20 nl of water or Xlcyclin E2 mRNA (0.5 mg/ml) according to the method of Matthews and Colman (1991). Batches of 50 injected oocytes were incubated for 2 hours at 19°C in 0.5 ml OR2 supplemented with 10 μCi of [^{35}S]methionine (Amersham) and then overnight in 4 ml OR2 with or without 10 μg/ml of progesterone (Sigma). Oocyte maturation was estimated by the appearence of a white spot at the animal pole. Protein extracts from oocytes and in vitro matured oocytes were prepared as previously described (Aoufouchi et al., 1995).

Xenopus egg extract preparation, depletion procedure and incubation

Fresh egg extracts were prepared according to the method of Hutchison et al. (1988). Samples (20 μl) of fresh egg extracts containing 2×10^3 demembranated sperm heads/μl of extract (Hutchison et al., 1988) and 1 μl of [^{35}S]Xlcyclin E were incubated at 21°C and portions analysed as described (Chevalier et al., 1995). Frozen egg extracts were supplemented and depleted with BSA or suc1 beads as previously described (Chevalier et al., 1995). Samples (15 μl) were supplemented with 0.8 μl containing 2×10^4 sperm heads together with 1 μCi of [^{32}P]dCTP or biotin-11-dUTP (Hutchison et al., 1988) and incubated for 3 hours at 21°C. Samples were taken for both protein and DNA analysis. To prepare RNase-treated extracts, fresh extracts were treated for 9 minutes at 21°C with 0.1 μg/ml RNase A (Boehringer), then supplemented with RNasin (Promega, 1 unit/μl of extract), incubated for an extra minute at 21°C and stored on ice. Then 50 μg/ml calf liver tRNA (Boehringer), 3 mM phosphocreatine, 7.5 μg/ml creatine phosphokinase and 50 μg/ml mRNA were added to RNase-treated extracts before 90 minutes incubation at 21°C. RNase-treated extracts were depleted using anti-cdk2 antibody as previously described (Chevalier et al., 1995).

Protein analysis and quantification of DNA synthesis

Samples of supernatant and washed beads were taken after depletion for comparison with untreated extract (Chevalier et al., 1995). cdks were revealed by western blotting using anti-PSTAIRE monoclonal antibody kindly provided by Dr M. Yamashita (Yamashita et al., 1991; Chevalier et al., 1995). Anti-Xlcyclin E blots were blocked for 2 hours at 22°C with BRBT (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20) containing 3% skimmed milk and 1.5% BSA, incubated overnight at 4°C in 1/1,000 antibody l or 1/500 antibody 2 in BRBT/milk/BSA, washed with BRB, incubated with alkaline phosphatase goat anti-rabbit IgG and developed (Harlow and Lane, 1988). Molecular mass markers were standard (45 kDa) or prestained (49.5 kDa; Bio-Rad). ^{32}P-labelled samples (10 μl cytoplasm per track) were analysed as previously described (Hutchison et al., 1988).

Cytological analysis

Samples were biotin-11-dUTP labelled, fixed and processed according to the method of Hutchison et al. (1988). Nuclei were incubated with anti-Xlcyclin E antibody l diluted 1/200 in phosphate buffered saline (PBS; Sambrook et al., 1989) containing 3% foetal calf serum overnight at 4°C. Total preimmune serum was diluted 1/100 in PBS. Second antibody was a goat anti-rabbit IgG coupled to fluorescein (Jackson Immunoresearch lab), diluted 1/100 in PBS containing 1% foetal calf serum. Biotin-dUTP was detected with Texas red streptavidin. Photographs were taken on a Zeiss Axiophot microscope using constant exposure times for each filter setting.

[^{35}S]radiolabelled Xlcyclin E was added to fresh egg extract (1 μl in 10 μl of extract) containing demembranated sperm heads (10^3/μl) and 4 μM biotin-11-dUTP and incubated and processed onto coverslips (Hutchison et al., 1988). After rinsing in PBS (37°C), 0.5% TCA (4°C) and PBS (37°C), coverslips were stuck on slides and processed for indirect immunofluorescence as above except that anti-cyclin E antibody (1/100) was incubated at room temperature for 4 hours. After washing in PBS and air drying, slides were dipped in nuclear emulsion (Ilford K5), dried and stored in the dark at 4°C, for 90 hours. Slides were developed in Kodak Dl9 for 3 minutes, rinsed in 0.5% acetic acid, fixed 5 minutes in 30% sodium thiosulfate, rinsed in deionised water and mounted.

Whole-mount immunocytochemistry on mid blastula embryos (stage 8) was performed as described by Hemmati-Brivanlou and Harland (1989). Purified anti-cyclin E antibody 1 was diluted 1/100 in PBST (PBS, 0.2% BSA, 0.1% Triton X100). Goat anti-rabbit IgG coupled to horseradish peroxidase (Jackson Immunoresearch Lab.) diluted 1/500 was used as the second antibody. Final detection was performed with diaminobenzimide after 90 minutes incubation at 4°C. Observations were made on an Olympus inverted microscope.

RESULTS

Isolation and characterisation of several Xlcyclin E cDNA clones

A probe derived from the human cyclin E cDNA (Koff et al., 1991) was used to screen a lambda gt10 *Xenopus* unfertilized egg cDNA library (Paris et al., 1988). Analysis of restriction maps of the cDNAs inserted in the clones which gave positive hybridization signals, revealed the presence of three cDNAs called Xlcyclin E1A, E1B and E2 (Fig. 1). The three Xlcyclin E clones presented in Fig. 1 were entirely sequenced on both strands. Three other incomplete Xlcyclin E clones and two Xlcyclin B2 like clones were also found (data not shown).

The Xlcyclin E1A clone has an open reading frame of 1,224 nucleotides and a 3′UTR of 948 nucleotides (accession number L43512). This clone corresponds to that previously isolated by Rempel et al. (1995) but has longer 5′ and 3′UTRs. The 3′UTR of Xlcyclin ElA is unlikely to be a cloning artifact because we have isolated other Xlcyclin E1A clones which are shorter at

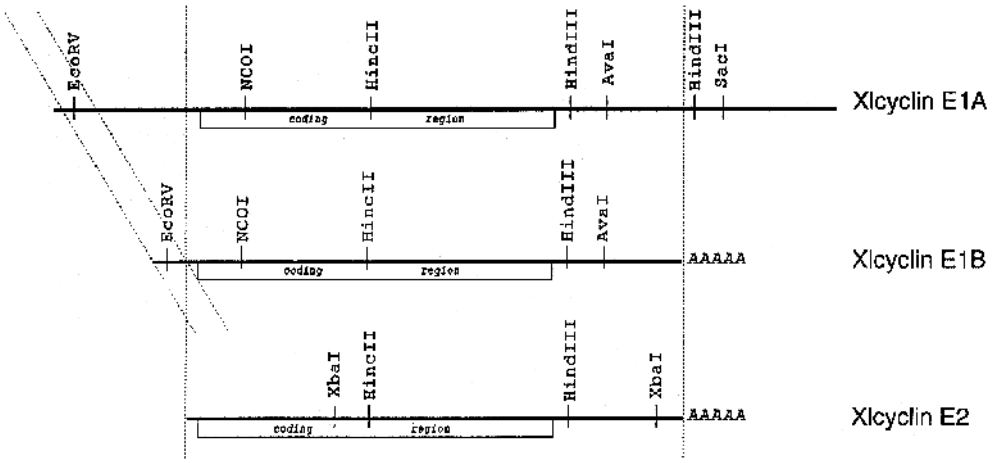

Fig. 1. Isolation and characterisation of several *Xenopus* cyclin E cDNA clones. Three *Xenopus* cyclin E clones isolated were 2,666, 1,822 and 1,700 nucleotides long (accession numbers L43512, Z13966 and L43513, respectively). Boxes indicate coding regions and thick lines represent the relative lengths of the three clones. AAAAA represents the poly(A) tail (60 adenyl residues for Xlcyclin E1B and 40 residues for Xlcyclin E2). The homologous sequences between the three clones are delimited by broken lines. Restriction sites used for restriction maps are indicated.

the 5′ end but have poly(A) tails (data not shown). Xlcylin E1A shows an unusual polyadenylation signal (ATTAAA, position 2,594-2,599) which could direct the polyadenylation of the corresponding RNA (Wickens, 1990). The Xlcyclin E1A and B clones share the same open reading frame and may be products of alternative splicing, though concensus splice sequences were not detected (Fig. 1). The Xlcyclin E2 clone differs from the E1 clones in its open reading frame (Fig. 1). All three clones contain cytoplasmic polyadenylation elements (TTTTAT) which have been reported to promote polyadenylation of other mRNAs during *Xenopus* oocyte maturation (Fox et al., 1989; McGrew et al., 1989). Southern blot analysis indicates that several genes coding for Xlcyclin E exist in the *Xenopus* genome (data not shown). This result is consistent with the identification of three cDNAs corresponding to mRNA synthesized from at least two different genes.

The predicted Xlcyclin E proteins are 408 amino acids long, slightly larger than human cyclin E (395) and rat cyclin E (396), but smaller than mouse cyclin E (491) and *Drosophila* cyclin EI and EII (601 and 708, respectively) (Koff et al., 1991; Tamura et al., 1993; Damjanov et al., 1994; Richardson et al., 1993). Comparison of Xlcyclin E1 with cyclin E2, human, rat, mouse and *Drosophila* E cyclins gave 91%, 59%, 57%, 57% and 37% identity, respectively. *Xenopus* E cyclins share sequence homology with a C-terminal region of human cyclin E identified as a PEST sequence (Lew et al., 1991). The key residues (prolines, serines and threonines) in the human sequence are conserved in the *Xenopus* sequence. As PEST sequences have been found preferentially in proteins with short half-lives (Rogers et al., 1986) *Xenopus* E cyclins may be unstable proteins.

Cyclin E mRNA expression

Total RNA extracted from oocytes, eggs and different stage embryos was subjected to northern blot analysis using the full length Xlcyclin E1B cDNA as a probe (Fig. 2). A major transcript of 2.4 kb was detected at all stages. In gastrulae, a minor transcript of 3.8 kb was also detected (data not shown). When a specific probe to Xlcyclin E1A was used, only the transcript at 3.8 kb was detected suggesting that this transcript was specific to Xlcyclin E1A (data not shown). Thus, the major 2.4 kb mRNA corresponds to Xlcyclin E1B and/or E2.

In order to analyse the polyadenylation status of Xlcylin E mRNA during oogenesis and early development, poly(A)$^+$ RNA was separated from poly(A)$^-$ RNA using oligo(dT) cellulose chromatography. Eg5 mRNA, which is polyadenylated during maturation and then deadenylated after fertilization was used as a control (Paris and Philippe, 1990; Le Guellec et al., 1991, and Fig. 2). *Xenopus* cyclin E mRNA, like Eg5 mRNA, was mostly present in the poly(A)$^-$ fraction from oocytes but became poly (A)$^+$ in eggs (Fig. 2). However, unlike Eg5 mRNA, Xlcyclin E mRNA remained in the poly (A)$^+$ fraction in embryos 5 hours after fertilization.

Identification of *Xenopus* cyclin E proteins

Two different polyclonal anti-Xlcyclin E antibodies have been produced: antibody 1 directed against the full length Xlcyclin E2 protein and antibody 2 against a specific carboxy-terminal peptide. On the western blot, antibody 1 detected recombinant Xlcyclin E2-(His)$_6$ (Fig. 3a) and immunoprecipitated a 45 kDa form of Xlcyclin E made in reticulocyte lysate, but not Xlcyclin A, Bl or B2 (Fig. 3b). We obtained the same results with antibody 2 (data not shown). Both purified antibodies detected several bands extending between 47 and 50 kDa in proteins affinity-precipitated with suc1 beads from unfertilized

Fig. 2. Northern blot analysis of total RNA, poly(A)$^+$ or poly(A)$^-$ fractions during oogenesis and early development. Samples of 10 μg of total RNA (T), 10 μg of poly(A)$^-$ RNA (–) and 200 ng of poly(A)$^+$ RNA (+) from stage I+II oocytes, stage V oocytes, unfertilized eggs (UFE) and embryos 5 hours after fertilization were separated on a formaldehyde agarose gel, transfered onto nylon membrane and hybridized firstly with full length Xlcyclin E1B cDNA probe. After autoradiography the blot was hybridized with a second probe corresponding to Eg5 cDNA (Le Guellec et al., 1991) and autoradiographed again to reveal both probes.

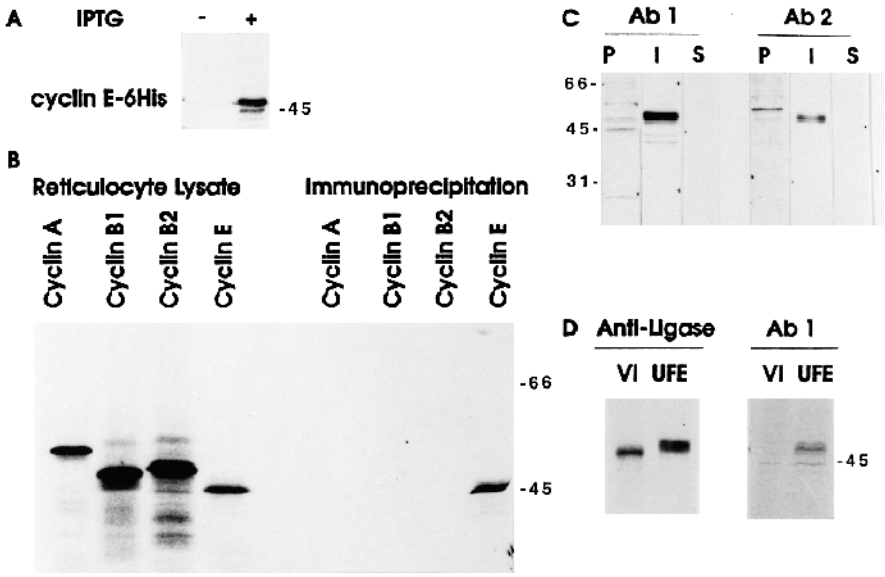

Fig. 3. Xlcyclin E is synthesized and postranslationally modified during oocyte maturation. Antibody characterization: (A) western blot of purified anti-Xlcyclin E antibody 1 on recombinant *Xenopus* cyclin E 6(His), induced (+) or not (–) with IPTG in BL21DE3. (B) Autoradiograph of *Xenopus* cyclin A, Bl, B2 and E3, produced in a rabbit reticulocyte lysate (left), which have been immunoprecipitated with anti-Xlcyclin E antibody 1 (Ab1, right). (C) Proteins precipitated on suc1 beads analysed by western blotting with preimmune serum (P), purified antibody (I) or purified antibody presaturated with recombinant Xlcyclin E2 (S) for both antibody 1 and 2. (D) Western blot of proteins from stage VI oocytes (Vl) and unfertilized eggs (UFE) probed with an anti-ligase antibody (Aoufouchi et al., 1995) or anti-cyclin E antibody 1. Size standards shown for each blot are in kDa.

egg extracts that were not observed with total preimmune sera (Fig. 3c). Antibody 1 detected two additional bands at 43 and 45 kDa that were not revealed with antibody 2 (Fig.3c) or with a third purified antibody directed against the full length Xlcyclin E (data not shown). In addition, these bands were not selectively precipitated by suc1 beads (see Fig. 9). These observations indicate that several forms of Xlcyclin E protein between 47 and 50 kDa are present in eggs. Xlcyclin E was not detected in oocytes before maturation (Fig. 3d). *Xenopus* DNA ligase I, which is present in oocytes and becomes phosphorylated during oocyte maturation (Aoufouchi et al., 1995) was detected on the same blot as a control for protein loading and in vitro maturation.(Fig. 3d). These results indicate that Xlcyclin E protein accumulates during oocyte maturation, when its mRNA also becomes polyadenylated.

cdk2 is necessary for Xlcyclin E phosphorylation

The capacity of *Xenopus* oocytes to induce post translational modification of Xlcyclin E was investigated by injecting in vitro transcribed Xlcyclin E2 mRNA into oocytes to force translation of Xlcyclin E. After incubation, oocytes contained several Xlcyclin E bands with apparent molecular masses of 45 to 49 kDa (Fig. 4a, lane Pg –). The same mRNA gave a product of 45 kDa when translated in rabbit reticulocyte lysate (Fig. 3b). However, following in vitro maturation essentially all the Xlcyclin E migrated at 49 kDa and the faster migrating

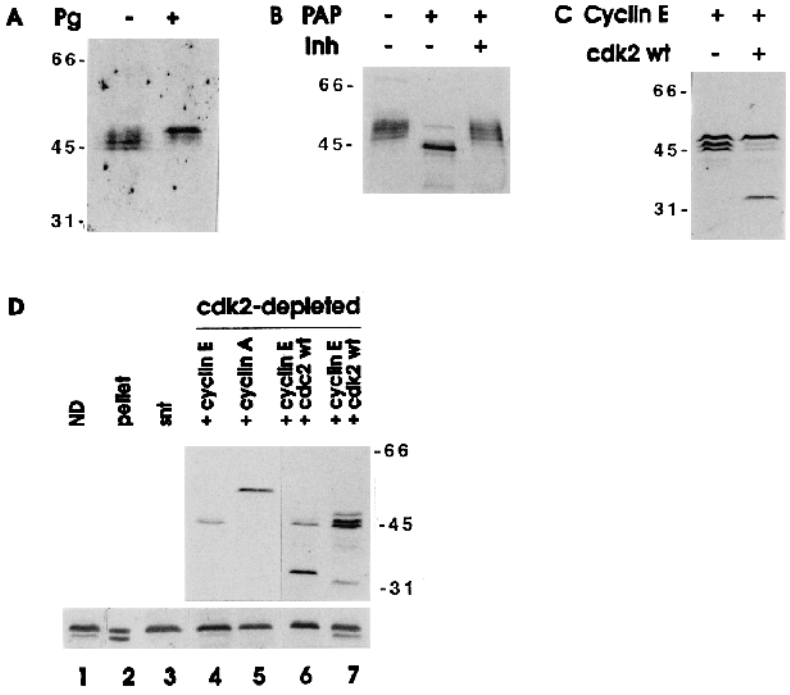

Fig. 4. cdk2 is necessary for Xlcyclin E phosphorylation in egg extract. (A) Stage VI oocytes were injected with Xlcyclin E2 mRNA and incubated with [^{35}S]methionine before in vitro maturation with progesterone (Pg). Proteins from oocytes incubated with (+) or without (–) progesterone were immunoprecipitated with anti-cyclin E antibody 1 and analysed by SDS-PAGE and autoradiography. (B) suc1 bead precipitates from egg extracts were treated with potato acid phosphatase (PAP) buffer, 0.5 unit of PAP, or with PAP supplemented with phosphatase inhibitor (Inh). Digestion products were analysed by western blotting with anti-Xlcyclin E antibody. (C) Xlcyclin E2 mRNA was translated with or without cdk2 mRNA in RNase-treated extract supplemented with [^{35}S]methionine. Protein samples were analysed by SDS-PAGE and autoradiography. Cdk2 protein migrated at 32 kDa. (D) RNase-treated extract was depleted with anti-cdk2 antibody beads before specific mRNAs were added together with [^{35}S]methionine and sperm heads. Non depleted extract (ND), pellet and supernatant (snt) after immunodepletion were analysed by western blotting using anti-PSTAIRE antibody (lower panel). Cdk2-depleted extracts supplemented with Xlcyclin E, Xlcyclin A, Xlcyclin E + cdc2 wt and Xlcyclin E + cdk2 wt mRNAs were analysed by western blotting using anti-PSTAIRE antibody (lower panel) and autoradiography (upper panel). Size standards shown for each blot are in kDa.

forms had disappeared (Fig. 4a, lane Pg +). This suggested that efficient post-translational modification of Xlcyclin E occurred during maturation but not in oocytes. That the modified forms of Xlcyclin E were phosphorylated is indicated by the sensitivity of suc1-precipitated egg cyclin E to potato acid phosphatase (PAP). A 45 kDa band was generated by PAP treatment and blocked by addition of a phosphatase inhibitor (Fig. 4b). Thus in unfertilized eggs, Xlcyclin E is a phosphorylated protein which is apparently modified at several sites.

Cyclin E has been shown to associate with cdk2 both in somatic cells (Lees et al., 1992) and in *Xenopus* embryos during early development (Jackson et al., 1995; Rempel et al., 1995). We have used RNase-treated extracts to determine whether cdk2 is required for Xlcyclin E phosphorylation. In such extracts containing endogenous cdk2 and Xlcyclin E proteins, added Xlcyclin E2 mRNA was translated into three radioactive forms, but when cotranslated with cdk2 mRNA, essentially only the slowest band was detected, suggesting that cdk2 stimulates Xlcyclin E phosphorylation (Fig. 4c). RNase-treated extracts lacking endogenous cdk2 were prepared by immunodepletion with anti-cdk2 antibody coupled to beads and cdks detected by western blotting with anti-PSTAIRE antibody (Fig. 4d; Yamashita et al., 1991; Chevalier et al., 1995). When synthesized in this extract, Xlcyclin E migrated as a single 45 kDa band. For comparison, Xlcyclin A migrated at 58 kDa (Fig. 4d, top panel). When cdk2 and Xlcyclin E mRNAs were cotranslated, Xlcyclin E protein appeared as three bands corresponding to the phosphorylated pattern observed in undepleted extracts. In contrast, when Xlcyclin E and cdc2 mRNAs were cotranslated, the phosphorylated forms of Xlcyclin E were not detected (Fig. 4d). Similarly, when cotranslated with an ATP-binding-site mutant cdk2 mRNA (cdk2.R33; Chevalier et al., 1995), Xlcyclin E protein was not modified (data not shown). Thus active cdk2 protein is necessary for Xlcyclin E phosphorylation in egg extracts.

Xenopus cyclin E is not degraded in egg extracts

The stability of Xlcyclin E during the cell cycle was tested by adding radiolabelled Xlcyclin E, made in rabbit reticulocyte lysate, to fresh egg extracts. Phosphorylation of added 45 kDa Xlcyclin E was detected by 10 minutes (Fig. 5a). Essentially all of the added cyclin E is modified during 90 minutes incubation whereas the apparent molecular mass of Xlcyclin A did not vary (Fig. 5a). Since only phosphorylated forms of cyclin E are precipitated with suc1 beads, and cdk2 is required for cyclin E phosphorylation (Fig. 4), this suggests that the majority of added cyclin E associates rapidly with cdk2 in the extract. [^{32}P]dCTP pulse-labelling detected two rounds of DNA replication separated by mitosis during 200 minutes incubation (Fig. 5b). By 15 minutes modification of added cyclin E was again detected, appearing as 46.5 and 48 kDa bands on a high resolution gel (Fig. 5c). During mitosis, further slower migrating forms appeared (Fig. 5c, 115, 135 minutes) and remained during the second S phase. No sudden loss of radiolabelled cyclin E was detected at any point during the incubation. Thus, in vitro translated Xlcyclin E was phosphorylated, but not transiently unstable through replication or mitosis in egg extracts. This suggests that endogenous Xlcyclin E, the level of which does not change from fertilization to MBT (data not shown), is not rapidly degraded in each cell cycle.

Nuclear localisation of *Xenopus* cyclin E in vivo and in vitro

After fertilization, *Xenopus* eggs undergo twelve rapid cell cycles without G_1 and G_2 phases. Xlcyclin E localisation in whole mount stage 8 embryos was investigated using purified anti-Xlcyclin E antibodies (Fig. 6). Antibody l gave a strong nuclear signal in embryonic cells (Fig. 6B), that was not observed with either preimmune serum or antibody 1 preabsorbed with recombinant Xlcyclin E2 (Fig. 6A and C). Similar results were obtained with antibody 2 and also on stage 6 embryos (data not shown). Thus Xlcyclin E protein appears to be nuclear in early embryos as it is in serum stimulated somatic cells (Pagano et al., 1994; Ohtsubo et al., 1995).

Immunocytological studies were also performed on nuclei

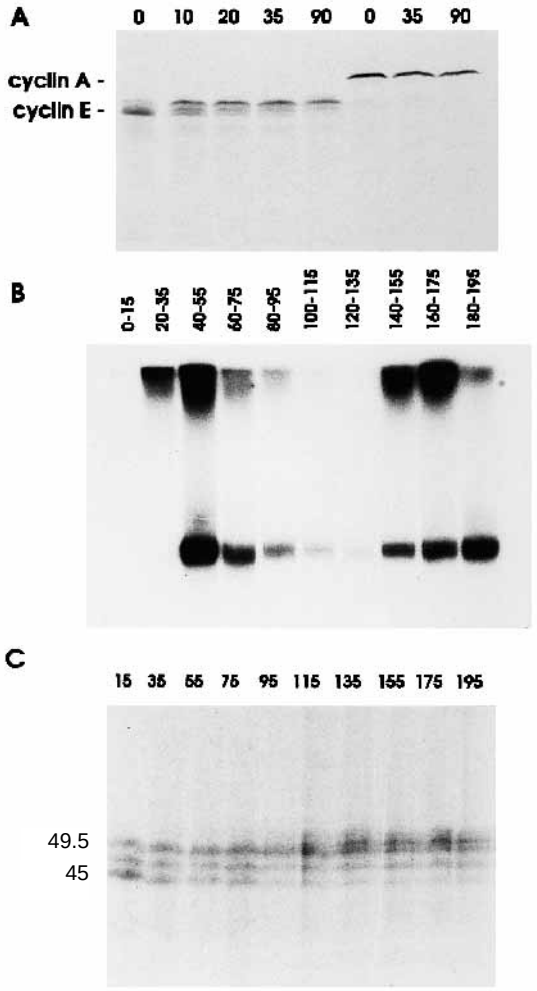

Fig. 5. Xlcyclin E protein is post translationally modified and remains stable when added to egg extracts. (A) ^{35}S-radiolabelled Xlcyclin E or Xlcyclin A translated in vitro were added separately to fresh egg extract with cycloheximide and sperm heads, and incubated. Radiolabelled proteins were autoradiographed after separation on SDS-PAGE. (B) Analysis of replication through two cell cycles. [^{32}P]dCTP was added every 20 minutes to extract supplemented with Xlcyclin E translated in reticulocyte lysate and sperm heads, incubated for a further 15 minutes and analysed on a 1% agarose gel and autoradiographed. (C) Parallel samples were analysed by SDS-PAGE and autoradiographed. Positions of 45 and 49.5 kDa molecular mass markers are indicated.

formed in cycling egg extracts pulse-labelled with biotin-11-dUTP for 10 minutes just before fixing. At the start of the incubation, sperm heads added to the egg extract did not stain with anti-cyclin E antibody 1 (Fig. 7, 0). After 10 minutes incubation, when the chomatin had partly decondensed but before replication had started, clear uniform nuclear Xlcyclin E staining was detected (Fig. 7, 10). Nuclear Xlcyclin E and chromatin staining were both uniform during S phase when replication occurs (Fig. 7, 30). As replication terminates, nuclei swell and chromatin condenses into fibres typical of G_2/prophase (Hutchison et al., 1988). At this time (60-80 minutes) Xlcyclin E staining was distributed throughout the nucleoplasm and not specifically chromatin associated (Fig. 7, 80). After nuclear envelope breakdown, condensed chromatin was observed without Xlcyclin E staining (Fig. 7, 110). When mitosis was completed, a second S phase started and Xlcyclin E was again detected in replicating nuclei (Fig. 7, 130). Antibody 2 gave similar staining patterns (data not shown). These results indicate that Xlcyclin E enters decondensing nuclei rapidly before initiation of DNA replication and apparently redistributes to the nucleoplasm in G_2.

Xlcyclin E is not detected with mitotic chromatin. However, Xlcyclin E may be present, but not accessible to antibody in condensed chromatin. We have used radiolabelled Xlcyclin E, which is rapidly phosphorylated in egg extracts (Fig. 5), to follow nuclear localisation. Radiolabelled Xlcyclin E, biotin-11-dUTP and sperm heads were added to egg extracts and incubated for different times before autoradiographic analysis. Like the endogenous protein, radiolabelled Xlcyclin E associated rapidly with decondensing pronuclei before replication started (data not shown) and stayed in the nucleus during S and G_2 phases (Fig. 8, left). Xlyclin E was not detected associated with mitotic chromosomes (Fig. 8, right). When Xlcyclin E was added for 10 minutes during S phase or in G_2 before fixation, nuclei were strongly radiolabelled (data not shown). Thus in vitro translated Xlcyclin E protein is able to translocate into nuclei during S and G_2 phases but is excluded from chromatin in mitosis.

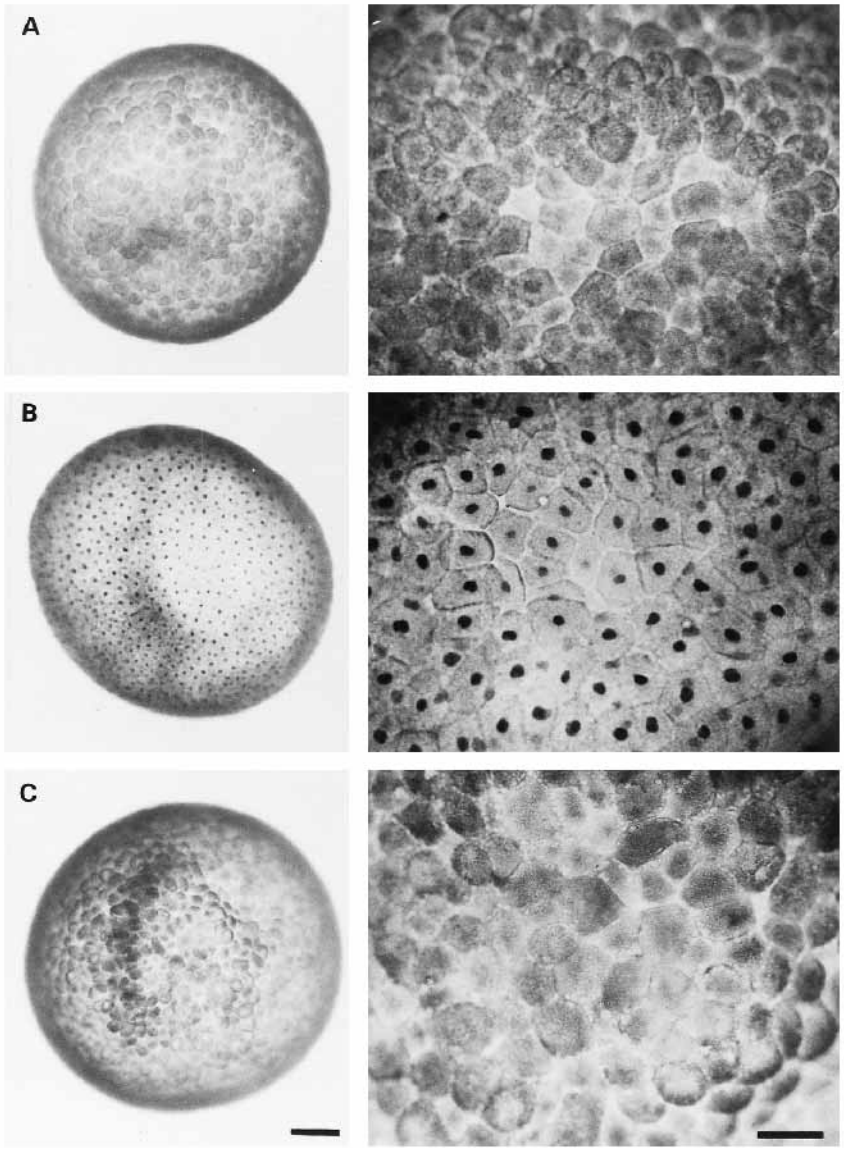

Fig. 6. Xlcyclin E is localised in nuclei of *Xenopus* embryos. Whole-mount embryos at mid blastula stage were incubated with (A) preimmune serum 1, (B) anti-Xlcyclin E antibody 1 and (C) anti-Xlcyclin E antibody 1 preabsorbed with recombinant Xlcyclin E2. Detection was performed with a goat anti-rabbit IgG coupled to horseradish peroxidase and revealed with diamino benzidine. Similar results were obtained with antibody 2 and on stage 6 embryos. Bars: lower magnification, 50 μm; higher magnification, 20 μm.

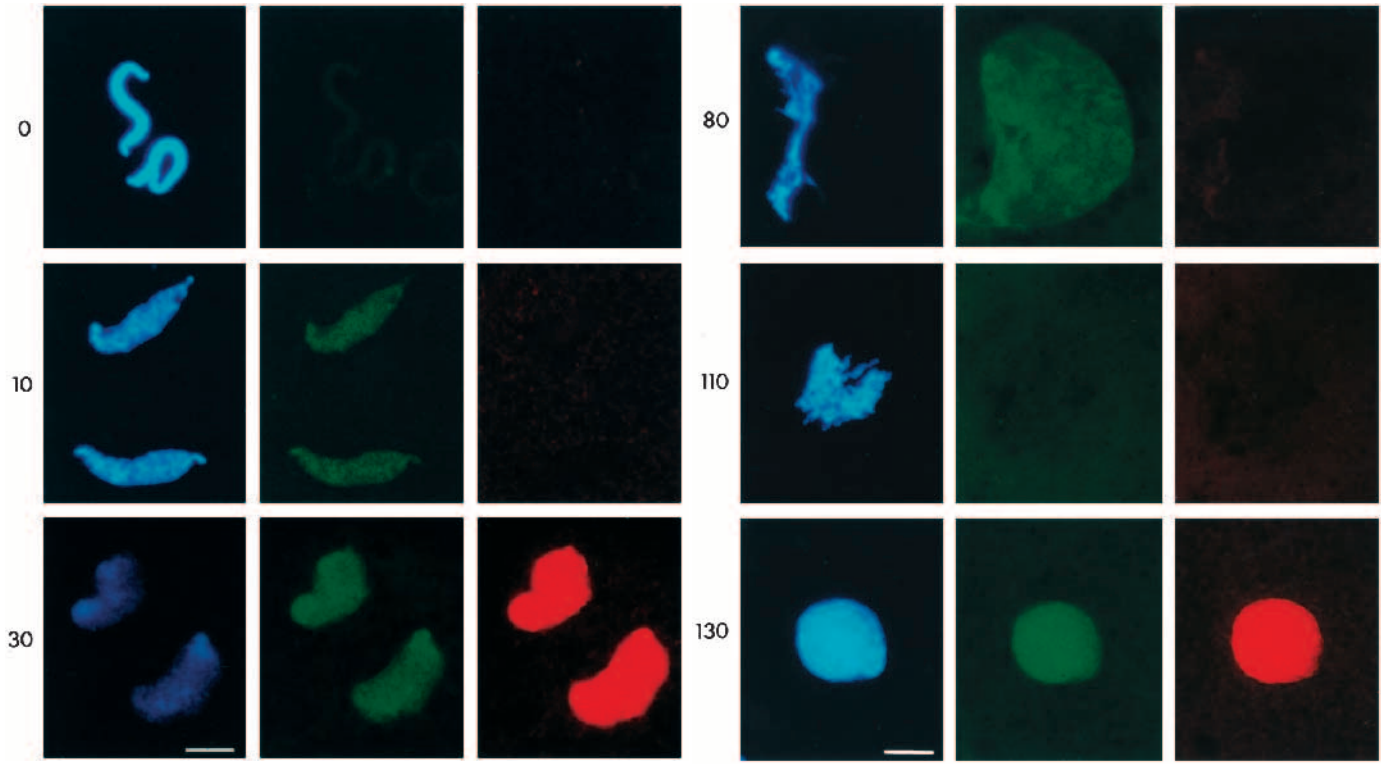

Fig. 7. *Xenopus* cyclin E is nuclear before replication starts. Fresh egg extract containing sperm heads was pulse labelled with biotin-11-dUTP at 10 minute intervals during two cycles. Nuclei were fixed at the times (in minutes) indicated and samples analysed by indirect immunofluorescence microscopy (blue, DAPI staining of DNA; green, FITC conjugated anti-rabbit IgG, detection of Xlcyclin E; red, Texas red streptavidin, detection of replicating nuclei). Neither preimmune serum nor antibody presaturated with recombinant Xlcyclin E produced any nuclear staining (data not shown). Bar, 3 μm.

Nuclear localisation of Xlcyclin E correlates with replication in suc1-depleted egg extracts

Cyclin E protein is involved in initiation of DNA replication both in *Xenopus* egg extracts and in somatic cells (Jackson et al., 1995; Ohtsubo et al., 1995). Depletion of cdks from egg extracts with suc1 beads severely inhibits replication (Blow and Nurse, 1990; Fang and Newport, 1991; Chevalier et al., 1995). Under these conditions a small fraction of nuclei still undergo S phase, implying that initiation of replication is rate-limiting in cdk-depleted extracts (Chevalier et al., 1995). If the reduced levels of cdk2/cyclin E in suc1-depleted extracts was limiting initiation, cyclin E localisation should be restricted to the small fraction that replicated. Western blots confirmed that phosphorylated Xlcyclin E was depleted from the extract by suc1 beads but not by control BSA beads (Fig. 9, lanes suc1 pellet and BSA pellet). The cross-reacting 45 kDa band detected by antibody 1 remains predominantly in the supernatant. Following incubation of these extracts with sperm pronuclei, replication and Xlcyclin E localisation were assessed cytologically. In BSA depleted extracts, 93% of nuclei replicated strongly and showed clear Xlcyclin E labelling (Fig. 9B, BSA depl.+). In suc1-depleted extracts, as previously observed (Chevalier et al., 1995), only 9% of nuclei had clearly undergone S phase, while 59% were unreplicated (data not shown). These unreplicated nuclei lacked clear Xlcyclin E staining (Fig. 9B). In contrast, the few nuclei that had replicated showed clear labelling with either antibody 1 or 2, indicating that this restricted set of nuclei contained Xlcyclin E (Fig. 9B and data not shown). Thus, in frozen extracts depleted with suc1-beads, nuclear localisation of Xlcyclin E correlated closely with those nuclei which replicated their DNA.

DISCUSSION

Cyclin E mRNA becomes polyadenylated and phosphorylated protein accumulates during maturation

We have identified three cDNAs corresponding to *Xenopus* cyclin E. Coding regions of Xlcyclin ElA/B and Xlcyclin E2 differ by 9% in the amino acid sequence. The Xlcyclin E1 cDNA recently described (Rempel et al., 1995) is essentially identical to part of the Xlcyclin E1A cDNA presented in this paper but is shorter in both the 5′ and 3′UTRs. Using a probe to the coding region they detected the major 2.4 kb transcript(s) but using a specific probe to Xlcyclin E1A we found only a minor 3.8 kb mRNA which appears at the beginning of oogenesis (data not shown). The major 2.4 kb mRNA corresponds to Xlcyclin E1B and/or E2 cDNAs. Though the 5′UTR sequences of Xlcyclin E1 might be products of alternative splicing, we have not detected any cDNA with a coding region altered by alternative splicing, unlike the observations made for *Drosophila* and humans (Richardson et al., 1993; Sewing

et al.,1994; Ohtsubo et al., 1995). In *Drosophila*, Dmcyclin E type I and II proteins differ in their amino-terminal regions through alternative splicing. Dmcyclin E type I protein is only synthesized zygotically while the type II protein is maternally supplied (Richardson et al., 1993). In humans, two cyclin E forms differing by a fifteen amino-acid extension of the N terminus are detected in cultured cells (Ohtsubo et al., 1995). Sequence analysis of the three *Xenopus* cyclin E cDNA clones indicates that each mRNA contains both a putative nuclear polyadenylation signal and a CPE, consistent with the polyadenylation of the predominant mRNA during oocyte maturation. There is a correlation between the adenylation status of an mRNA and its association with polysomes (Paris and Philippe, 1990). The observation that Xlcyclin E mRNA is mostly in the poly(A)$^-$ fraction during oogenesis and found in the poly(A)$^+$ fraction both in the unfertilized eggs and during early development suggests that synthesis of Xlcyclin E starts during maturation and is still active after fertilization.

Like Xlcyclin E mRNA, cdk2 (Egl) mRNA also becomes polyadenylated during oocyte maturation (Paris et al., 1991). This is consistent with the accumulation of both Xlcyclin E and cdk2 proteins during oocyte maturation, leading to the formation of active cyclin E/cdk2 complexes (Gabrielli et al., 1992; Chevalier et al., 1995; Rempel et al., 1995). Though stage VI oocytes contain stockpiles of many proteins necessary for DNA replication (Davidson, 1986), the oocyte has the ability to initiate replication only after maturation, when both Xlcyclin E and cdk2 proteins have been synthesised. This may

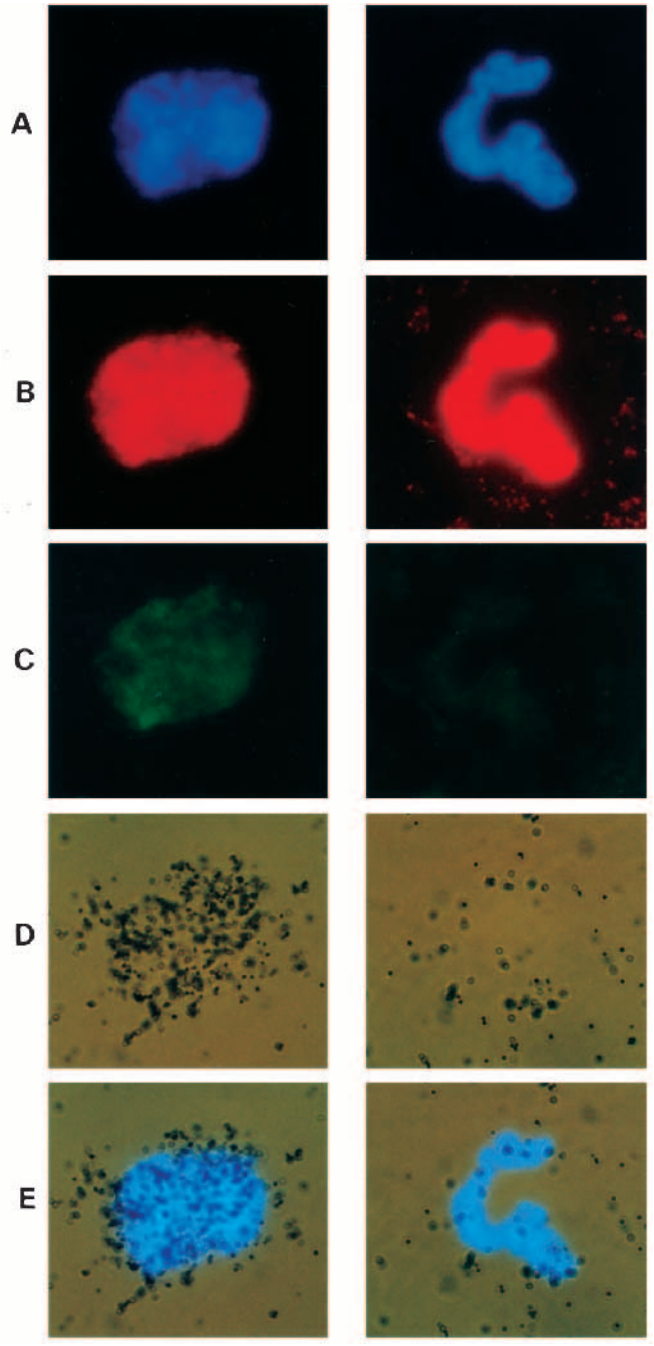

Fig. 8. Exogenous Xlcyclin E accumulates in the nucleus in S phase and G_2 and is not associated with condensed chromatin during mitosis. Radiolabelled Xlcyclin E and biotin-11-dUTP were added to a fresh egg extract containing sperm heads. Nuclei were fixed in G_2 (left) or M phases (right). (A) DAPI, staining of DNA; (B) Texas red streptavidin, detection of replication; (C) FITC-conjugated anti-rabbit IgG, detection of Xlcyclin E; (D) Bright field, detection of ^{35}S-labelled exogenous Xlcyclin E; (E) dual bright field and DAPI image. Control radioactive reticulocyte lysate lacking cyclin E, when added to extracts did not produce nuclear-localised autoradiographic grains.

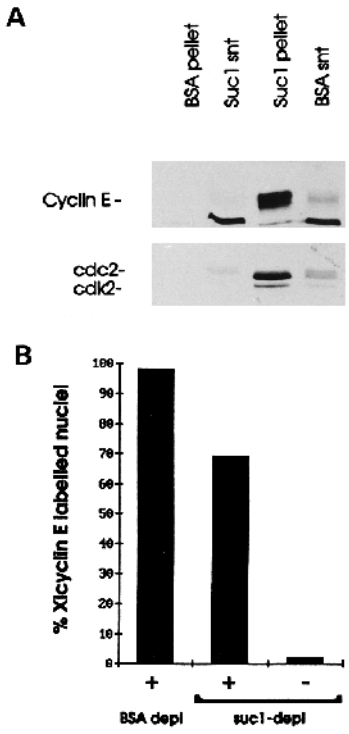

Fig. 9. Nuclear localisation of Xlcyclin E correlates with replication in suc1-depleted extract. Following depletion with suc1- or BSA-beads, extract supernatants were incubated with sperm heads and biotin-11-dUTP for 3 hours. (A) Detection of cdks with anti-PSTAIRE antibody and Xlcyclin E with Ab1 in supernatant (snt) and pellet fractions taken immediately after depletion. (B) Nuclei with strong (+) or no (–) biotin incorporation were scored for positive Xlcyclin E labelling. For the BSA treated extract, 128 nuclei were scored. For the suc1-treated extract 118 and 518 nuclei were scored with strong and no biotin, respectively.

help to ensure that the oocyte, during its growth, can accumulate large quantities of replicative enzymes without initiating replication. We suggest that the immature oocyte lacks the capacity to initiate replication in part because Xlcyclin E is absent and consequently active cdk2/cyclin E complexes are not formed. This contrasts with B cyclins which are present in inactive complexes with cdc2 in full grown oocytes (Kobayashi et al., 1991). Both the presence of cdc2/cyclin B and the absence of cdk2/cyclin E may ensure that endoreduplication does not occur during the prolonged growth of the prophase oocyte.

Purified antibodies to Xlcyclin E, which recognised the 45 kDa in vitro translation products of the Xlcyclin E clones, detected several phosphorylated forms of Xlcyclin E in unfertilized eggs but not in oocytes. These phosphorylated forms were coprecipitated with suc1 beads and were converted to a 45 kDa form by phosphatase treatment. Phosphorylation of cyclin E required the presence of cdk2 (Fig. 4). In addition, immunoprecipitates of active cdk2/cyclin E contain phosphorylated forms of Xlcyclin E (Jackson et al., 1995; Rempel et al., 1995). These observations may be explained if cdk2 must be bound to Xlcyclin E for the latter to be phosphorylated and the complex active.

Cyclin E stability

The mitotic cyclins accumulate prior to mitosis but are rapidly degraded during mitotic exit, leading to the loss of cdc2 kinase activity. This selective degradation requires an N-terminal destruction sequence which is present in A and B-type cyclins but is not detected in cyclin E. However, the E cyclins contain PEST sequences, which are found frequently, but not exclusively, in proteins with short half-lives (2 hours; Rogers et al., 1986). All vertebrate E cyclins have a single PEST sequence at the C terminus, while *Drosophila* cyclin E type I and II have 7 and 11, respectively, found at both N and C termini (Richardson et al., 1993). We observed that radiolabelled Xlcyclin E was stable through mitosis in egg extracts and, indeed, was not transiently unstable at any time in these extracts. These observations suggest that the half life of Xlcyclin E is longer than one cell cycle in early embryos. In syncitial *Drosophila* embryos, cyclin E protein is present throughout the nuclear division cycle, while in later embryos it is expressed periodically only in proliferating cells with a G_1 phase and in endoreduplication cycles (Richardson et al., 1993; Sauer et al., 1995). This transient expression is regulated by negative feedback in endoreduplication cycles, but not in mitotic cycles (Sauer et al., 1995). Cyclin E expression in mammalian cells in culture is also periodic such that cyclin E/cdk2 kinase activity is maximal in late G_1 and declines through S and G_2 phases (Dou et al., 1993; Ohtsubo et al., 1995). In rat cells released from serum starvation, increased expression of both cyclin E and cdk2 in G_1 appears, in part, to reflect increased mRNA stabilisation (Oda et al., 1995). Thus different modes of regulation could contribute to cyclin E protein levels in different cell cycle contexts. Though it has not been measured, a half-life of 3-4 hours for cyclin E protein would accomodate both the rise and fall in protein level seen in the 16 hours between G_1 and mitosis in serum stimulated cells (Ohtsubo et al., 1995) and the presence of cyclin E throughout the short cycle in egg extracts and early *Drosophila* embryos. Presumably the presence of substantial levels of Xlcyclin E and active Xlcyclin E/cdk2 complexes (Rempel et al., 1995) at exit from mitosis allows S phase entry without an intervening G_1 and without further protein synthesis.

Cyclin E localisation and DNA replication

Using purified anti-Xlcyclin E antibody, we have shown that Xlcyclin E is nuclear in *Xenopus* embryos and that Xlcyclin E localises in the nucleus before replication occurs in egg extracts. This pattern is entirely consistent with a requirement for Xlcyclin E in the initiation of S phase. Depletion of cdk2/cyclin E complexes using either suc1-beads or cdk2 or Xlcyclin E antibodies inhibits initiation of S phase (Blow and Nurse, 1990; Fang and Newport, 1991; Chevalier et al., 1995; Jackson et al., 1995). Using extracts treated with suc1 beads to remove phosphorylated cyclin E and cdk2, we observed that unreplicated nuclei lacked Xlcyclin E localisation. Only those few nuclei which managed to replicate extensively showed clear Xlcyclin E staining (Fig. 9). These observations suggest that nuclear uptake of Xlcyclin E is essential for replication and, since cdk2 is required for cyclin E phosphorylation, it is this complex that enters nuclei.

In serum-stimulated human cultured cells, cyclin E protein accumulates in late G_1 nuclei and remains in the nuclei during S phase though the levels are reduced in mid to late S phase. Early G_1 nuclei lack cyclin E staining (Ohtsubo et al., 1995). These changes correlate with changes in cyclin E/cdk2 kinase activity. In contrast, in egg extracts, Xlcyclin E is detected in nuclei throughout interphase and does not obviously decrease in level as S phase proceeds (Fig. 5). It does relocate predominantly to the nucleoplasm as chromatin condenses in G_2/prophase. In addition, Xlcyclin E/cdk2 complexes are active through interphase and mitosis (Rempel et al., 1995). The continued presence of active cdk2/Xlcyclin E in G_2, as in *Drosophila* early embryos (Sauer et al., 1995), is not sufficient to induce rereplication. Presumably the chromatin-associated targets for cdk2/cyclin E binding in G_1 and S phases are no longer available in G_2. *Xenopus* egg extracts should allow identification of substrates of this complex, particularly as DNA replication occurs without transcription or translation.

We much appreciate the gifts of antibodies from Drs S. Aoufouchi, M. Yamashita and T. Hunt, and cDNA clones from Drs J. Gautier, A. Koff and J. Roberts. We thank the Fondation pour la Recherche Médicale for a short-term fellowship (S.C.). This work was supported by grants from the Ligue Départementale d'Ille et Vilaine de recherche sur le cancer and the French government (GREG) (M.P.), and the Cancer Research Campain (C.F.). The nucleotide sequences reported for Xlcyclin E1A, B and E2 have been submitted to the GenBank™/EMBL data bank with accession numbers L43512, Z13966 and L43513, respectively.

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(Received 17 October 1995 - Accepted 8 March 1996)