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# Study of the cell cycle-dependent assembly of the DNA pre-replication centres in *Xenopus* egg extracts

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**RPA is a cellular, three-subunit, single-stranded (ss) DNA binding protein, which assists T-antigen in the assembly of the pre-priming complex in the SV40 replication system. By immunodepletion and complementation, we have identified RPA as an essential factor for cellular DNA replication in *Xenopus* extracts. RPA assembles post-mitotically on the decondensing chromosomes into numerous subnuclear pre-replication centres (preRCs) which serve, upon formation of the nuclear membrane, as RCs for the initiation of DNA synthesis. By a variety of experiments including the use of isolated components, we demonstrate that an inactive cdc2–cyclin B kinase complex is essential to allow post-mitotic assembly of the preRCs. In contrast, the active cdk2–cyclin A kinase does not impede or facilitate the assembly of preRCs. Digestion analysis using the single-strand-specific P1 nuclease as well as competition experiments with ssDNA, reveal that replication-associated unwinding of the DNA, assisted by RPA, requires the formation of the nuclear membrane. The p21 cdk-interacting protein Cip1 appears to inhibit DNA replication prior to the unwinding DNA step, but after assembly of preRC and nuclear reconstruction.**

*Key words:* cdc2 kinase/DNA unwinding/replication centres/RPA

## Introduction

Cell-free extracts derived from *Xenopus* eggs allow efficient replication of chromosomal DNA *in vitro* (reviewed by Almouzni and Wolffe, 1993). In low-speed supernatant (LSS) extracts made from crushed *Xenopus* eggs, exogenously added demembranated sperm nuclei decondense, acquire a nuclear membrane–lamina structure and subsequently replicate their DNA only once before mitosis (Lohka and Masui, 1983, 1984; Blow and Laskey, 1986; Hutchison *et al.*, 1987). Assembly of an intact nuclear structure has been shown to be a prerequisite for the initiation of replication on added chromosomal DNA using this egg extract system. Removal of membrane vesicles (high-speed supernatant, HSS) or nuclear lamins from an LSS yields extracts which are defective in DNA replication (Newport, 1987; Sheehan *et al.*, 1988; Newport *et al.*, 1990; Leno and Laskey, 1991; Meier *et al.*, 1991; Jenkins *et al.*, 1993). Continuous nuclear transport also seems to be needed for nuclear DNA replication, since wheat germ agglutinin, an inhibitor of nuclear transport, blocks replication (Cox, 1992).

Biochemically, eukaryotic DNA replication is best understood in the SV40 *in vitro* replication system. A number of cellular factors have been identified which are essential for the replication of DNA containing an SV40 origin in conjunction with the SV40 large T-antigen (for reviews see Challberg and Kelly, 1989; Stillman, 1989). Replication protein A (RPA, also called RF-A) has been identified as a factor implicated in the initiation of SV40 replication *in vitro*. RPA is composed of three subunits of 70, 32 and 14 kDa, and binds preferentially to single-stranded (ss) DNA (Fairman and Stillman, 1988; Wold and Kelly, 1988; Kim *et al.*, 1992). RPA cooperates with SV40 large T-antigen and facilitates the generation of a ssDNA region prior to the initiation of SV40 DNA synthesis (Fairman and Stillman, 1988; Kenny *et al.*, 1989; Wold *et al.*, 1989).

We have reported that in *Xenopus* egg extracts, the 70 kDa subunit of RPA (p70) is associated with subnuclear foci (~200) which we call pre-replication centres (preRCs). The punctated preRCs exist transiently, forming post-mitotically on the decondensing chromosomes prior to the assembly of the nuclear membrane–lamina complex. The preRCs are thought to comprise an assembly of a large number of pre-initiation replication complexes poised for initiation at discrete subnuclear regions prior to nuclear reconstruction (Adachi and Laemmli, 1992). Upon assembly of the nucleus, initiation of DNA synthesis commences, initially confined to the preRCs, resulting in a punctated replication pattern consisting of RCs which have been previously observed (Nakamura *et al.*, 1986; Mills *et al.*, 1989; Nakayasu and Berezney, 1989; Fox *et al.*, 1991; Leno and Laskey, 1991; O' Keefe *et al.*, 1992). Ongoing DNA synthesis induces a redistribution of p70: its punctated staining pattern disappears and p70 becomes homogeneously distributed over chromatin. (Mitotic chromosomes are negative for p70.)

To understand the regulatory mechanisms that control the S phase of the cell cycle, it is necessary to examine precisely the cell cycle stages at which the replication machinery assembles. A biochemical characterization of the preRCs could provide an avenue towards this goal. As an initial step along this line, we demonstrate biochemically in the *Xenopus* egg extract system a requirement for RPA in cellular DNA replication and we provide evidence that RPA-dependent unwinding of DNA requires the assembly of the nuclear membrane. We also observe that the assembly/disassembly of RPA into preRCs is regulated by cyclin-dependent kinases.

## Results

### *Purification of RPA from Xenopus egg extracts*

We examined the requirement for RPA in chromosomal DNA replication by immunodepletion of *Xenopus* egg extracts and subsequent complementation with purified RPA. For this purpose, we set out to purify RPA from *Xenopus* egg extracts and to raise high titre antibodies against this complex to permit efficient immunodepletion.

RPA is a major protein complex in egg extracts which resists elution by 2 M salt from DNA–Sepharose and is thought to contain ssDNA regions (Adachi and Laemmli, 1992). RPA was eluted from DNA–Sepharose by 1.5 M NaCl plus 50% ethyleneglycol (Fairman and Stillman, 1988) and further purified by hydroxylapatite chromatography (Figure 1). The resulting fraction was nearly homogeneous, consisting of three proteins of 70, 32 and 14 kDa (p70, p32 and p14, respectively), similar to RPA complexes from human and budding yeast (Figure 1, lane 3; Fairman and Stillman, 1988; Wold and Kelly, 1988; Brill and Stillman, 1991). In this preparation, p32 migrated as multiple bands, which is probably due to phosphorylation as we used mitotic egg extracts as starting material; p32 is known to be phosphorylated upon the $G_1$/S transition and until the end of mitosis (Din *et al.*, 1990; Fang and Newport, 1993; Y.Adachi, unpublished result). By scanning the gel of the final RPA fraction (called purified RPA), we estimated a >90% purity and a molar ratio of ~1:1:1 for the three subunits (lane 3).

The specific antiserum raised against purified RPA reacted strongly with p70 and p32, and weakly with p14 in immunoblots (Figure 2A, lane 2). The rabbit anti-RPA antibodies also detected p70 and p32 in crude LSS extracts of *Xenopus* eggs (lane 1). Note that p32 migrates as a single band in the interphase extract used here. The anti-RPA antibodies cross-reacted weakly with several other proteins in LSS extracts (lane 1), but these proteins (e.g. a band at ~50 kDa in lane 1) were not observed as components of purified RPA (lane 2).

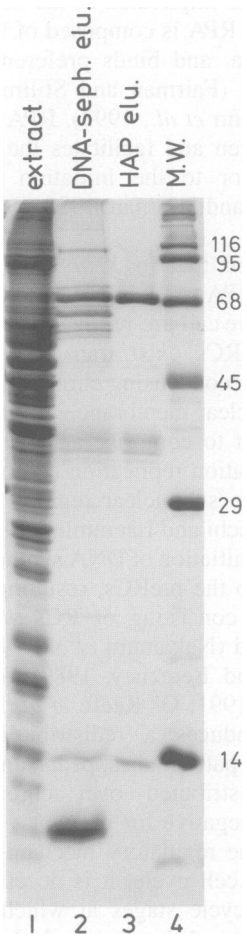


**Fig. 1.** Purification of RPA from *Xenopus* egg extracts. RPA is purified from a HSS derived from *Xenopus* eggs (lane 1) by fractionation on a DNA–Sepharose column. After a 1 M NaCl wash of this column, RPA was eluted with 1.5 M NaCl plus 50% ethyleneglycol (lane 2). This eluate is strongly enriched in RPA; additional purification is achieved by fractionation on a hydroxylapatite column and RPA was eluted with 70 mM potassium phosphate (lane 3). Samples were separated on a 15% SDS–polyacrylamide gel and stained with Coomassie blue. Purified RPA is composed of 70, 32 and 14 kDa proteins (p70, p32 and p14, respectively). p32 runs as multiple bands because of mitotic phosphorylation. Lane 4: molecular weight markers (sizes are in kDa).

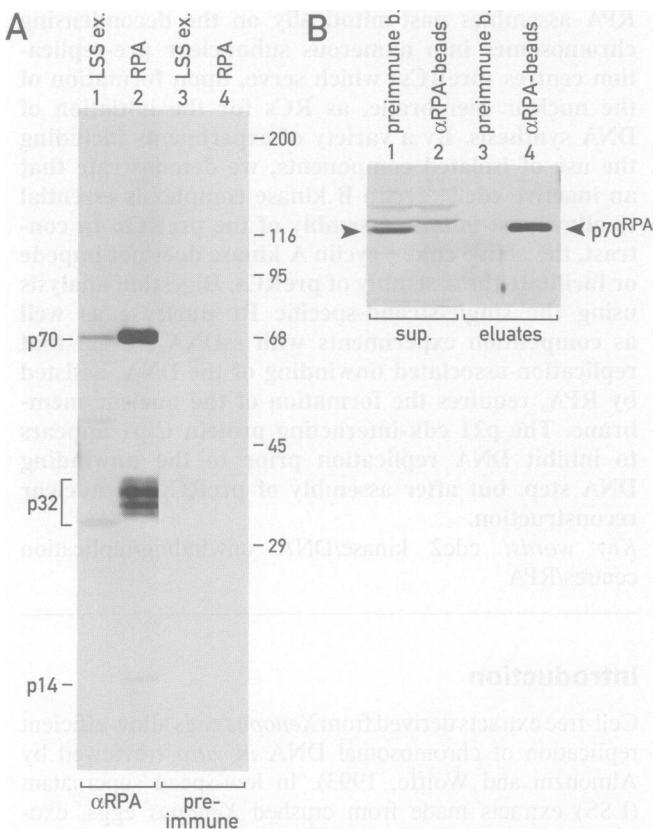


**Fig. 2.** Immunodepletion of RPA from egg extracts. (**A**) A high affinity antibody was prepared against purified RPA to study its role in cellular DNA replication and tested by immunoblotting against samples separated by SDS–PAGE. The anti-RPA, but not the preimmune serum, specifically detects the subunits of the purified RPA (lanes 2 and 4) also in the crude LSS derived from *Xenopus* extracts (lanes 1 and 3). The mutliple bands of the p32 proteins observed in the purified RPA, derived from a mitotic extract, are due to mitotic phosporylation. (**B**) The anti-RPA serum efficiently depletes RPA from LSS extracts. Protein A–Sepharose loaded with preimmune serum (preimmune b.) or anti-RPA antiserum (α-RPA-beads) were used to deplete LSS extracts as indicated (sup.). Proteins bound to the beads were eluted with SDS and also analysed (eluates), all samples were fractioned by SDS–PAGE. The immunoblot with a chicken antiserum directed against the 70 kDa subunit of RPA was used to reveal the efficiency of RPA depletion.

### *Sperm nuclei do not replicate in RPA-depleted LSS extracts: complementation by addition of purified RPA*

LSS extracts prepared from *Xenopus* eggs can replicate exogenously added demembranated *Xenopus* sperm nuclei efficiently (Blow and Laskey, 1986; Hutchison *et al.*, 1987; Lohka and Masui, 1983). LSS extracts were immunodepleted for RPA by mixing the extracts with a 25–30% vol of protein A−Sepharose beads coated with anti-RPA antiserum. As a control, we used beads precoated with preimmune serum. We verified the depletion of RPA by immunoblotting with chicken anti-*Xenopus* p70 antibodies. Figure 2B shows the efficient depletion of p70 from the extracts treated with anti-RPA beads compared with the preimmune control (lanes 1 and 2). p70 was recovered in the SDS eluate from the anti-RPA beads, but not from the preimmune beads (lanes 3 and 4). We estimate that depletion for RPA was >98% since the antiserum used could detect p70 in 50-fold diluted extract (data not shown).

Demembranated *Xenopus* sperm nuclei were added to the extracts treated with preimmune beads or with anti-RPA beads. DNA synthesis was measured by incorporation of a radiolabelled deoxynucleotide into high molecular weight sperm DNA (Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison *et al.*, 1987). The extracts treated with preimmune beads showed efficient replication of added sperm nuclei, the level of synthesis reached a maximum plateau at 60–90 min (Figure 3A, $RPA^+$ extracts; we note that most of the added sperm nuclei fully replicated to achieve the maximum incorporation counts shown, as estimated from the reported endogenous concentration of dATP in egg extracts; Blow and Laskey, 1986; data not shown). In the extracts depleted with anti-RPA beads (Figure 3A, $RPA^-$ extracts, 0 ng/μl RPA), very little chromosomal DNA synthesis was detected. At 120 min, the radioactivity of the $RPA^-$ extract sample was 3.5% of that of the control $RPA^+$ extract (Figure 3B). Under these experimental conditions, sperm nuclei decondensed and nuclear membrane assembly occurred within the first 30 min. Morphological examination of nuclear assembly by phase contrast microscopy did not show any differences in either extract (data not shown). We also demonstrated by immunofluorescence that sperm nuclei in the $RPA^-$ extracts did not acquire any RPA stainable foci (data not shown; see also Adachi and Laemmli, 1992).

To confirm further that the replication defects of $RPA^-$ extracts were specifically due to the lack of RPA rather than the loss of some other essential factors during the depletion process, we examined whether the addition of purified RPA to $RPA^-$ extracts would be sufficient to restore DNA replication. Increasing amounts of purified RPA were added to $RPA^-$ extracts and their replication efficiency was examined (Figure 3A and B). Replication of $RPA^-$ extracts was efficiently restored by addition of purified RPA. The $RPA^-$ extracts supplemented with 15 ng/μl of purified RPA showed incorporation levels equivalent to that of $RPA^+$ control extracts at 120 min, although a lag is noted in the complemented extract; at 60 min their incorporation level was approximately half that of the control. Addition of purified RPA to 22.5 or to 37.5 ng/μl improved the incorporation level at 60 min, but did not change significantly the final level of incorporation achieved at 120 min. We estimate that the concentration of RPA in intact LSS extracts is ~15 ng/μl (data not shown; Adachi and Laemmli, 1992). Thus, readdition of RPA to the original concentration is required to regain equivalent levels of final replication products.

It is worth pointing out that it is possible to complement RPA-depleted extract after assembly of the nuclear membrane−lamina structure. In one such experiment, demembranated sperm nuclei were exposed to a RPA-depleted extract for 30 and 60 min to allow reconstruction of the nuclei prior to the addition of RPA. Over 90% of added sperm nuclei completed nuclear membrane assembly by 30 and 60 min as observed by phase contrast microscope and exclusion of fluorescently labelled dextran sulfate. However, despite the late addition of RPA, we observed a >65% complementation of the replication potential (data not shown). This appears to eliminate RPA as a serious contender for the so-called licensing factor

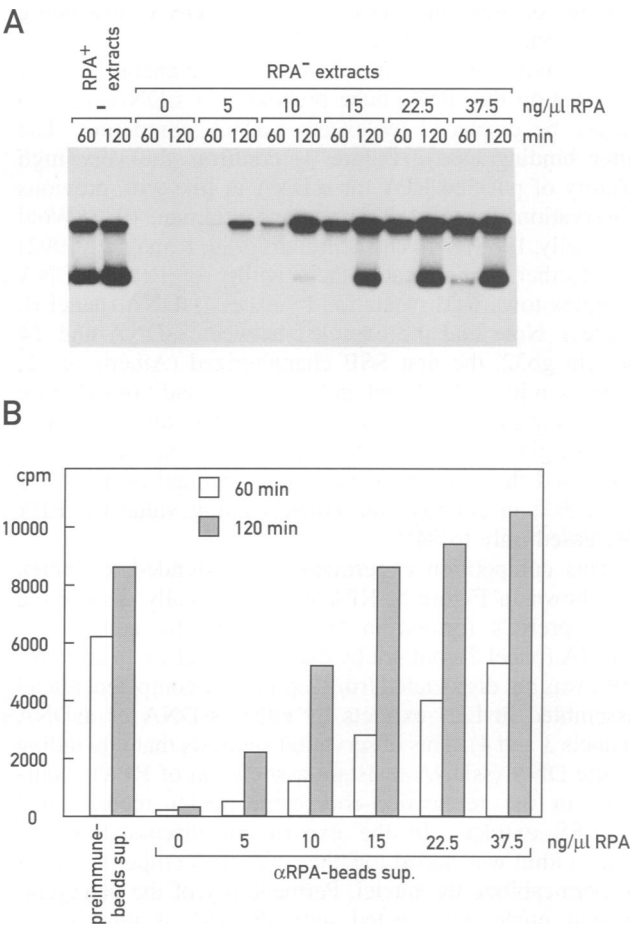


**Fig. 3.** RPA-depleted extracts are defective in DNA replication; complementation by the addition of purified RPA. (**A**) Demembranated *Xenopus* sperm nuclei were incubated in LSS extracts immunodepleted with anti-RPA beads ($RPA^-$ extracts) or with preimmune beads ($RPA^+$ extracts). [α-$^{32}$P]dATP was added to monitor the replication of added sperm nuclei. $RPA^-$ extracts supplemented with the indicated amounts of purified RPA were tested (0–37.5 ng/μl RPA). Samples were taken at 60 and 120 min, treated with proteinase, run on an agarose gel and autoradiographed. The position of the upper bands corresponds to the origin of the gel. The lower bands represent DNA that entered the gel. (**B**) The radioactivity incorporated into the high molecular weight DNA shown in panel A were quantified by liquid scintilation counting.

discussed by Laskey and collaborators (Blow and Laskey, 1988; Leno *et al.*, 1992), since access of the licensing factor to chromosomes is proposed to occur only following breakdown of the nuclear membrane at mitosis.

### *RPA can be dissociated from preRCs formed in HSS extracts by adding exogenous ssDNA competitor*

The formation of preRCs (p70 foci) occurs in cycling extracts immediately upon decondensation of chromosomes, but before the onset of DNA synthesis (Adachi and Laemmli, 1992). Efficient formation of preRCs is also observed on added sperm nuclei in membrane-free HSS extracts which are unable to initiate DNA synthesis. Assembly of the nuclear membrane lamina structure is required for the initiation of DNA synthesis (Newport, 1987; Sheehan *et al.*, 1988). Thus the following question arises: do preRCs formed in the HSS extracts include regions of unwound DNA, or does DNA unwinding require nuclear assembly?

To study this question, we took advantage of our observation that RPA, once prebound to ssDNA, can no longer be displaced by excess ssDNA competitor. The filter binding assay (Figure 4) confirms the very high affinity of purified RPA for ssDNA in line with previous observations (panel A; Fairman and Stillman, 1988; Wold and Kelly, 1988; Brill and Stillman, 1989; Kim *et al.*, 1992) and further demonstrates the stability of RPA–ssDNA complex towards dissociation by excess ssDNA (panel B, circles). Note that the complex between ssDNA and T4 protein gp32, the first SSB characterized (Alberts *et al.*, 1968), could be displaced under similar conditions (Figure 4B, triangles). In the case of gp32, addition of a 1000-fold weight excess of heat-denatured salmon sperm DNA decreased the fraction of the probe retained on the filter to 12.5%; in contrast, the corresponding value for RPA decreased only to 94%.

This competition experiment was extended to nuclei. As shown in Figure 5, RPA was specifically dissociated from preRCs formed in HSS extracts by addition of ssDNA (panel 2), but not by dsDNA (panel 1). In contrast, RPA was not dissociated from replication-competent nuclei assembled in LSS extracts by either ssDNA or dsDNA (panels 3 and 4). This observation suggests that unwinding of the DNA (ssDNA-resistant association of RPA) occurs only in the replication-competent nuclei reconstituted in LSS extracts. In the experiment discussed above, lysolecithin was added together with the competitor DNA to permeabilize the nuclei. Permeability of the detergent-treated nuclei was tested with the aid of fluorescein-labelled dextran sulfate of 150 kDa molecular weight (Newmeyer *et al.*, 1986). While nuclei assembled in LSS extracts excluded the dextran sulfate, we observed in the presence of lysolecithin an identical fluorescent signal from the nuclear interior or the surrounding solution (data not shown). The detergent-treated nuclei should be permeable to the competitor. Indeed, the experiment discussed below demonstrates that it has been possible to extract RPA from the detergent-treated LSS nuclei with the help of ssDNA, if DNA replication is inhibited with the cdk-interacting protein Cip1 (see below; Figures 5 and 9). Cip1 is a potent inhibitor of cyclin-dependent kinases

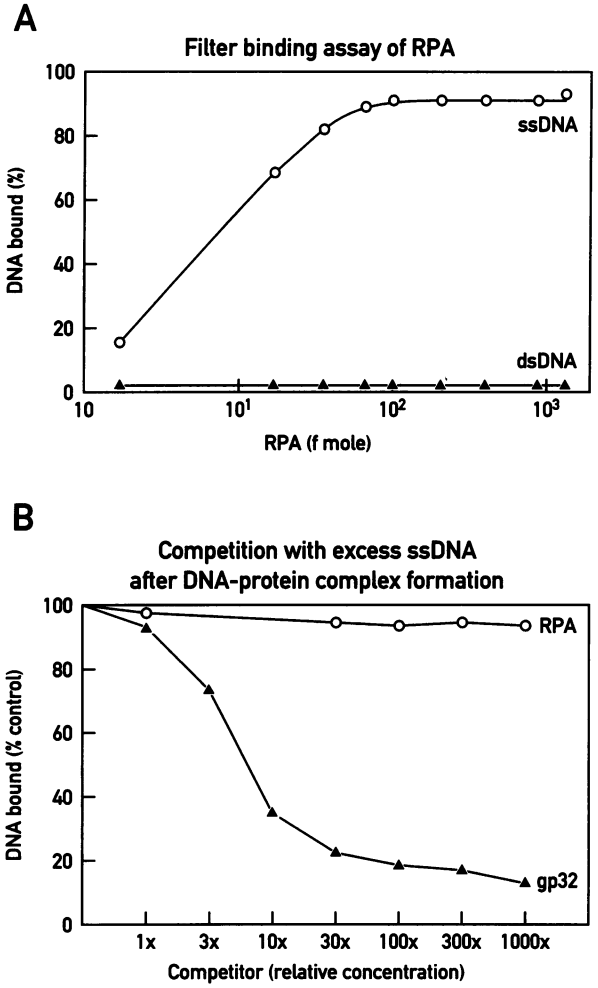


**Fig. 4.** The RPA–ssDNA complex resists dissociation by competitor ssDNA. (**A**) A 3.6 kb linear plasmid DNA was end-labelled. Heat-denatured (ssDNA, open circles) or native (dsDNA, closed triangles) DNA probe (5 ng, 2.2 f mol) was titrated with increasing amount of purified RPA and the resulting complex was determined by nitrocellulose filter binding. Each point, representing per cent filter bound, is an average of duplicate samples. (**B**) Five nanograms of end-labelled probe (3 kb, 2.6 f mol) were heat-denatured and incubated either with 10 ng (86 fmol) of RPA (open circles) or 100 ng of T4 gp32 (closed triangles). After a 30 min incubation, excess heat-denatured salmon sperm DNA was added to assess the stability of the complex. After 30 min, samples were filtered and the amount of the probe retained was determined as above.

implicated in the $G_1$ to S phase transition (Harper *et al.*, 1993).

### *Assembly of the nuclear membrane is required for the RPA-dependent unwinding of DNA*

The competitive displacement experiments for RPA described above suggest that DNA unwinding occurs following assembly of the nuclear membrane–lamina structure. To obtain independent evidence for this notion, we used the single-strand-specific nuclease P1; P1 has been successfully used to detect duplex DNA unwound by the dnaA protein at the replication origin of *Escherichia coli* (Bramhill and Kornberg, 1988; see also Schnos *et al.*, 1988).

*Xenopus* sperm nuclei were incubated either in HSS or LSS extracts for 90–120 min, then exposed to P1 nuclease

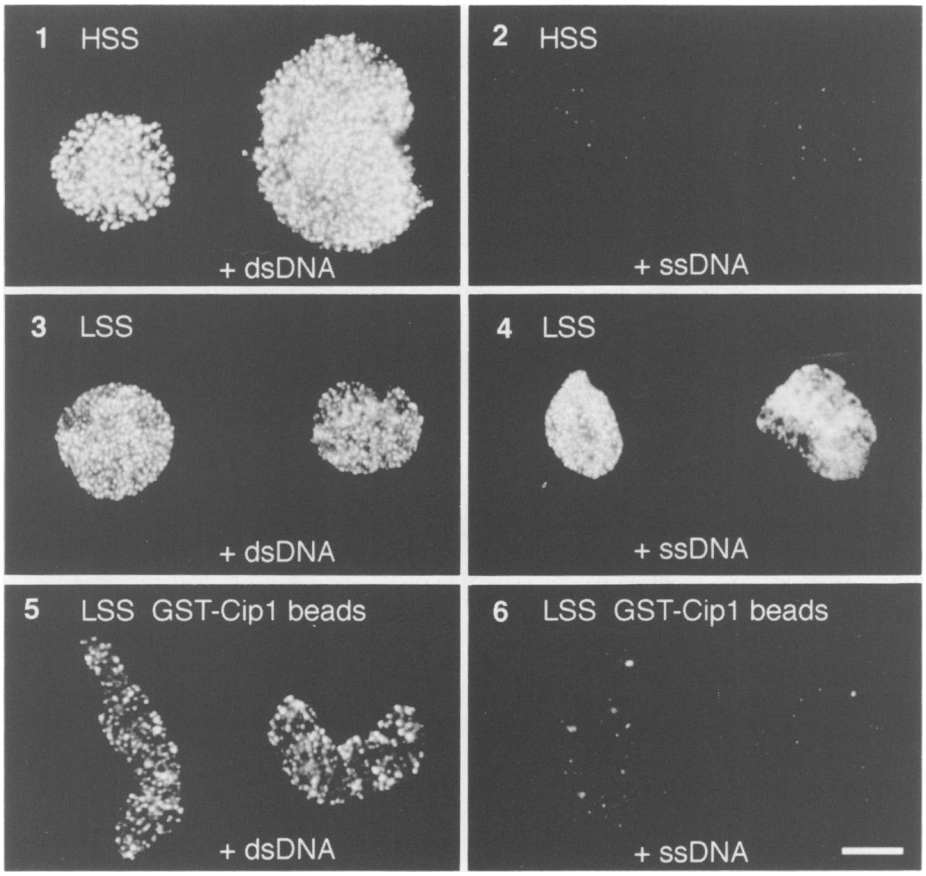


**Fig. 5.** RPA resists extraction by ssDNA following nuclear assembly, a step inhibited by Cip1. Sperm nuclei were incubated for 60 min either in HSS (panels 1 and 2), LSS (panels 3 and 4) or LSS treated with GST−Cip1 beads (panels 5 and 6). Then native (dsDNA; panels 1, 3 and 5) or heat-denatured (ssDNA; panels 2, 4 and 6) salmon sperm DNA was added to 40 ng/μl. Lysolecithin (500 μg/ml) was also added to LSS samples together with competitor DNA (panels 3, 4, 5 and 6). Incubation was continued for 30 min with the competitor DNA. Samples were fixed and subjected to immunofluorescence with affinity-purified anti-p70 antibodies to score for preRCs. Scale bar: 10 μm.

and the gently isolated DNA was subjected to field-inversion gel electrophoresis (FIGE; Carle *et al.*, 1986), blotted, and hybridized to *Xenopus* genomic DNA. Under the electrophoretic conditions used, DNA molecules up to 150 kb were well separated (Figure 6). Nuclei incubated in HSS extracts did not show significant sensitivity to P1 nuclease (Figure 6, lane 1, −P1 and lane 2, +P1). However, P1 treatment of nuclei incubated in LSS extracts resulted in the striking appearance of a DNA subfraction consisting of 20–50 kb-sized fragments (lane 4); most of the DNA remained undigested (>150 kb in length). We do not know whether the P1-dependent conversion of the bulk DNA band to the 20–50 kb smear was maximal under our experimental condition; but a fractional conversion of the bulk DNA band by P1 to the 20–50 kDa smear was not unexpected. Initiation of DNA replication was temporally staggered (early to late replication regions), consequently, only a fractional unwinding of the replication origins may have occurred.

If the P1 sensitivity of LSS nuclei is specifically due to the unwinding of the DNA prior to the initiation of DNA synthesis, it should be dependent on the presence of RPA. Indeed, in the LSS extracts depleted for RPA, the DNA smear induced by P1 was much reduced (lane 8). Readdition of purified RPA restored P1 sensitivity at least partially and the peak of digested DNA (~20–50 kb) was again observed (lane 10). Densitometric scanning of the autoradiographs indicates that lanes 9 and 10 are underloaded by approximately half compared with those in lanes 5–8. Thus, complementation by RPA (lane 10) is better than a cursory inspection of Figure 6 would indicate.

The results of these experiments using P1 nuclease are consistent with those of the ssDNA competition experiments and suggest that preRCs formed in HSS extracts do not contain unwound ssDNA region and that unwinding occurs only after assembly of nuclear membrane−lamina structure. Important for this conclusion is the absence or presence of the 20–50 kb smear in the HSS (lanes 1 and 2) versus the LSS samples (lanes 3–6) of Figure 6. The P1 nuclease-derived DNA subfraction is proposed to reflect replicon-sized (or multiples thereof) DNA fragments derived from neighbouring unwound initiation regions which are physically clustered in the nucleus into RCs.

### *Assembly and disassembly of preRCs are controlled by the cell cycle state of the extracts*

The low affinity of purified RPA for double-stranded (ds) DNA and the apparent absence of unwound DNA in the preRCs suggest that the post-mitotic association of RPA is driven through protein−protein interactions (see Discussion). As demonstrated next, this cell cycle-dependent behaviour of RPA is controlled by cyclin-dependent phosphokinases.

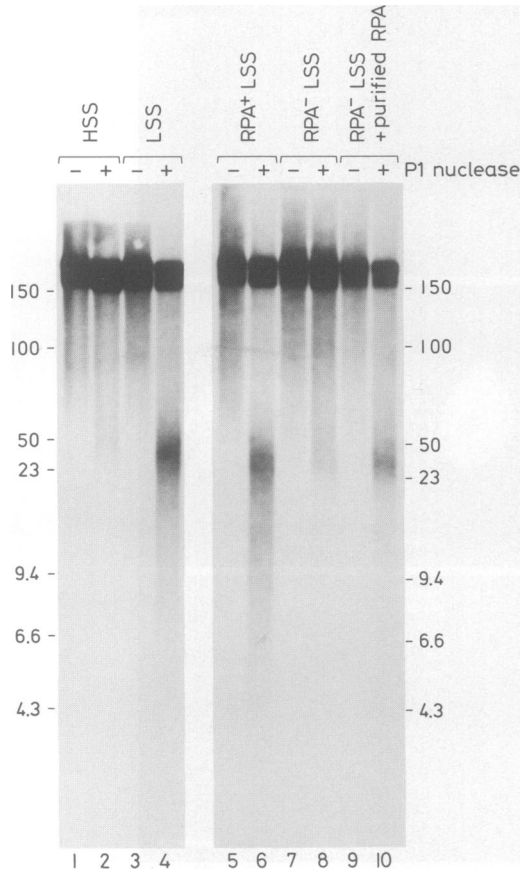


**Fig. 6.** Nuclei assembled with a nuclear membrane–lamina structure show sensitivity to ssDNA-specific nuclease P1; P1 sensitivity is dependent on RPA. Sperm nuclei were incubated in HSS or LSS extracts for 60 or 120 min, respectively. Samples were divided into two: one aliquot was exposed to the ssDNA-specific nuclease P1 and the other was not. Samples were treated with proteinase K, subjected to FIGE, Southern blotted and hybridized to a total *Xenopus* DNA probe. To examine whether the P1 sensitivity of nuclei incubated in LSS (lane 4) is dependent on RPA, LSS extracts were treated with preimmune beads ($RPA^+$ LSS) or with anti-RPA beads ($RPA^-$ LSS). Purified RPA was added back to one of the RPA-depleted LSS extracts. Sperm nuclei were added to these extracts and analysed as above. Note that the amount of DNA loaded in the samples complemented with purified RPA (lanes 9 and 10) is approximately half of those on lanes 5–8 as determined by densitometry.

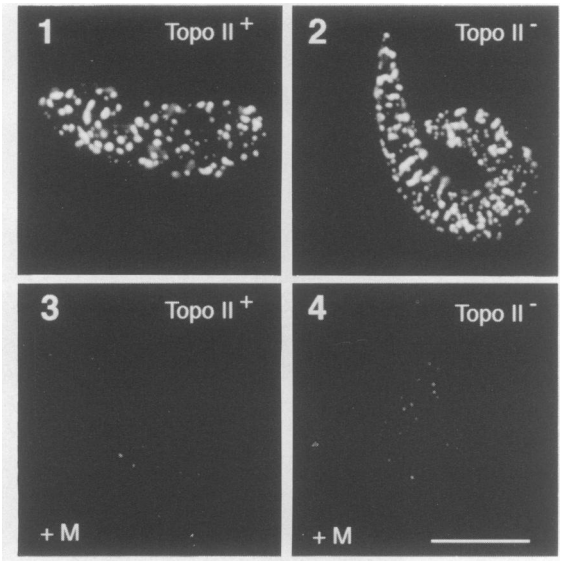


**Fig. 7.** Addition of mitotic extracts induces disasssembly of preRCs. *Xenopus* sperm nuclei were added to high speed supernatant (HSS) extracts to allow assembly of preRCs as scored by immunofluorescence using affinity-purified anti-p70 antibodies (panel 1). PreRCs assemble normally in HSS extract immunodepleted for topoisomerase II (panel 2). Dissociation of RPA from preRCs could be induced by the addition of complete (panel 3) or topoisomerase II-depleted (panel 4) mitotic extracts (+M). Topoisomerase II is neither implicated in the assembly nor the disassembly of preRCs. Scale bar: 10 μm.

For this purpose, added sperm nuclei are first exposed to an interphase HSS extract which results in the decondensation and efficient assembly of the preRCs (Figure 7, upper panels 1 and 2). Upon the subsequent addition of mitotic extracts, we observe a strongly reduced p70 staining pattern (lower panels 3 and 4). This disassembly is not simply brought about by chromosome condensation which is induced by the addition of the mitotic extract, since an identical observation is made with extracts immunodepleted for topoisomerase II (compare panels 1 and 2 with 3 and 4). Extracts immunodepleted for topoisomerase II are known to be defective in chromosome condensation (Adachi *et al.*, 1991; data not shown). These results also imply that topoisomerase II is not essential for the assembly of preRCs.

Conversely, formation of preRCs can be induced in mitotic extracts by treatment with the $p13^{suc1}$ protein coupled to Sepharose beads (Figure 8, row 2). The protein $p13^{suc1}$ complexes with the cyclin-dependent kinase cdc2, a key component of maturation promoting factor (MPF), and cdk2, which is thought to be implicated in the $G_1$ to S phase transition (Dunphy *et al.*, 1988; Blow and Nurse, 1990; Fang and Newport, 1991). Under our experimental conditions, depletion of the mitotic extract with Sepharose beads charged with $p13^{suc1}$ reduced the histone H1 kinase activity to the level equivalent to that of interphase extracts or even less (Table I). Conversion of the extracts from mitosis to interphase is manifested by the morphology of the added sperm chromatin stained for DNA as shown next. Sperm chromatin added to the mitotic extract ($M^+$) condensed into fibrous (mitotic-like) chromosomes (DNA stain is red) and preRCs (as white dots) are absent in mitotic extracts (Figure 8, row 1). In contrast, in the extracts treated with p13 beads ($M^-$), nuclei are decondensed and acquire preRCs (row 2).

p13 beads are known to complex with a number of other proteins besides the cyclin kinases (e.g. Blow and Nurse, 1990). In order to identify the cyclin kinase that is possibly implicated in the dissociation of the preRCs, we used different isolated kinase and cyclin materials kindly provided by David Morgan (UCSF). The following experiments were performed to demonstrate that preRC assembly is specifically due to the depletion of cyclin B–cdc2 kinase complex by p13 beads.

We employed insect cell extracts containing ~1–2% total protein of either cyclin B1 or cdc2 kinase which had been overexpressed by recombinant baculovirus (Desai *et al.*, 1992). cdc2 kinase in the lysate is inactive, but can be activated by adding the lysate containing cyclin B1 (Desai *et al.*, 1992). We observed that addition to the $M^-$ (p13 bead-depleted) extract of either the cyclin B1- or cdc2-containing lysate had no inhibitory effect on the

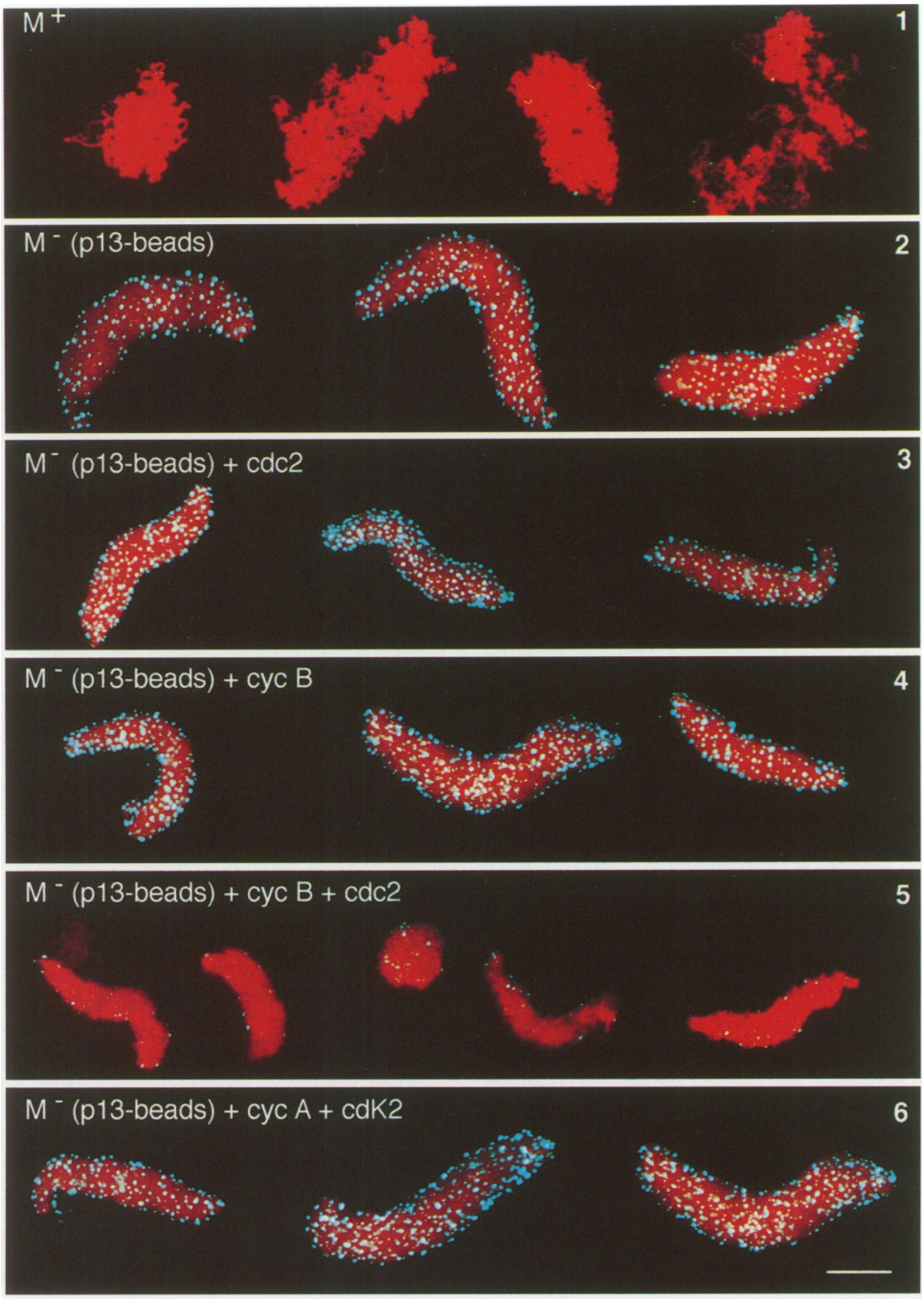


**Fig. 8.** Depletion of a mitotic extracts with p13 beads allows preRC assembly; specific inactivation of the cdc2 kinase is required. Mitotic extracts were treated either with mock-Sepharose beads (row 1, $M^+$) or p13 beads (row 2, $M^-$ p13 beads). The depleted extracts (rows 2–6) were supplemented with the insect cell lysates containing either cdc2 (row 3), cyclin B1 (row 4) or both (row 5) as indicated. The $M^-$ extract was also complemented with partially purified active cdk2−cyclin A1 complex (row 6). Sperm nuclei were added to these extracts and after 60 min samples were scored by immunofluorescence for preRCs with affinity-purified anti-p70 antibodies. DNA is stained with propidium iodide as shown in red and prePCs appear in blue/white. Scale bar: 10 μm.

preRC assembly (Figure 8, rows 3 and 4) and the H1 kinase activities measured in these complemented extracts remained low at the level of the uncomplemented $M^-$ (see Table I). In contrast, addition of both the cyclin B1 or cdc2 containing lysate to the $M^-$ extract raised the H1 kinase activity to that of the mitotic $M^+$ extract (Table I). This complemented cyclin B1−cdc2 kinase activity led to a dramatic inhibition of the punctated RPA staining (Figure 8, row 5). To study further the specificity of the negative role played by the active cdc2 kinase in the assembly of the preRCs, we also tested partially purified cyclin A−cdk2 kinase. In contrast, this complex does not, despite the resulting elevated H1 kinase activity (Table I), interfere with the preRC formation if added to the $M^-$ extract (Figure 8, row 6).

In conclusion, the interphase (post-mitotic) preRCs can be disassembled by the addition of mitotic extracts and the assembly of preRCs in mitotic extracts can be induced

**Table I.** H1 kinase activity and efficiency of preRCs assembly on sperm chromatin

| | H1 kinase activities[a] | preRCs |
|---|---|---|
| $M^+$ | 26 | − |
| $M^-$(p13 beads) | 1.8 | +++ |
| $M^-$(p13 beads) + cdc2 | 1.0 | +++ |
| $M^-$(p13 beads) + cycB | 1.4 | +++ |
| $M^-$(p13 beads) + cdc2 + cycB | 23 | − |
| $M^-$(p13 beads) + cdk2 + cycA | 17 | +++ |

Mitotic extracts ($M^+$) were depleted with p13 beads ($M^-$ p13 beads). The depleted extracts were added with insect cell lysates containing either cdc2 or cyclin B1 (cyc B) alone, reassembled cdc2−cyclin B complex or partially purified cyclin A−cdk2 kinase. H1 kinase activities and preRC formation on added sperm chromatin were examined. See also Figure 8.
[a]pmol phosphate transferred to histone H1/min/μl extract.

by depletion with p13 beads. Addition of active cyclin B1−cdc2 kinase is sufficient to suppress the assembly of preRCs in the mitotic extracts depleted with p13 beads. Thus, the assembly of RPA into preRCs is negatively regulated by the activity of the mitotic cdc2 kinase.

It is noteworthy to point out that addition of cyclin B1−cdc2 kinase did not restore the full chromosome condensation potential of the $M^-$ extract. We observed, however, some compaction of the nuclei, but no prophase-like fibre (Figure 8, row 5), despite the high re-established histone H1 kinase activity (Table I). The insect lysates appear not to contain an inhibitory activity on chromosome condensation as tested by addition to intact mitotic extracts. Conceivably, the p13 beads may have depleted the extract of essential non-kinase components.

### *The p21 cdk-interacting protein Cip1 inhibits DNA replication in LSS extracts, but not the assembly of preRCs*

Recently, several groups have identified a 21 kDa protein (Cip1, also called Sdi1, Waf1, CAP20 or Pic1) which interacts with cyclin-dependent kinases and inhibits their catalytic activities (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). Cip1 is thought to regulate the cell cycle negatively as potent $G_1$ cyclin−cdk kinase inhibitor. Overexpression of Cip1 inhibits DNA synthesis in human fibroblasts and this inhibition can be counteracted by co-expression of SV40 T antigen (Harper *et al.*, 1993). With the aim of defining further steps in the assembly and activation of preRCs, we have examined the effects of Cip1 on DNA replication and preRC formation in the *Xenopus* egg extracts.

LSS or HSS extracts were exposed for 1 h to glutathione−Sepharose beads loaded either with glutathione *S*-transferase (GST) or the GST−Cip1 fusion proteins (Harper *et al.*, 1993). Following the removal of the beads (some GST−Cip1 might dissociate from the beads due to glutathione in the extract), we tested the replication potential of treated extracts using demembranated sperm nuclei as described above (Figure 3). We observed efficient DNA replication in the control LSS extracts exposed to GST beads (Figure 9, lanes 1 and 2). In contrast, exposure to the GST−Cip1 beads resulted in no significant (<5%) incorporation of the radioactive precursor (Figure 9, lanes 3 and 4). Microscopic examination showed that decondensation of sperm chromatin and assembly of nuclear membranes appeared normal in either extract (data not shown). Interestingly, some basic component(s) of the replication machinery and/or chromatin assembly process appears to be a sensitive target of the Cip1-mediated block; treatment of HSS extracts with GST−Cip1 beads is inhibitory on the replication/chromatinization of the ss M13 to the ds supercoiled form (Figure 9, lanes 5 and 6). This process is known to occur in the membrane-free HSS extract without assembly of the nucleus (Mechali and Harland, 1982; Blow and Nurse, 1990).

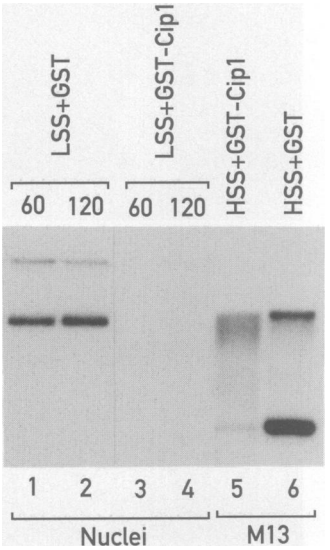


**Fig. 9.** GST−Cip1 (p21) beads inhibits the replication of added sperm chromatin in LSS extracts. LSS extracts were treated with GST beads (lanes 1 and 2) or GST−Cip1 beads (lanes 3 and 4) and replication of sperm chromatin in these extracts was examined by incorporation of radioactive dATP into high molecular weight DNA. Samples were taken after 60 (lanes 1 and 3) and 120 min (lanes 2 and 4) incubation, digested with proteinase K, run on a agarose gel and autoradiographed. HSS extracts were also treated as LSS extracts above with GST−Cip1 (lane 5) or GST (lane 6) beads and they were examined for their ability to synthesize DNA on M13 ssDNA template. 50 ng fo M13 ssDNA was added to 5 μl of the beads-treated HSS extracts and incubated for 60 min. In lanes 5 and 6, lower bands represent replicated supercoiled form (resulting from chromatin assembly) and upper bands the nicked circle molecule.

As shown next, the Cip1-mediated block on DNA replication occurs post-assembly of the preRCs, but appears to occur prior to the unwinding of the DNA. Figure 5 (panel 5) shows that exposure of LSS or HSS extracts (not shown) to GST−Cip1 beads does not inhibit the assembly of preRCs. Does the Cip1-mediated block occur prior to DNA unwinding? To answer this question, we repeated the RPA extraction experiment using ds or ssDNA as described above using LSS extracts treated with GST−Cip1 beads or with control GST beads. As is evident from Figure 5, competition with ssDNA (panel 6), but not dsDNA (panel 5) leads to a significant reduction of the RPA-stainable centres. In contrast, in nuclei assembled in LSS extracts treated with GST beads, we observed no dissociation of RPA by the ssDNA competitor (data not shown, but see panel 4). In support of the assigned specificity of Cip1 for cdks, we observed that GST−Cip1 does not inhibit chromosome condensation in treated mitotic extracts (not shown).

In summary, treatment with GST−Cip1 beads neither inhibits the assembly of preRCs nor the formation of nuclei. DNA replication, however, is inhibited in LSS extracts using nuclei as substrate. The competition experiments with ss or dsDNA suggest that inhibition by Cip1 beads occurs prior to the unwinding stage of DNA replication.

## Discussion

Native, functionally active RPA was purified from *Xenopus* egg extracts. This protein complex has similar basic characteristics to the RPAs from human and budding yeast (Fairman and Stillman, 1988; Brill and Stillman, 1989; Wold *et al.*, 1989; Kim *et al.*, 1992) in that it consists of three subunits of 70, 32 and 14 kDa (Figure 1), and binds ssDNA specifically as a very stable complex which resists dissociation by excess ssDNA competitor (Figure 4). This low off-rate of RPA might be useful to cells for marking ssDNA regions on which DNA synthesis must be completed prior to the onset of mitosis.

RPA was originally identified as an essential cellular factor for the large T-antigen-mediated unwinding of DNA containing the SV40 replication origin (Wold and Kelly, 1988). RPA also stimulates DNA polymerases α and δ (Kenny *et al.*, 1989, 1990) and is reported to interact with DNA polymerase α (Dornreiter *et al.*, 1992). RPA has been proposed to function as a eukaryotic SSB analogue and a number of experimental observations support this notion. Firstly, in budding yeast, deletion of any subunit of RPA is lethal; mutants accumulate multiply budded cells with a single nucleus, consistent with a defect in the S phase of the cell cycle (Heyer *et al.*, 1990; Brill and Stillman, 1991). Second, RPA colocalizes with punctated DNA RCs which can be visualized by labelling replicating DNA with bromodeoxyuridine or biotinylated-dUTP (Wilcock and Lane, 1991; Adachi and Laemmli, 1992; Cardoso *et al.*, 1993). Third, RPA is required for human DNA excision repair *in vitro* (Coverley *et al.*, 1991).

In this report, we present direct biochemical evidence that RPA is required for the replication of intact sperm nuclei in *Xenopus* egg extracts, an observation also recently reported by Fang and Newport (1993). We have raised antibodies against RPA and used them to immunodeplete endogenous RPA from LSS egg extracts. RPA-depleted extracts are unable to replicate added sperm nuclei. The replication defect is efficiently restored by the addition of purified RPA to the depleted extracts (Figures 2B and 3), providing direct evidence that the replication defect is specifically due to the lack of RPA.

After an initial lag, RPA-complemented extracts reach a similar final level of dATP as control extracts (Figure 3A and B). This initial lag might arise as follows. The 32 kDa subunit of RPA preparations from mitotic extracts migrates as multiple bands, presumably reflecting the phosphorylation observed from S phase to the end of mitosis which has been reported by others (Din *et al.*, 1990; Dutta and Stillman, 1992; Fang and Newport, 1993). The mitotic RPA might have to be dephosphorylated to the active interphase form, resulting in a delay in the initiation process. We have also noted several proteins with apparent affinity for RPA in the eluates from the anti-RPA beads used for immunodepletion (Y.Adachi, unpublished data). Thus, partial depletion from the extracts of proteins with selective affinity for RPA might slow down the replication kinetics.

Initiation of DNA synthesis is initially confined to preRCs, they become RCs following assembly of the nuclear membrane−lamina (Adachi and Laemmli, 1992). The competition and nuclease digestion experiments presented here demonstrate that RPA-dependent unwinding of the DNA occurs predominantly following the reconstruction of the nuclear membrane. The competition experiments with excess ssDNA reveal by immunofluorescence the displacement of RPA from the preRCs, but not from the RCs (Figure 5).

Experiments with the ssDNA-specific nuclease P1 provide good evidence for the above hypothesis. Sperm nuclei incubated in HSS extracts (assembly of preRCs) show little sensitivity to P1 nuclease, whereas the same treatment of the nuclei incubated in LSS extracts (assembly of RCs) yields digestion products averaging 20–50 kb in length (Figure 6). Assembly of the nuclear membrane−lamina structure is required to acquire sensitivity to the P1 nuclease as well as resistance towards a displacement of RPA by ssDNA. In our P1 assay, most of the genomic DNA of LSS nuclei is not digested and remains >150 kb, even in the presence of lysolecithin (Figure 6; data not shown). This partial P1 sensitivity is likely to reflect the notion that replication initiation is regional as well as physically clustered into foci in the eukaryotic genome. The P1 fragments are proposed to represent replicons (or multiples thereof), a notion consistent with the following considerations. (i) In early *Xenopus* embryos, one round of DNA synthesis requires 10–16 min (Miake-Lye *et al.*, 1983; Hyrien and Mechali, 1993). (ii) If the overall rate of fork movement is 0.6 kb/min *in vivo* (Callan, 1972; Mahbubani *et al.*, 1992), then an origin would exist every 12–20 kb. (iii) Recently Hyrien and Mechali (1993) have reported that in *Xenopus* early embryos the ribosomal DNA replicon is 9–12 kb in length.

Given the low affinity of purified RPA for dsDNA and the apparent absence of unwound DNA in the preRCs, it would be reasonable to suggest that the post-mitotic association of RPA into preRCs occurs via protein−protein interactions. The *Xenopus* extracts appear to contain the necessary proteins. While we observed a very weak affinity of purified RPA for dsDNA, we noted that RPA, loaded in the presence of the crude HSS extracts, binds to dsDNA−cellulose (containing no ssDNA regions) and can be eluted with 1.5 M salt (data not shown; note that RPA is not eluted from ssDNA affinity column by 1.5 M salt alone). This retention of RPA on dsDNA−cellulose is likely to be brought about by other protein components present in the crude extract. Indeed, RPA is reported to bind the acidic activation domains of VP16, p53 and GAL4, and Gal4 can stimulate BPV-1 DNA replication *in vitro* with the appropriate template (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993). Both p53 and RPA are localized at replication sites of herpes simplex virus (Wilcock and Lane, 1991). Proteins, such as p53, might act as tethers for the association of RPA with preRCs.

The assembly of preRCs in the post-mitotic extract is not a spontaneous phenomenon, but requires ATP and an ambient temperature (Adachi and Laemmli, 1992). The putative protein−protein interactions proposed to be

involved in the assembly of RPA into preRCs are under the control of the cell cycle. Dissociation of RPA from preRCs formed in HSS extracts can be reversibly induced by addition of mitotic extracts (Figure 7). This dissociation is independent of mitotic chromosome condensation and occurs in condensation-defective extracts immunodepleted for topoisomerase II. Conversely, depletion of the cdc2-like kinases with $p13^{suc1}$ beads leads to the assembly of preRCs (Figure 8, row 2). Using isolated components, we demonstrate that specific inactivation of the cdc2–cyclin B complex is essential to allow the assembly of the preRCs, since addition of the active cdc2–cyclin B complex to a mitotic extract depleted with $p13^{suc1}$ beads prevented the assembly of preRCs (Figure 8). In contrast, addition of either cdc2, cyclin B or the active cdk2–cyclin A complex did not impede normal preRCs assembly (Figure 8, rows 3, 4 and 6). Clearly, cdc2–cyclin B specifically prevents the assembly of preRCs in egg extracts.

The cyclin-dependent kinase, cdk2, is known to be required for the initiation of DNA replication, this kinase as well as cyclin A were reported to colocalize to RCs (Blow and Nurse, 1990; Fang and Newport, 1991; Cardoso *et al.*, 1993). The p21 cdk-interacting protein Cip1 (for a review, see Hunter, 1993) was found, supposedly acting as a kinase inhibitor, to block DNA replication, but not the assembly of preRCs or the formation of the nuclear membrane, as tested in LSS *Xenopus* egg extracts (Figure 8). This inhibition of DNA replication in egg extracts is consistent with the previous results by Harper *et al.* (1993), carried out with fibroblasts and NIH3T3 cells. The Cip1-mediated block of replication appears to occur prior to the DNA unwinding step as suggested by the ssDNA competition experiment; RPA could be significantly extracted by competition with ssDNA from nuclei exposed to an extract treated with GST–Cip1 beads compared with the control (see Figure 5, panels 5 and 6).

The preRCs are proposed to consist of a large number of pre-initiation RCs poised for initiation and assembled at discrete subnuclear regions. Assembly of the preRCs requires specifically inactivation of the mitotic cdc2 kinase. In contrast, activation of the preRCs requires (not exclusively) nuclear reconstruction and a Cip1-inhibited activity, supposedly an active $G_1$ cyclin-dependent kinase. The experiments reported here dissect a number of post-mitotic steps, and they will help to provide an experimental base to study the molecular details of these key events.

## Materials and methods

### *Preparation of Xenopus egg extracts*

*Xenopus laevis* females were primed and ovulation was induced as described (Murray, 1991). Interphase extracts were prepared essentially as described previously (Finlay and Forbes, 1990; Adachi and Laemmli, 1992). Extraction buffer consisted of 250 mM sucrose, 10 mM HEPES–KOH, pH 7.5, 50 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, 10 μg/ml cytochalasin B, 1× protease inhibitor cocktail. Protease inhibitor cocktail was prepared as 1000× stock which contains 10 mg/ml each of leupeptin, chymostatin, pepstatin A and antipain in DMSO. After washing with extraction buffer, eggs were crushed by a 10 min centrifugation in a Beckman SW50.1 rotor at 12 000 r.p.m. (15 000 *g*). The cytoplasmic fractions were spun again (15 000 r.p.m. for 10 min) to eliminate contaminating lipids and pigments, divided into 100 μl aliquots, frozen in liquid nitrogen and stored at −70°C (LSS). HSS was prepared by additional centrifugation at 45 000 r.p.m. (200 000 *g*) in the SW50.1 for 2 h at 2°C. The cleared cytoplasmic layer was recovered, divided into aliquots, frozen and stored at −70°C. Mitotic extracts were prepared as described (Lohka and Maller, 1985; Newport and Spann, 1987; Adachi *et al.*, 1991; Adachi and Laemmli, 1992). Demembranated *Xenopus* sperm nuclei were prepared according to Lohka and Masui (1983).

### *Purification of RPA from Xenopus egg extracts and antibody preparation*

Mitotic extracts were thawed, supplemented with PMSF to 0.4 mM and spun at 10 000 *g* for 10 min. Twenty milliltres of the supernatant were loaded onto a DNA–Sepharose column (1.6×8 cm) equilibrated with HE-B (5 mM HEPES–KOH, pH 7.5, 1 mM EDTA, 10 mM β-glycerolphosphate, 1 mM DTT, 0.1 mM PMSF, 0.1× protease inhibitor cocktail) plus 12.5 mM KCl. The column was washed with six column volumes of the same buffer and proteins were eluted stepwise with 2.5 column volumes of H-B (10 mM HEPES–NaOH, pH 7.5, 10 mM β-glycerolphosphate, 1 mM DTT, 0.1 mM PMSF) plus 0.5 M NaCl and 1 mM EDTA, then H-B plus 1 M NaCl and finally H-B plus 1.5 M NaCl and 50% ethyleneglycol. RPA was eluted in H-B plus 1.5 M NaCl and 50% ethyleneglycol as described previously (Fairman and Stillman, 1988). The fractions containing RPA were loaded directly onto a hydroxylapatite column (BioGel HTP; Bio-Rad; 1×2.5 cm) equilibrated with 10 mM β-glycerolphosphate, pH 7.5, 1.5 M NaCl, 0.5 M sucrose, 1 mM DTT, 0.1× protease inhibitor cocktail. The column was washed with four column volumes of 10 mM β-glycerolphosphate, pH 7.5, 20 mM KCl, 0.5 M sucrose, 1 mM DTT, 0.1× protease inhibitor cocktail. RPA was eluted with 2.5 column volumes of 70 mM potassium phosphate, pH 7.5, 0.5 M sucrose, 1 mM DTT, 0.1× protease inhibitor cocktail. The final fraction contained ~300 μg of nearly homogeneous RPA. The protein was devided into aliquots, frozen in liquid nitrogen and stored at −70°C.

Immunization and bleeding of rabbits were performed essentially according to Harlow and Lane (1988). Fifty micrograms of purified RPA were injected three times into each rabbit. The first injection was performed with complete Freund's adjuvant. The second and third injections were performed with incomplete adjuvant containing 5 mg of poly(A)•poly(U) (Sigma) as described (Hovanessian *et al.*, 1988).

### *Filter binding assays*

Filter binding assays were performed according to Wold *et al.* (1989). Five nanograms (2.2 fmol) of intact (ds) or heat-denatured end-labelled DNA probe were incubated with purified RPA in HKM buffer (10 mM HEPES–KOH, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) plus 1 mM DTT, 0.1% digitonin at 23°C for 30 min as described in the figure legends. RPA was diluted with HKM buffer plus 1 mM DTT, 0.1% digitonin and 50 μg/ml bovine serum albumin (Sigma; fraction V). Reactions were filtered through nitrocellulose membranes (Millipore HA, 0.45 μm) and the membranes were washed three times with 0.5 ml of HKM buffer. The membranes were dried and the amount of probe retained on the membranes was determined by liquid scintillation counting. T4 gp32 was a gift from Dr M.Hurley.

### *RPA immunodepletion and complementation*

Protein A–Sepharose CL4-B (Pharmacia Fine Chemicals) was swollen and washed three times with phosphate-buffered saline (PBS). The resin was divided into aliquots and mixed with an equal volume of antiserum directed against *Xenopus* RPA or preimmune serum in Eppendorf tubes. The mixtures were incubated at room temperature for 2 h on a rotator. The resins were washed three times with 30 vol of PBS, twice with 30 vol of 10 mM HEPES–KOH, pH 7.5, 2.5 mM magnesium acetate, 50 mM potassium acetate, 250 mM sucrose 1 mM DTT, 1× protease inhibitor cocktail and once with 2 vol of the same buffer supplemented with an ATP-regenerating system (final concentrations of 2 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine kinase). Four vol of LSS extracts supplemented with an ATP-regenerating system were added to the protein A–Sepharose coupled as described above and incubated at 4°C for 1 h on a rotator. The Sepharose was pelleted in an Eppendorf microfuge for 5 s and the supernatant was collected as the depleted extract. Demembranated *Xenopus* sperm nuclei prepared according to Lohka and Masui (1983) were added to 500 nuclei/μl of depleted extract. Purified RPA was added as described in the text. Incubation was performed at 22°C. To monitor DNA replication, [α-$^{32}$P]dATP was also included in the reactions at 0.1 μCi/μl of extract. Samples were taken at various time points, quenched with SDS–EDTA, treated with proteinase K and run on agarose gel as described (Hutchison *et al.*,

1987; Fang and Newport, 1991). Incorporation of radioactive dATP into chromosomal DNA was examined by autoradiography of the dried gel or by cutting out and counting the gel bands by liquid scintillation counting using the tritium channel.

### *Competition of nuclei in extracts with ssDNA and P1 nuclease treatment*

HSS or LSS extracts were thawed and supplemented with an ATP-regenerating system and aphidicolin (40 μg/ml final concentration). Cycloheximide was also added to LSS extracts to 20 μg/ml. *Xenopus* sperm nuclei were added to 1000 nuclei/μl and incubated at 22°C for 1 h. Samples were divided into two aliquots. Each aliquot received 40 μg/ml intact or heat-denatured salmon sperm DNA (sonicated) and incubation was continued for a further 30 min. Lysolecithin (final concentration, 0.5 mg/ml) was also added with competitor DNA to the LSS nuclei to permeabilize their nuclear membranes. Permeability was examined by the addition of fluorescein-labelled dextran sulfate (average mol. wt 150 kDa; Newmeyer *et al.*, 1986). Samples were fixed and the localization of RPA in the nuclei was examined by immunofluorescence using affinity-purified anti-p70 antibodies as described previously (Adachi and Laemmli, 1992). The fluorescence micrographs were recorded on an MRC 600 confocal microscope (Bio-Rad) equipped with a krypton–argon laser and printed to papers using a Kodak dye sublimation printer XLS 8300.

For P1 nuclease experiments, sperm nuclei were incubated in HSS extracts (1500 nuclei/μl) for 1 h or in LSS extracts (900 nuclei/μl) for 2 h in the presence of aphidicolin as described above. When LSS extracts depleted for RPA were used, 700 sperm nuclei/μl were added. For the complementation of RPA-depleted LSS extracts, purified RPA was added to 25 ng/μl at the beginning of the incubation. Samples containing 13 500 nuclei were taken, diluted with HMN buffer (5 mM HEPES–NaOH, pH 7.5, 8 mM $MgCl_2$, 100 mM NaCl) to bring the volume to 30 μl and incubated for 2 min at 37°C. One unit of P1 nuclease (Pharmacia; 1 unit/μl stock in 8.5 mM sodium acetate, pH 6.0, 50% glycerol) was added and the reaction was stopped after 10 s by adding EDTA to 20 mM and SDS to 1%. Sixty μg of proteinase K were added and samples were incubated for 60 min at 37°C. Then another 60 μg of proteinase K were added and incubation was continued for an additional 60 min. The proteinase-treated samples were loaded on 0.8% agarose gels cast in 0.5× TBE. Samples were allowed to run into the gel for 90 min at 10 V/cm, after which time FIGE was performed for 17.5 h at 5 V/cm at room temperature using program 3 of a PPI-200 programmable power inverter (MJ Research, Cambridge, MA). Southern blotting was performed as described (Käs and Laemmli, 1992) using total *Xenopus* genomic DNA as a probe.

### *p13 and GST–Cip1 beads*

$p13^{suc1}$ protein was overexpressed in *E.coli* and purified according to Labbé *et al.* (1991). p13 was conjugated to Affi-gel 10 (Bio-Rad) at 10 mg/ml following the manufacturer's instructions. Depletion of mitotic extracts with p13 beads was performed essentially as described for immunodepletion of topoisomerase II (Adachi *et al.*, 1991). Mitotic extracts were mixed with an equal volume of p13 beads and incubated at 4°C for 1 h on a rotating wheel, spun on a microfuge and the supernatants were recovered. H1 kinase activity was measured according to Felix *et al.* (1989). Activity was indicated as pmol phosphate transferred to H1 per min per μl of extract. Insect cell lysates containing human cdc2 or cyclin B1 were kind gifts from Dr D.Morgan (Desai *et al.*, 1992). These lysates contain ~5 mg/ml total protein, 1–2% of which is cdc2 or cyclin B1 and shows little H1 kinase activities. Active cdc2–cyclin B complex was formed by mixing equal amounts of the two lysates (Desai *et al.*, 1992). Ten microlitres of mitotic extracts depleted with p13 beads were complemented with 2 μl of the mixed lysate containing active cdc2–cyclin B to achieve the nearly equivalent total H1 kinase activity of the original mitotic extract (see Table I). The same amount of buffer or insect cell lysates containing cyclin B or cdc2 alone was added to the controls. Demembranated sperm nuclei were added to 2000/μl and incubated at 22°C for 1 h. Samples were taken, fixed and stained with affinity-purified anti-$p70^{RPA}$ antibodies as described to score for preRCs (Adachi and Laemmli, 1992).

GST–Cip1(p21) was expressed in *E.coli* using a plasmid pGST–Cip1 which was generously provided by Dr J.W.Harper (Harper *et al.*, 1993). The fusion protein GST–Cip1 was prepared from inclusion bodies, solubilized by the Sarcosyl–Triton X-100 method described by Grieco *et al.* (1992) and attached to glutathione–Sepharose 4B (Pharmacia) beads. Control beads (GST beads) loaded with glutathione *S*-transferase were prepared identically using the Sarcosyl–Triton X-100 step from a soluble cell lysate. The loaded GST and GST–Cip1 beads were washed with 10 mM Tris–HCl, pH 8.5, 1 mM EDTA, 100 mM NaCl, 0.1% digitonin and 1× protease inhibitor cocktail several times, divided into aliquots and stored at −70°C. The control beads contain ~5 μg of GST per μl of beads, the GST–Cip1 beads have approximately one-tenth of that load. Exposure of LSS extracts to GST or GST–Cip1 beads was performed essentially as described above for RPA immunodepletion using 40% volume of the beads to LSS extracts. Competition experiments with ss or dsDNA were performed as described above in the presence of aphidicolin.

### *Other methods*

SDS–PAGE was carried out as described (Laemmli, 1970). Western blotting was performed as described (Towbin *et al.*, 1979), using a 1:500 dilution of chicken anti-p70 antibodies (Adachi and Laemmli, 1992) or the same dilution of rabbit anti-RPA antibodies. Alkaline phosphatase-conjugated anti-chicken IgG was obtained from Sigma and used as a secondary antibody. Protein concentration was estimated by the method of Bradford (1976) using BSA as a standard.

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