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# RCC1, a Regulator of Mitosis, Is Essential for DNA Replication

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**Temperature-sensitive mutants in the RCC1 gene of BHK cells fail to maintain a correct temporal order of the cell cycle and will prematurely condense their chromosomes and enter mitosis at the restrictive temperature without having completed S phase. We have used *Xenopus* egg extracts to investigate the role that RCC1 plays in interphase nuclear functions and how this role might contribute to the known phenotype of temperature-sensitive RCC1 mutants. By immunodepleting RCC1 protein from egg extracts, we find that it is required for neither chromatin decondensation nor nuclear formation but that it is absolutely required for the replication of added sperm chromatin DNA. Our results further suggest that RCC1 does not participate enzymatically in replication but may be part of a structural complex which is required for the formation or maintenance of the replication machinery. By disrupting the replication complex, the loss of RCC1 might lead directly to disruption of the regulatory system which prevents the initiation of mitosis before the completion of DNA replication.**

During the cell cycle, numerous processes must be temporally coordinated. In particular, the timing of mitosis must be carefully controlled with respect to the completion of DNA replication. If a cell were to initiate mitosis before replication was complete, the result would be chromosome damage or loss or, in more extreme cases, complete fragmentation of the chromosome through inappropriate condensation (45; reviewed in reference 21). We have previously shown that blocking the replication of nuclear DNA with aphidicolin inhibits the onset of mitosis in *Xenopus* egg in vitro cycling extracts by preventing the activation of the MPF (M-phase-promoting factor) kinase (9). However, the mechanism by which regulatory systems recognize incomplete replication is still poorly understood. Mutants of the *cdc25* gene in *Schizosaccharomyces pombe*, the *nimA1* and *bimE7* genes in *Aspergillus nidulans*, and the RCC1 gene in hamster cells all enter mitosis before replication is complete (15, 39, 42, 43) and thus must be aberrant in either the system recognizing unreplicated DNA, the system monitoring the extent of replication, or the signalling pathway which suppresses the onset of mitosis in the presence of unreplicated DNA. *cdc25* encodes a tyrosine phosphatase, which functions to control the tyrosine phosphorylation of the kinase subunit of MPF (11, 18, 24). *nimA1* encodes a mitosis-inducing kinase, distinct from the kinase subunit of MPF (43), and *bimE7* encodes a putative membrane-spanning protein of unknown function (14). RCC1 encodes a nuclear, chromatin-bound protein whose function is not fully understood at a biochemical level (35).

In order for a control system to detect incomplete replication, there must be some molecule which recognizes incomplete replication at the level of the DNA and acts as the source of the inhibitory signal preventing premature mitosis. This molecule would be expected to have several characteristics. First, it would likely be chromatin associated. Second, in the absence of this molecule, the cell would be unable to detect incomplete replication. Thus, mutants lacking it would initiate mitosis before completing DNA replication. Third, the molecule could either measure the amount of unreplicated DNA directly by being displaced or modified as replication proceeds or measure some parameter associated with replication, such as the persistence of replication complexes. Among the mutants known to act in the pathway coupling the completion of S phase to the initiation of mitosis, the only protein product known to be chromatin associated is the RCC1 protein (40). Moreover, BHK cells which possess a temperature-sensitive (*ts*) mutation in the RCC1 gene prematurely activate MPF at the nonpermissive temperature and enter mitosis before they finish S phase (39). RCC1 has also been found to be highly conserved in both sequence and function in organisms as diverse as yeasts and humans (41). Therefore, RCC1 is a very likely candidate for being a molecule directly involved in monitoring the completion of DNA replication in the cell cycle.

*Xenopus* extracts replicate DNA very efficiently and have therefore been used extensively to examine DNA replication in the cell cycle. Nuclei will form when DNA is added to *Xenopus* egg extracts (26), and these nuclei are functional by a number of different criteria: they form nuclear envelopes, form a nuclear lamina, and replicate their DNA (6, 27, 32, 34, 46). When single-stranded naked DNA or sperm chromatin is added to *Xenopus* extracts, the DNA undergoes a single round of replication during which it is replicated essentially to completion (2, 6, 32). Nuclei formed in *Xenopus* extracts are also capable of nuclear disassembly when MPF is added to the extract (8, 12, 25, 30, 33). These activities can also be performed by partially fractionated extracts. High-speed centrifugation separates the crude extracts into several fractions. Recombining two of these, the soluble cytoplasmic and particulate membrane fractions, results in a reconstituted extract which forms nuclei, replicates their DNA, and undergoes nuclear disassembly in response to added MPF (32, 33, 48). Use of reconstituted extracts allows the manip-

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ulation of the individual fractions in order to characterize the reactions of the cell cycle.

To investigate the biochemical function of RCC1, we undertook an examination of the role of the RCC1 protein in *Xenopus* extracts. We depleted fractionated *Xenopus* extracts of the RCC1 protein by serial immunoprecipitations with affinity-purified anti-RCC1 antibodies and examined the ability of these extracts to form nuclei from sperm chromatin and to replicate the DNA in these nuclei. We find that RCC1 is required for neither chromatin decondensation, chromatin formation on naked templates, nor nuclear formation but that it is absolutely required for the replication of added sperm chromatin DNA. Our results further suggest that RCC1 does not participate enzymatically in replication but may be part of a structure which is a required for the formation or maintenance of the replication machinery.

## MATERIALS AND METHODS

**Preparation of extracts; nuclear formation and replication assays.** Fractionated extracts were prepared by a variation of methods described elsewhere (16). Alterations are as followed. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was omitted from the lysis buffer. After the first 200,000 × *g* centrifugation, the membrane and soluble fractions were collected and each subjected to an additional centrifugation. The soluble fraction was recentrifuged for 20 min at 200,000 × *g* for 20 min to remove any remaining membranes. The membrane fraction was diluted greater than fivefold in lysis buffer, and the diluted membranes were layered upon a 0.5-ml cushion of lysis buffer that had additional sucrose to a final concentration of 0.5 M. The membranes were then pelleted through the sucrose cushion for 25 min at 20,000 rpm a Beckman TL-55 rotor. The membrane pellet was resuspended in a minimal volume of lysis buffer–0.5 M sucrose. Reconstitution of extracts was performed just as previously described. All replication assays were carried out exactly as described by Dasso and Newport (9).

**Immunodepletion of RCC1 from *Xenopus* extracts.** Affinity-purified antibodies were prepared from polyclonal serum raised against *Escherichia coli*-expressed RCC1 protein by using standard methods (20). Protein A-Sepharose beads (Sigma) were blocked by two 15-min room temperature incubations with 3.5 volumes of bovine serum albumin (10 mg/ml) in phosphate-buffered saline (PBS). They were then washed three times in 3.5 volumes of PBS. After washing, 600 μl of purified anti-RCC1 antibodies (250 μg/ml) was added to 100 μl of packed beads, and the mixture was allowed to incubate at room temperature for 1 h with rotation. To prepare beads for mock treatment of extracts, preimmune serum containing an approximately equivalent amount of immunoglobulin G was diluted with PBS to 600 μl and incubated with 100 μl of packed beads. After incubations with antibodies, the beads were washed three times in 400 μl of XB. To deplete the cytosol fraction, 4 volumes of cytosol and 1 volume of packed beads were incubated at 4°C for 1.5 h with rotation. The beads were pelleted by centrifugation, and the cytosol was removed and reincubated for an additional 1.5 h with 1 volume of fresh beads. PBS contains 125 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, and 8.1 mM $Na_2HPO_4$. XB contains 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, and 10 mM HEPES (pH 7.7).

**Preparation of *E. coli*-expressed RCC1 protein.** The protein used in Fig. 1A was prepared as described in Nishitani et al. (38). We have engineered a plasmid which can be used to produce protein with the authentic N terminus of RCC1, and this protein was used for the experiment shown in Fig. 3B. This plasmid was produced by introducing a *Bsp*HI site at the translation start site of the *Xenopus* RCC1 clone, using the polymerase chain reaction, and subcloning the *Bsp*HI-*Bgl*II fragment of the *Xenopus* clone (containing the entire coding sequence) into *Nco*I- and *Bam*HI-cut pET8c. This plasmid was transformed into *E. coli* LysS, and expression of the protein was induced with isothiopropylthiogalactoside (IPTG) for 3 h, after which the bacteria were pelleted by centrifugation and frozen at −70°C. The RCC1 protein expressed by induced bacteria fractionated to the inclusion bodies, and it was purified as follows. The cells were thawed and resuspended in 5 ml of buffer A (2.4 M sucrose, 40 mM Tris-HCl [pH 8.0], 10 mM EDTA) and allowed to sit on ice for 30 min; 20 ml of buffer B (50 mM Tris-HCl [pH 7.4], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg of lysozyme per ml, 75 μg of phenylmethylsulfonyl fluoride) was then added, and incubation on ice continued for 2.5 h. Then 20 μg of DNase I per ml, 10 mM $MgCl_2$, and 0.1% deoxycholate were added, and incubation on ice continued until the viscosity of the mixture decreased (about 30 min). The preparation was then centrifuged in an Beckman SW28 rotor for 30 min at 8,000 rpm to collect the inclusion bodies. The pellets were carefully resuspended in wash buffer (buffer B plus 10 mM EDTA and 0.5% Triton X-100) and allowed to sit at room temperature for 10 min. The inclusion bodies were centrifuged again in an SW28 rotor as described above and then solubilized in 10 ml of buffer B with 8 M urea. The preparation was incubated at 37°C for 30 min and then centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 20 min to remove bacterial debris. The urea was remove by stepwise dialysis in buffer C (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1 mM 2-mercaptoethanol) with 4, 2, and 0 M urea. The protein to be added to the extract was further dialyzed into XB* buffer (100 mM KCl, 1 mM $MgCl_2$, 50 mM sucrose, 10 mM HEPES [pH 7.7], 10% glycerol) and stored in small aliquots at −70°C. Protein prepared from inclusion bodies by this method and protein prepared by the method of Nishitani et al. (38) functioned equally well in in vitro assays.

**Determination of the RCC1 complement in reconstituted nuclei.** To determine the amount of RCC1 per nucleus, nuclei were formed in standard reconstitution reactions except that the proportion of membranes to cytosol was increased and aphidicolin (50 μg/ml) was added to the reaction mixtures. In standard reconstituted extracts, the ratio of membranes to cytosol is 1:9, whereas for this experiment, the ratio was 1:5. Sperm nuclei were prepared as described in Wilson and Newport (48) and added at the indicated concentrations. The reaction mixtures were incubated at room temperature for 90 min. After incubation, 10-μl aliquots of each reaction mixture were then diluted fivefold with XB and centrifuged for 5 min in a Eppendorf Microfuge. The upper 20 μl of the supernatant were reserved for Western immunoblots, and the remainder of the supernatant was drawn off and discarded. The pellet (which was approximately 3 μl in volume) was washed again with 50 μl of XB and then evenly resuspended in 15 μl of XB. Bradford protein assays were performed on the supernatants and resuspended pellets to check that the protein concentrations in supernatants and the pellets were roughly equal for all nuclear concentrations. Equal volumes of supernatants and of resuspended pellets were Western blotted by standard methods (20). Bovine serum albumin was used as a standard for any determinations of protein concentration.

**Chromatin assays.** Micrococcal nuclease assays were per-

formed by the method of Almouzni et al. (3). Briefly, 2 mM ATP, 20 mM creatine phosphate, 5 μg of creatine kinase per ml, and 10 μg of single-stranded M13 DNA (U.S. Biochemicals) per ml were added to depleted or mock-treated cytosol. The reaction mixtures were incubated at room temperature for 3 h to allow replication of the single-stranded DNA, and a sample was taken at the end of the incubation to be run on a 0.8% agarose–Tris-borate-EDTA (TBE) gel to ensure that the DNA had replicated to completion; 3 mM $CaCl_2$ and micrococcal nuclease (300 U/μg of input DNA) were added to the remaining sample, which was then incubated at room temperature. Aliquots were taken from the mixture at the indicated times, digestion was stopped by the addition of 30 mM EDTA, and the samples were placed on ice. After all samples were collected, RNase A (0.3 mg/ml) was added and the samples were incubated for 30 min at room temperature. The samples were then diluted with 2 volumes of 10 mM Tris–11 mM EDTA–0.5% sodium dodecyl sulfate (SDS) (pH 7.0), and pronase K was added to 0.3 mg/ml. Incubation was continued for 1 h at 37°C, after which the samples were extracted once with phenol and once with chloroform and then ethanol precipitated. The samples were resuspended in Tris-EDTA (TE; pH 7.0) and run on a 1.5% agarose–TAE gel (2), and the gel was stained with ethidium bromide to visualize the digestion products.

Supercoiling assays were performed as follows. Seven micrograms of pBSIIKS- plasmid DNA (in 1 mM Tris–180 mM sucrose–1.8 mM $MgCl_2$–35 mM KCl–5 mM ATP [pH 7.5]) was treated with 87 U topoisomerase I (gift of P. Laybourn and J. Kadonaga) and 1,400 U of T4 DNA ligase (Bethesda Research Laboratories) at 37°C for 40 min to give a relaxed population of supercoiled plasmids. By using depleted or mock-treated cytosol, reaction mixtures containing 20 μg of relaxed plasmid per ml, 2 mM ATP, 20 mM creatine phosphate, 5 μg of creatine kinase per ml, and 50 μg of aphidicolin per ml were made. (Cytosol constituted 80% of the final reaction volume.) The reaction mixtures were incubated at room temperature. Aliquots were removed at the indicated times, added to 1.8 volumes of stop buffer (1% SDS, 20 mM EDTA, 50 mM Tris [pH 8.0], 1 μg of RNase A per ml), and kept on ice. After all of the samples were collected, they were incubated at 37°C for 15 min. Pronase K (0.8 mg/ml) was then added to the samples, and incubation continued for 45 min at 37°C, after which the samples were extracted once with phenol and then ethanol precipitated. The samples were resuspended in TE (pH 7.0) and run on a 1.2% agarose–TBE gel, and the gel was stained with ethidium bromide to visualize the products.

## RESULTS

**Depletion of RCC1 from *Xenopus* extracts.** For our studies, crude soluble extracts were prepared by crushing *Xenopus* eggs by centrifugation, and these extracts were further fractionated by centrifugation at 200,000 × *g* to separate the membrane and cytosol fractions. When demembranated sperm chromatin was added to a mixture of these two fractions, the chromatin decondensed, formed nuclei, and then underwent a single, efficient round of replication (32, 48). To elucidate the role of RCC1 protein in *Xenopus* extracts, we prepared an affinity-purified antibody against the *Xenopus* RCC1 protein, which we had previously cloned and expressed in *E. coli* (38). Western blot analysis of the membrane and cytosol fractions and of the sperm chromatin showed that approximately 98% of the RCC1 protein is contributed by the cytosol fraction (Fig. 1A). A small

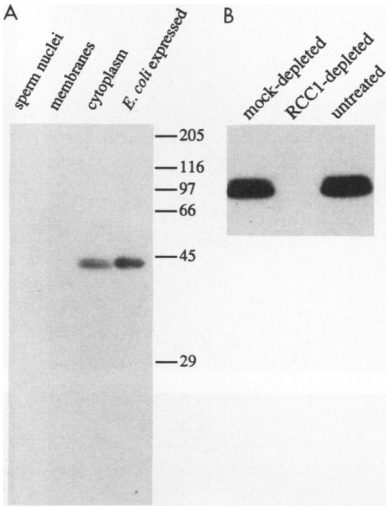


FIG. 1. Depletion of RCC1 protein from *Xenopus* extracts. (A) Fractionation of RCC1 to the cytosol with ultracentrifugation. Amounts of membranes, sperm nuclei, and cytosol equivalent to those in 1 μl of reconstituted extract (0.9 μl of cytosol, 0.1 μl of membranes, and 10,000 sperm nuclei) were run on an SDS-gel, transferred to polyvinylidene difluoride membranes, probed with purified anti-RCC1 antibodies, and visualized by using $^{125}$I-protein A. The RCC1 in reconstituted extracts is contributed almost entirely by the cytosol. A lane containing 6 ng of *E. coli*-expressed RCC1 is shown for comparison. (B) Depletion from the cytosol. Fractionated cytosol was mock depleted, RCC1 depleted by using affinity-purified anti-RCC1 antibodies, or left untreated. Equal volumes of all samples were run on an SDS-acrylamide gel, blotted, and probed as described above.

amount of the RCC1 in reconstituted extracts was contributed by the membranes, probably reflecting some contamination of the membrane fraction with cytosolic components, but no RCC1 was detectable in preparations of sperm chromatin. The lack of RCC1 on the chromatin added to our assays is probably due to the highly condensed state of sperm DNA, in which the chromatin does not possess the full complement of protein normally present on chromosomes (44). Since almost all of the RCC1 protein is present in the soluble fraction of the extract, this allowed us to remove essentially all RCC1 from reconstituted extracts by immunodepletion from the soluble cytosol without subjecting the particulate membrane fraction to the additional manipulations and centrifugations necessary for immunodepletion. Using anti-RCC1 antibodies bound to protein A-Sepharose beads, we depleted the RCC1 protein from our reaction mixtures by serial immunoprecipitations from the cytosol (Fig. 1B). We estimate that greater than 98% of the RCC1 was removed from the cytosol by this method. These RCC1-depleted extracts were used for the studies described below.

In mutant BHK cells, the loss of the RCC1 protein in *ts* mutants upon a temperature shift causes chromosome condensation and nuclear envelope breakdown before DNA replication is completed (36, 37, 39). On the basis of this observation, it was proposed that RCC1 might be directly involved in chromosome condensation and decondensation. To test whether RCC1 is directly involved in chromatin decondensation and nuclear formation, we added demembranated sperm chromatin to RCC1-depleted extracts and looked for the ability of the extract to make nuclei. We found that RCC1-depleted extracts were competent to decondense

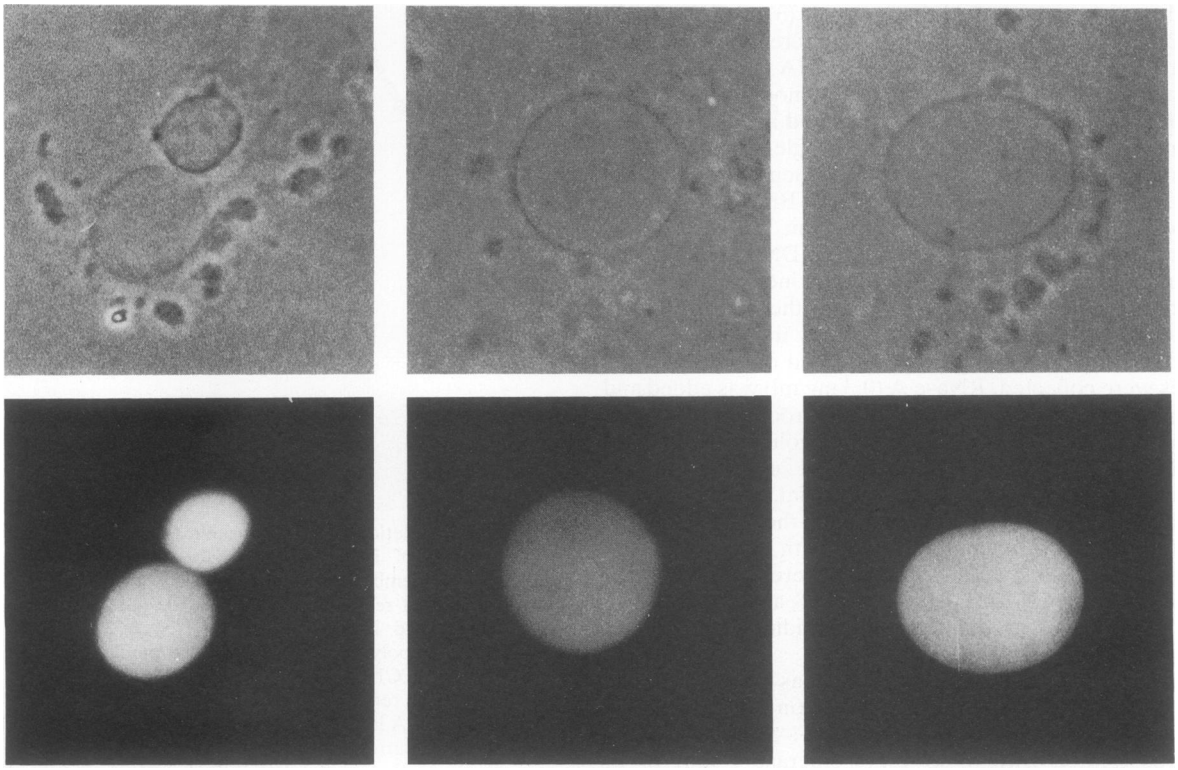

FIG. 2. Formation of nuclei in RCC1-depleted extracts. Demembranated sperm chromatin was added at 1,000 nuclei per μl to extracts reconstituted with RCC1-depleted (left panels), mock-depleted (center panels), and untreated (right panels) cytosol. Nuclei were photographed after being allowed to form for 2 h. The upper panels show phase-contrast images of nuclei; the lower panels show DNA staining with Hoechst 33258 DNA dye.

sperm chromatin and to form nuclei, although these nuclei were slightly smaller than those in mock-depleted and untreated extracts (Fig. 2). This finding suggests that the premature chromosome condensation observed in mutant BHK cells is not directly due to the absence of RCC1. Rather, it seems more likely that MPF is inappropriately activated when RCC1 is lost in these mutants, leading to the premature onset of mitosis. Our extracts were not competent to carry out the synthesis of the cyclin subunit of MPF (31) because they were prepared in the presence of cycloheximide, an inhibitor of translation. Thus, the removal of RCC1 protein from our extracts did not cause premature chromosome condensation as it does in *ts* RCC1 mutants because MPF is absent.

**RCC1-depleted extracts are incapable of DNA replication.** RCC1 mutants enter mitosis prematurely when shifted to the restrictive temperature. Therefore, if RCC1 had a function in interphase nuclei, this function might be difficult to observe at the restrictive temperature. However, fractionated extracts lack MPF and are thus arrested in interphase and unable to enter mitosis. This characteristic permitted us to investigate what role, if any, RCC1 plays during interphase. We were particularly interested in determining whether RCC1 has a role in DNA replication. If it does, this might imply some coordination between the replication machinery and systems detecting unreplicated DNA. The stable assembly of nuclei in RCC1-depleted extracts allowed us to examine DNA replication directly in the absence of RCC1. To do this, extracts were examined for their ability to incorporate radioactive deoxynucleoside triphosphates (dNTPs) into DNA by pulse-labelling aliquots of extract at 30-min intervals. The incorporation of dNTPs into sperm chromatin templates was assayed by running the replication products on agarose gels. These experiments clearly showed that the RCC1-depleted nuclei failed to replicate their DNA. Both the mock-depleted and undepleted extract replicated added sperm chromatin efficiently (Fig. 3A). The mock-depleted and undepleted extracts both initiated replication within 60 min of the beginning of the reaction, showing a peak in dNTP incorporation at between 90 and 120 min. In contrast, there was no detectable DNA replication in the RCC1-depleted extract at any time during the experiment.

To prove that the replication deficiency was directly due to the depletion of RCC1, RCC1 protein expressed in *E. coli* (38) was added back to depleted extracts. When purified *E. coli*-expressed RCC1 protein was added back to the depleted extract, the ability to replicate sperm chromatin was largely restored (Fig. 3B). At the highest concentration of added protein shown, replication was restored to approximately 32% of the control level. We consistently observed this level of rescue with the addition of *E. coli*-expressed proteins and saw little inhibition or enhancement of deoxynucleotide incorporation in undepleted extracts below 50 μg of added protein per ml. The incorporation of dNTPs by extracts supplemented with bacterially expressed RCC1 was aphidicolin sensitive (data not shown), demonstrating that this synthesis was mediated by polymerases α and δ rather than by repair enzymes (19, 47). Our results demonstrates that the effect of immunodepletion on replication is specific to RCC1 and that RCC1 is absolutely required for the replication of sperm chromatin. The inability of RCC1-depleted extracts to replicate added sperm chromatin suggests that RCC1 either

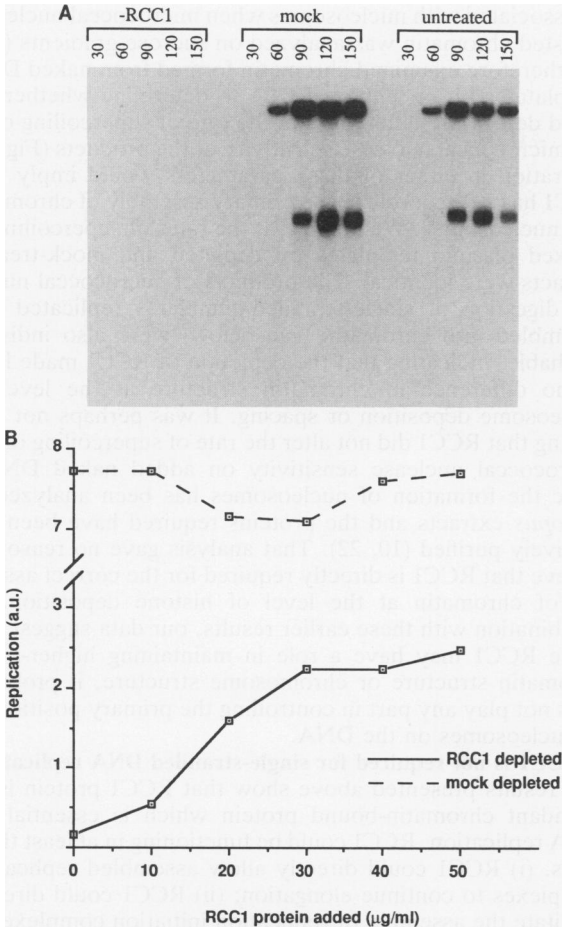


FIG. 3. Replication in RCC1-immunodepleted extracts. (A) Nuclei formed from demembranated sperm chromatin fail to replicate DNA in RCC1-depleted extracts. Extracts were reconstituted with RCC1-depleted (−RCC1), mock-depleted, or untreated cytosol, and sperm chromatin was added to a concentration of 1,000 nuclei per μl. Aliquots were removed at 30-min intervals and pulse-labelled with [α-$^{32}$P]dATP for 30 min, and the labelling was terminated by the addition of sample buffer. The incorporation of label into high-molecular-weight DNA was visualized by running the products of the pulse-labelled reactions on a 0.8% agarose gel. (B) The addition of *E. coli*-expressed RCC1 protein rescues the replication of chromatin in depleted extracts. *E. coli*-expressed RCC1 protein was added at the indicated concentrations to extracts containing sperm chromatin (1,000 nuclei per μl) which had been reconstituted from either RCC1-depleted or mock-treated cytosol. These reactions were constitutively labelled with [α-$^{32}$P]dCTP for 4 h and stopped by the addition of sample buffer, and the products were run on an agarose gel. The amount of replication was quantitated by scanning an autoradiogram of the dried gel with an LKB Ultrascan scanning densitometer. Two different exposures of the gel were scanned to ensure that the X-ray film was within its linear range.

participates directly in replication or performs some function which is a prerequisite for replication.

It is possible that RCC1 exists in a complex with other proteins in *Xenopus* extracts and that those proteins are also required for efficient replication. We rarely saw rescue exceed 40% of the incorporation of control extracts, and adding concentrations of expressed protein above 50 μg/ml caused increasing inhibition of replication in both depleted and control extracts with higher concentration of RCC1 protein. If another protein were being codepleted, the inability of bacterially produced RCC1 to restore replication to control levels might reflect the low concentration of the associated protein. We have looked extensively for RCC1-associated proteins, but we have not found evidence for any such proteins. On silver-stained or Coomassie blue-stained SDS-polyacrylamide gels, there are some differences between the profiles of proteins eluted from anti-RCC1 and mock-treated beads, but these differences are relatively minor and have not been reproducible between batches of extract. We have also investigated the association between RCC1 and the Ran protein, since they are known to exist in a chromatin-bound complex (4, 5). We found very little association of Ran with RCC1 in the absence of chromatin (data not shown), and we think it unlikely that Ran depletion had any significant impact upon replication. There are a number of alternative reasons why the exogenous RCC1 protein might have failed to fully restore replication, even when added back at five- to eightfold the original concentration; for example, the *E. coli*-expressed protein may not be fully functional because of failure to renature properly or because of the absence of some modification required for full activity. The present data suggest that these alternatives are more likely, but the possibility that RCC1 is associated with a minor species required for replication deserves further consideration and investigation.

**RCC1 is present at high concentrations on chromatin of reconstituted nuclei.** Previous reports have demonstrated that the RCC1 protein present in cells is located within the nucleus, bound to chromatin (40). To further examine the role of RCC1 in DNA replication, we determined the amount of RCC1 protein found in reconstituted *Xenopus* nuclei. This was done to determine whether the stoichiometry of RCC1 bound to DNA was consistent with RCC1 playing an enzymatic role in DNA synthesis. In this experiment, increasing amounts of demembranated sperm chromatin were added to extracts and allowed to form nuclei. The extracts were then centrifuged to separate the nuclei from the cytoplasm, and the amount of RCC1 protein in the two fractions was assayed by Western blotting. Results of this experiment showed that with increasing concentrations of sperm chromatin, more RCC1 was in the pellet, which contained the nuclei, and less was in the supernatant (Fig. 4A). When demembranated sperm chromatin was incubated with cytosol alone and centrifuged to separate the chromatin from the cytosol, the RCC1 protein also fractionated increasingly to the pellet with higher sperm concentrations (data not shown). Since nuclei do not form under these conditions, this result shows that RCC1 binds directly to the chromatin rather than accumulating in the nucleus solely by transport.

A plot of the amount of RCC1 remaining in the supernatant as a function of nuclear concentration demonstrated that the amount of residual RCC1 in the supernatant was linearly dependent on the number of nuclei (Fig. 4B). Extrapolation of this linear relationship to high nuclear concentrations indicated that all of the RCC1 protein in the extract would be bound to chromatin at a concentration of approximately 6,000 sperm nuclei per μl. It was not possible to confirm this number by direct experimentation, because above approximately 4,000 nuclei per μl, other components of the extract required to assemble nuclei become limiting (48). This was also reflected by decreased nuclear size at concentrations above 5,000 nuclei per μl. To determine how much RCC1 protein was present in each nucleus, we determined the total amount of RCC1 present in 1 μl of the soluble fraction of a extract and then divided this amount by 6,000. The concen-

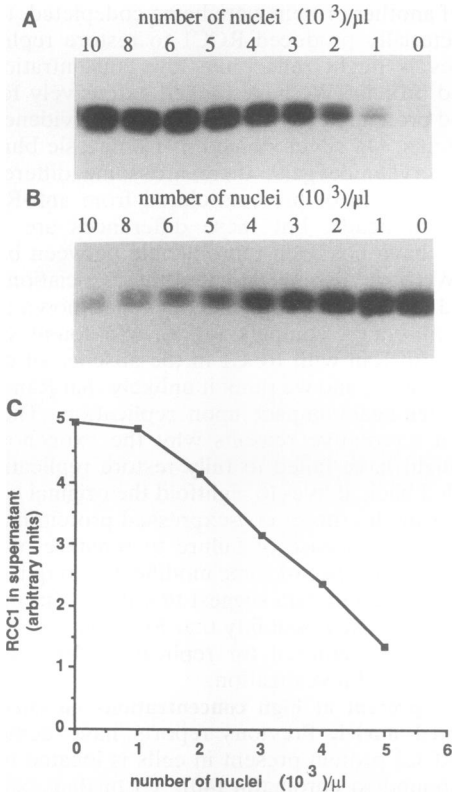


FIG. 4. Estimation of the amount of RCC1 protein per nucleus. (A and B) RCC1 protein increasingly fractionates to the pellet with increasing sperm chromatin concentrations. Sperm chromatin was added to a reconstituted extract at the indicated number of nuclei per microliter. After nuclei were allowed to form for 1 h, the reaction mixtures were crudely fractionated into an insoluble pellet (A) which contained the DNA and a soluble supernatant (B) which contained free RCC1 protein. Approximately equal amounts of pellet protein and equal amounts of supernatant protein were run on SDS-acrylamide gels, immunoblotted with affinity-purified anti-RCC1 antibodies, and visualized with $^{125}$I-protein A. (C) The RCC1 protein in the supernatant allows for the formation of 6,000 nuclei per μl. The supernatant blot shown in panel B was quantitated by scanning densitometry, and the relative amount of free RCC1 protein was plotted as a function of the nuclear number. An estimate of the amount of RCC1 per nucleus was taken from the linear portion of this curve. The inability of even very high nuclear concentrations (10,000 nuclei per μl) to exhaust the free RCC1 in the supernatant may reflect the aberrant formation of nuclei at such excessive chromatin concentrations.

tration of RCC1 per microliter of extract was quantitated by Western blotting, using purified *E. coli* RCC1 protein as a standard (data not shown), and found to be 6 ng/μl, or 1 pg per nucleus. On the basis of these data and a haploid *Xenopus* genome size of $2.8 \times 10^9$ bp (7), we estimate that RCC1 is present on the DNA at the surprisingly high frequency of one RCC1 monomer per 210 bp of DNA, or approximately one RCC1 monomer per nucleosome. Although this high concentration of RCC1 on the DNA does not rule out an enzymatic role in replication, it would be more consistent with a role of RCC1 in chromatin or nuclear structure.

**Depletion of RCC1 does not alter nucleosome assembly.** The high concentration of RCC1 on the chromatin of reconstituted nuclei suggests that RCC1 is a structural component of the chromosome. Further, RCC1 was previously shown to be associated with nucleosomes when micrococcal nuclease-digested chromatin was analyzed on sucrose gradients (17). We therefore examined chromatin formed from naked DNA templates with or without RCC1 to determine whether we could detect any differences in the rate of supercoiling or in the micrococcal nuclease sensitivity of the products (Fig. 5). Alteration in either of these parameters would imply that RCC1 has a direct role in the primary assembly of chromatin into nucleosomes. We found that the rates of supercoiling of relaxed plasmid templates by depleted and mock-treated extracts were identical. The products of micrococcal nuclease digestion of single-stranded templates replicated and assembled into chromatin (see below) were also indistinguishable, indicating that the depletion of RCC1 made little or no difference in chromatin structure at the level of nucleosome deposition or spacing. It was perhaps not surprising that RCC1 did not alter the rate of supercoiling or the micrococcal nuclease sensitivity on added naked DNAs, since the formation of nucleosomes has been analyzed in *Xenopus* extracts and the proteins required have been extensively purified (10, 22). That analysis gave no reason to believe that RCC1 is directly required for the correct assembly of chromatin at the level of histone deposition. In combination with these earlier results, our data suggest that while RCC1 may have a role in maintaining higher-order chromatin structure or chromosome structure, it probably does not play any part in controlling the primary positioning of nucleosomes on the DNA.

**RCC1 is not required for single-stranded DNA replication.** The results presented above show that RCC1 protein is an abundant chromatin-bound protein which is essential for DNA replication. RCC1 could be functioning in at least three ways. (i) RCC1 could directly allow assembled replication complexes to continue elongation; (ii) RCC1 could directly facilitate the assembly of replication initiation complexes at replication origins, or (iii) RCC1 could be required for the integrity of a chromatin or nuclear structure which is essential for DNA replication but does not participate enzymatically in replication. Although it is currently difficult to differentiate between the last two possibilities, it is possible to investigate whether an assembled replication complex can elongate in the absence of RCC1 protein. To address this question, we took advantage of the observation that replication complexes will assemble onto and replicate single-stranded DNA templates added to *Xenopus* extracts (29). The replication of chromatin templates displays a strong requirement for the prior formation of nuclear structures (34, 46). By contrast, single-stranded DNAs display very efficient (>95%), aphidicolin-sensitive replication in *Xenopus* extracts, but they do not require nuclear structures in order to replicate (2, 3). Further, single-stranded DNAs replicate under conditions in which closed nuclear envelopes could not form (in the presence of cytosol alone), unlike double-stranded templates. To determine whether RCC1 is essential for the elongation phase of replication, we determined whether extracts depleted of RCC1 were able to replicate single-stranded DNA templates. RCC1- and mock-depleted extracts were assayed for the ability to replicate single-stranded M13 DNA (Fig. 6). We found that the replication of single-stranded M13 DNA was unaltered by the depletion of RCC1. The results of this experiment argue against a role for RCC1 as an essential component of the replication complex. This experiment does not address whether RCC1 has other activities required for replication fork movement through double-stranded DNA, such as a helicase function. However, earlier experiments on *ts* RCC1 mutants, which exam-

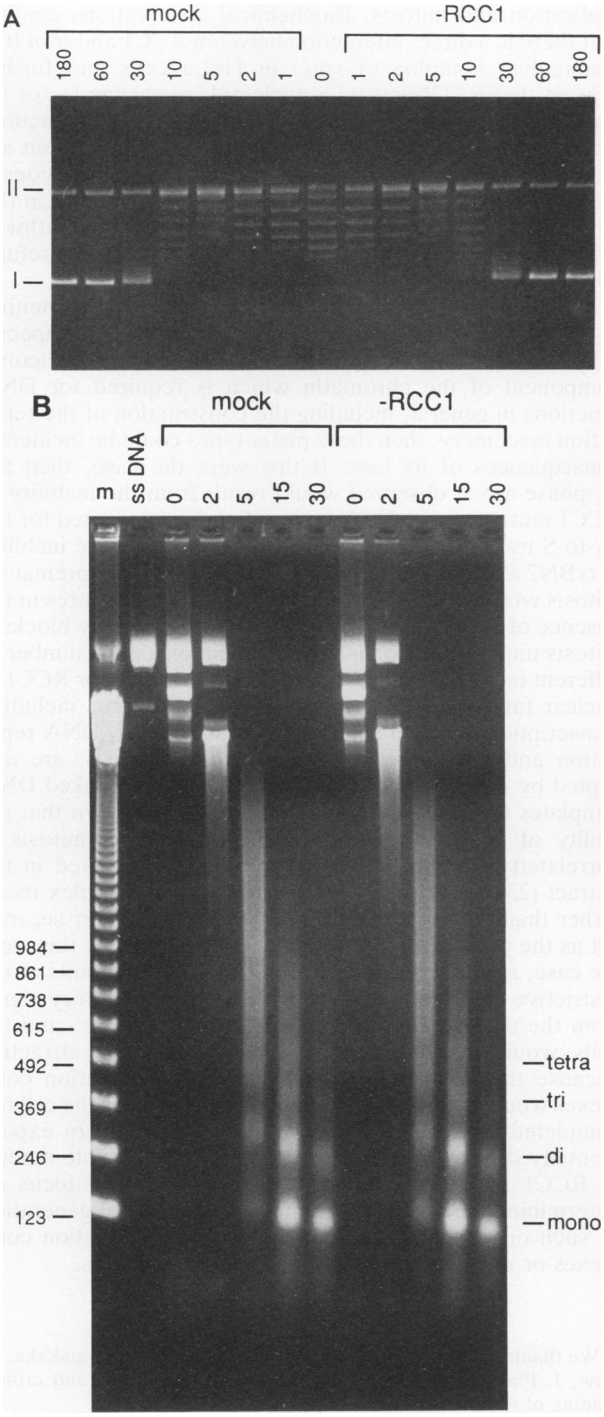


FIG. 5. Evidence that RCC1 depletion does not alter chromatin structure at the level of nucleosomes. (A) Supercoiling of plasmid templates is independent of RCC1. Relaxed plasmid (lane 0) was added to reaction mixtures with RCC1-depleted (−RCC1) or mock-treated cytosol as described in Materials and Methods. Aliquots of the reaction mixtures were removed at the times indicated (in minutes) and treated as described in the text. There were no visible differences in the rate or the extent of supercoiling of the added template. (B) Nucleosome assembly onto replicating single-stranded DNA is independent of RCC1. Almouzni and Mechali (2) demonstrated that spaced chromatin is efficiently assembled onto single-stranded DNAs as they are replicated in *Xenopus* extracts. Since single-stranded DNAs replicated efficiently in the absence of RCC1 (Fig. 6), we examined chromatin assembly on replicating M13mp18 DNA. The DNA was allowed to replicate for 3 h in reactions with depleted (−RCC1) or mock-treated cytosol and then treated with micrococcal nuclease for the times indicated (in minutes). Lane m is a 123-bp ladder DNA marker (Bethesda Research Laboratories). Micrococcal nuclease patterns were unaltered by assembly in the absence of RCC1. We did not find any difference in the chromatin produced through unreplicative (A) and replicative (B) pathways, suggesting that RCC1 does not affect the association of nucleosomes with DNA by either pathway of assembly. Sizes are indicated in base pairs.

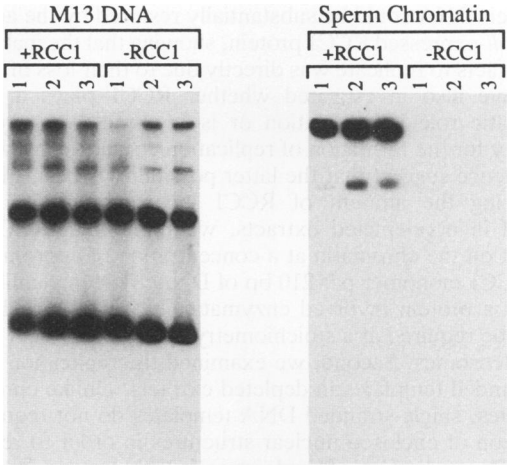


FIG. 6. Evidence that replication of M13 single-stranded DNA is not inhibited by RCC1 depletion. Extracts were reconstituted from mock-depleted (+RCC1) or RCC1-depleted (−RCC1) cytosol and then divided in half. To one half (left), M13 DNA was added at a concentration of 3 ng/μl, and to the other half (right), demembranated sperm chromatin was added to a concentration of 1,000 nuclei per μl. These reactions were constitutively labelled with [α-$^{32}$P]dATP. Samples were taken at 1, 2, and 3 h, stopped with sample buffer, and run on agarose gels.

ined the shutoff of replication as cells prematurely condensed their chromosomes, have suggested that RCC1 is not directly involved in the elongation phase of chromosomal replication (13). It thus appears that RCC1 is not essential for movement of replication complexes along either single- or double-stranded templates. These results indicate that RCC1 either plays an essential role during the formation of initiation complexes or is a structural component of the chromosome which is essential for the formation of these complexes. The abundant amounts of RCC1 found on the chromatin might suggest that the latter possibility should be considered the more likely.

## DISCUSSION

We have used partially fractionated *Xenopus* extracts to investigate the function of RCC1, a protein known to be essential for the coupling of the completion of DNA synthesis to the initiation of mitosis. By immunodepletion of the RCC1 protein from egg extracts, we have found that RCC1 is not required for the decondensation of sperm chromatin, the formation of nucleosomes onto naked DNA templates, or the formation of nuclei. However, RCC1 was absolutely required for the replication of sperm chromatin templates in *Xenopus* extracts, as its depletion abolished the competence of extracts to replicate. The ability of depleted extracts to repli-

cate their DNA could be substantially restored by the addition of *E. coli*-expressed RCC1 protein, showing that the inability of the extracts to replicate was directly due to their loss of RCC1. We have also investigated whether RCC1 plays a direct, enzymatic role in replication or is required in a structural capacity for the formation of replication complexes. Two lines of evidence suggest that the latter possibility is true. First, by examining the amount of RCC1 incorporated into nuclei formed in nondepleted extracts, we found that RCC1 was present on the chromatin at a concentration of approximately one RCC1 monomer per 210 bp of DNA. It seems unlikely to us that a protein involved enzymatically in DNA replication would be required at a stoichiometry nearly equivalent to that of nucleosomes. Second, we examined the replication of single-stranded templates in depleted extracts. Unlike chromatin templates, single-stranded DNA templates do not require the formation of enclosed nuclear structures in order to replicate (2, 3). The replication of single-stranded DNA was not affected by RCC1 depletion, showing both that these templates required neither nuclear structures nor RCC1 function for replication and that RCC1 does not play any enzymatic role in the replication of these templates. Together with results of earlier reports, our results suggest that RCC1 is a structural component of chromatin required for authentic nuclear DNA replication and that RCC1's function is also essential for the transmission of the signal preventing premature mitosis before the completion of DNA replication. Our results provide the first direct link between the DNA replication machinery and systems detecting incomplete replication in the cell cycle.

There are several phenotypes associated with cells carrying mutations in the RCC1 protein. The original genetic screen in which RCC1 was isolated selected for BHK cell mutants which arrested at the restrictive temperature in the $G_1$ phase of the cell cycle prior to replication. It was subsequently observed that a cell line mutated in the RCC1 gene (*ts*BN2 cells) was not only subject to $G_1$ arrest but also defective in its ability to prevent mitosis prior to the completion of S phase (36). It was originally unclear how RCC1 could be involved both in the $G_1$-to-S transition and in monitoring the progression of S phase. There are at least two possible ways in which RCC1 could function to give these phenotypes. One possibility presented by our data is that RCC1 performs some function in the initiation of replication and that this function is necessary to trigger a signal characteristic of S phase. This function could be responsible for both phenotypes. First, without RCC1, the cell would not be able initiate replication and enter S phase. Therefore, the cell would remain arrested in $G_1$ phase. Second, RCC1 would be necessary throughout S phase for the generation of signals that keep mitotic functions (i.e., MPF) from becoming prematurely active. If RCC1 were destroyed after the cell had entered S phase, existing replication forks would elongate and terminate normally but no new initiation complexes would form. As a consequence, the RCC1-dependent signal would attenuate, MPF could activate before replication is complete, and the cell would enter mitosis prematurely. This model proposes that the activation and inactivation of the RCC1 signalling pathway is tightly coupled to the presence of DNA replication complexes and that inactivation of this pathway would define the end of S phase. Studies in *S. pombe* (28) may suggest a direct role for RCC1 in signal transduction. Mutations in *pim1*, the *S. pombe* homolog of RCC1, are suppressed by the overexpression of *spi1*, a small Ras-like G protein. Mutations in *spi1* show a premature mitosis phenotype similar to that of *pim1*, so *spi1* may also function in a signal transduction pathway which couples replication and mitosis. Biochemical observations confirm that there is a direct interaction between RCC1 and *Ran* (the mammalian homolog of *spi1*) in HeLa cells and further suggest that RCC1 acts as a nucleotide exchange factor for Ran (4). This interaction may facilitate signal transduction. However, it should be noted that both RCC1 and Ran are extremely abundant in mammalian cells, Ran protein constituting approximately 0.36% of the total cellular protein (5). Given this high concentration, a signal transduction pathway relying upon RCC1 and Ran would have to be carefully constructed to ensure adequate sensitivity.

A second possibility is that RCC1's primary role is neither in the $G_1$-to-S transition nor in monitoring S phase specifically. Rather, if RCC1 functioned as an essential structural component of the chromatin which is required for DNA functions in general, including the construction of the replication machinery, then these phenotypes could be incidental consequences of its loss. If this were the case, then the $G_1$-phase arrest observed would result from the inability of RCC1 mutants to perform nuclear functions required for the $G_1$-to-S transition, including DNA replication. The inability of *ts*BN2 at the restrictive temperature to prevent premature mitosis would be due to aberrant chromatin structures in the absence of RCC1 which disrupt the signal normally blocking mitosis until replication is complete. Studies on a number of different organisms might support a general role for RCC1 in nuclear functions, since many nuclear functions, including transcription (37) and splicing (1) in addition to DNA replication and the prevention of premature mitosis, are disrupted by the absence of RCC1. Studies using naked DNA templates in *Xenopus* cycling extracts have shown that the ability of a template to prevent the onset of mitosis is correlated with the extent to which it is replicated in the extract (23) and suggest that the replication complex itself, rather than the presence of unreplicated DNA per se, may act as the primary origin of the feedback signal. If this were the case, as replication is disrupted in RCC1 mutants at the restrictive temperature, there would be no inhibitory signal from the unreplicated DNA, MPF would activate, and the cells would enter mitosis. This hypothesis is attractive because the presence or absence of the replication complexes would be a very good indicator of whether the cell has completed replication and should enter mitosis. In experimentally distinguishing between these two possible models of RCC1 function, our future investigation will focus on determining whether RCC1 is involved in signal generation as such or in facilitating the formation of replication complexes or other structures on chromatin templates.

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