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cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication

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RPA is a single-stranded DNA binding protein complex purified from human cells and is essential for the initiation and elongation stages of SV40 DNA replication *in vitro*. In both human and yeast cells, the 34 kDa polypeptide subunit of RPA is phosphorylated in the S and G_2 phases of the cell cycle and not in G_1. One of the major RPA kinases present in extracts of human cells was purified and shown to be the cyclin B–*cdc2* complex. This purified kinase, and a closely related cyclin A associated *cdc2*-like kinase, phosphorylated RPA p34 on a subset of the chymotryptic peptides that were phosphorylated *in vivo* at the G_1–S transition. Two serines near the N-terminus of RPA p34 were identified as possible sites of phosphorylation by *cdc2* kinase. These same serines were necessary for RPA phosphorylation *in vivo*. The purified *cdc2* kinase stimulated SV40 DNA replication *in vitro* when added to G_1 cell extracts. The kinase also stimulated unwinding at the origin of replication, one of the earliest steps in DNA replication requiring RPA, but only in the presence of an additional factor present in G_1 cell extracts. Thus, one or more members of the cyclin–*cdc2* kinase family may be required for the initiation and maintenance of S phase, in part due to their ability to phosphorylate and activate a cellular DNA replication factor, RPA.

Key words: *cdc2* kinase/cell cycle/cyclin/DNA replication/RPA/S phase

Introduction

Rapid advances in the understanding of eukaryotic DNA replication have come from studies on SV40 DNA replication *in vitro*. A plasmid containing the SV40 origin of DNA replication (ori) is replicated faithfully *in vitro* in the presence of SV40 large T antigen (T Ag) and an extract of human cells, HeLa or 293 (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). Using this system, several human replication factors have been purified from the cellular extract (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990; Ishimi *et al.*, 1988; for reviews see Stillman, 1989; Challberg and Kelly, 1989). Since DNA replication is subject to cell cycle control and regulation by growth factors, we have begun to use our knowledge of cellular DNA replication factors to understand the mechanism of this regulation. It was anticipated that the regulatory pathways that directly affect the DNA replication factors would, in turn, be controlled by known cell cycle and growth factor pathways.

One of these cellular factors, replication protein A (RPA, previously known as RF-A or human ssb), is essential for SV40 DNA replication *in vitro*. It is a multi-subunit complex containing three polypeptides of 70, 34 and 11 kDa, of which the 70 kDa polypeptide has been shown to be a single-stranded DNA binding protein (Fairman and Stillman, 1988; Goetz *et al.*, 1988; Wold and Kelly, 1988; Kenny *et al.*, 1989; Wold *et al.*, 1989; Erdile *et al.*, 1991). The factor is required for T Ag dependent unwinding of the SV40 ori (Borowiec *et al.*, 1990). RPA promotes DNA synthesis by DNA polymerase α (pol α), inhibits non-specific initiation by pol α on the 3′ ends of Okazaki fragments, stimulates the leading strand polymerase complex comprised of DNA polymerase δ, PCNA and RFC, and physically interacts with pol α–primase (Tsurimoto *et al.*, 1989; Tsurimoto and Stillman, 1989a,b, 1991a,b; Collins and Kelly, 1991; Matsumoto *et al.*, 1991; Dornreiter *et al.*, 1992). Antibody inhibition studies have suggested that the p34 subunit is involved in the interaction of RPA with pol α, and in the initiation of DNA replication (Kenny *et al.*, 1990; S.-U.Din and B.Stillman, manuscript in preparation). The requirement for RPA in chromosomal DNA replication is underscored by the discovery that all three genes for the three subunits of RPA are essential for the viability of *Saccharomyces cerevisiae* (Brill and Stillman, 1989, 1991; Heyer *et al.*, 1990).

We have demonstrated previously that while the abundance of RPA remains unchanged throughout the cell cycle, the factor is modified by phosphorylation in a cell cycle phase specific manner (Din *et al.*, 1990). The 34 kDa subunit is phosphorylated in the S and G_2 phases of the cell cycle but is not in the G_1 phase. The phosphorylation is accompanied by a significant decrease in the mobility of RPA p34 during gel electrophoresis, suggesting that there is a change in the conformation of the polypeptide. Identical results were obtained with RPA purified from *S.cerevisiae* (Brill and Stillman, 1989; Din *et al.*, 1990). The strict cell cycle phase specific phosphorylation, the accompanying change in conformation of the protein and the evolutionary conservation of both these features from yeast to man, suggested a regulatory role of the phosphorylation in the activity of RPA and in DNA replication.

In this paper we address the following questions. How does the cell cycle phase specific phosphorylation of RPA correlate with the onset of DNA replication? What are the kinases involved? Does phosphorylation affect the activity of RPA as a replication factor? RPA was shown to be phosphorylated on multiple peptides at the G_1–S transition. RPA purified from asynchronously growing 293 cells was mostly in the dephosphorylated form presumably due to the action of phosphatases during the purification process. However, it was rapidly phosphorylated by at least three protein kinase activities present in the replication extract. One of the major RPA kinases was purified and found to be identical to cyclin B–*cdc2* kinase. The *cdc2* kinase stimulated DNA replication and origin unwinding in extracts

purified from G_1 phase cells. Furthermore, a cyclin A associated kinase which was activated earlier in the cell cycle than the cyclin B−*cdc2* kinase phosphorylated RPA on the same peptides as the cyclin B−*cdc2* kinase.

These results suggest a biochemical mechanism by which a cyclin−*cdc2* complex acts as an S phase promoting factor required for progression of the cell cycle from G_1 to S, and perhaps for the maintenance of S phase.

Results

RPA is phosphorylated at the G_1−S transition

To time the phosphorylation of RPA accurately with respect to the onset of DNA synthesis, HeLa cells were synchronized in the G_1 phase of the cell cycle. As these cells entered S phase (Figure 1), RPA phosphorylation was monitored by pulse-labeling the cells with [^{32}P]orthophosphate and immunoprecipitating RPA p34 from cell extracts. Entry into S phase was followed by pulse-labeling parallel cultures with [^{3}H]thymidine. Phosphorylation of RPA p34 began at the G_1−S transition.

To determine if the sites of phosphorylation changed at different stages of the cell cycle, chymotryptic phosphopeptide maps of RPA p34 obtained from cells pulse-labeled with ^{32}P in early S, mid S, late S and G_2−M stages were done (Figure 2). The same peptides were phosphorylated at all the stages examined.

Purification and identification of an RPA kinase

In order to identify protein kinases that phosphorylate RPA, purified human RPA was used as a substrate. More than 90% of the purified RPA p34 was in a more rapidly mobile, dephosphorylated form (S.-U.Din and B.Stillman, unpublished observations). Extracts prepared from 293 or HeLa cells by 0.1% Triton lysis or by hypotonic lysis (S100 extract) contained protein kinase activities that efficiently phosphorylated this dephosphorylated RPA in an *in vitro* kinase reaction. Fractionation of the S100 extract on a phosphocellulose column showed that the major RPA kinase activity was present in fraction PC IIA (0.2−0.33 M NaCl), a fraction that was essential for DNA replication and known to contain DNA polymerases α and δ (Tsurimoto *et al.*, 1990; Melendy and Stillman, 1991). The RPA kinase activity in fraction PC IIA was subsequently fractionated into three different activities. The fraction accounting for >50% of the total activity has been purified to apparent homogeneity through six additional fractionation steps and will be described here. The two other RPA kinases have also been purified; they phosphorylate RPA on sites not phosphorylated by the first kinase (unpublished results).

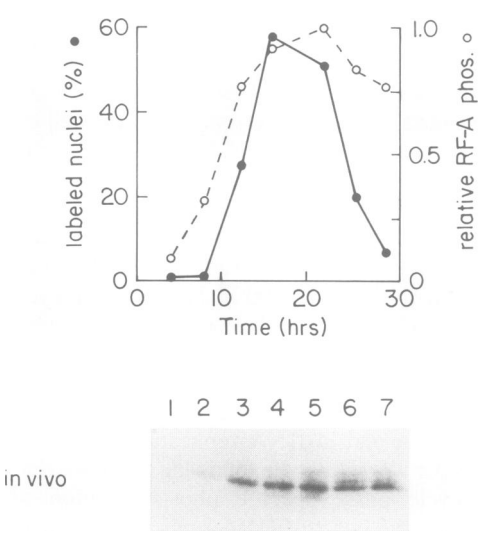

Fig. 1. Cell cycle regulation of RPA phosphorylation. HeLa cells in the G_1 phase of the cell cycle were put into culture and pulse-labeled for a 1 h period at the indicated times with either [^{32}P]orthophosphate or [^{3}H]thymidine. Phosphorylation of RPA p34 was determined by immunoprecipitation of the protein from equal numbers of cells with a monoclonal antibody (lane 1 from the first time-point, lane 2 from the second etc.). The amount of radioactivity in the p34 band in each lane was quantified with a phosphorimager and expressed as a fraction of the maximum observed (relative RF-A or RPA phosphorylation). This fraction is plotted along with the percentage of thymidine-labeled nuclei for each time point in the graph above.

Figure 3 shows a protein gel of fractions across a glycerol gradient, the final step in the purification. The RPA kinase sedimented in fractions 9−13, peaking in fraction 11 (Figure 3B). These same fractions contained polypeptides of 62 kDa, 45 kDa and a doublet of 31 kDa, all of which also peaked in fraction 11 (Figure 3A). The subunit composition of the RPA kinase was reminiscent of a cyclin−*cdc2* complex. When the substrate specificity of the RPA kinase was examined, these same fractions phosphorylated a known substrate of *cdc2* kinase, a bacterially produced fragment of T Ag of wild-type sequence containing Thr124 (Figure 3C), but not if the substrate Thr124 was mutated to alanine (Figure 3D; McVey *et al.*, 1989).

Immunoblots of fractions from another glycerol gradient supported the identification of the purified kinase as cyclin B−*cdc2*. The 62 kDa protein reacted specifically with a polyclonal antibody against cyclin B (Figure 4B) and not a monoclonal antibody against cyclin A (C160, data not shown). One of the 31 kDa bands was recognized by G8, a polyclonal antibody against the *cdc2* protein of *Schizosaccharomyces pombe* (Figure 4C) which recognizes human *cdc2* protein, and also by G6, a polyclonal antibody to the C-terminal six amino acids of the human *cdc2* protein (data not shown). An antibody raised against the PSTAIRE peptide common to several members of the *cdc2* kinase family recognized a doublet of 31 kDa, while an anti-cyclin B2 antibody did not recognize the 45 kDa polypeptide (data not shown).

Additional data which confirmed the identification of the RPA kinase as cyclin B−*cdc2* were as follows. The purified kinase complex autophosphorylated a protein of 62 kDa (marked cyclin B in Figure 3B) which was immunoprecipitated by anti-cyclin B and anti-*cdc2* antibodies (data not shown). Furthermore, the purified kinase had strong histone H1 kinase activity which was immunoprecipitated by an anti-cyclin B antibody but not by a polyclonal anti-cyclin A antibody. Finally, phosphorylation of RPA by the purified kinase was competitively inhibited by peptides that are specific substrates of the *cdc2* kinase complex (Figure 6A). These include the TPXKK peptide that mimics the phosphoacceptor sites of nucleolin (Peter *et al.*, 1990) and a peptide that is modeled after the *cdc2* phosphorylation site on SV40 T Ag (CSH103, Marshak *et al.*, 1991). In contrast, a peptide which has a sequence similar to CSH103 but is not a substrate of the *cdc2* kinase (CSH119) had little effect on the RPA kinase activity.

Phosphopeptide map of RPA phosphorylated in vivo and in vitro

To ensure that the purified kinase phosphorylated RPA p34 on peptides that were phosphorylated *in vivo*, phosphopeptide maps were performed. Cyclin B−*cdc2* kinase incorporated 1 mol phosphate/mol RPA. A chymotryptic phosphopeptide map of RPA labeled with phosphate in 293 cells (Figure 5A) was compared with that of RPA labeled in the *in vitro* kinase reaction (Figure 5B). Cyclin B−*cdc2* phosphorylated RPA p34 on peptides 1, 2 and 4, which are a subset of phosphopeptides obtained *in vivo* (determined by mixing the two sets of peptides, Figure 5C and D). One phosphopeptide, number 3 (Figure 5B) appeared to be an *in vitro* artifact. The presence of multiple phosphopeptides with RPA phosphorylated *in vitro* by cyclin B−*cdc2* kinase is at odds with the calculated incorporation of 1 mol phosphate/mol RPA p34. The discrepancy was probably due to incomplete digestion of the peptides, a hypothesis supported by results presented later which suggest that the four phosphopeptides were derived from phosphorylation by *cdc2* of two sites at

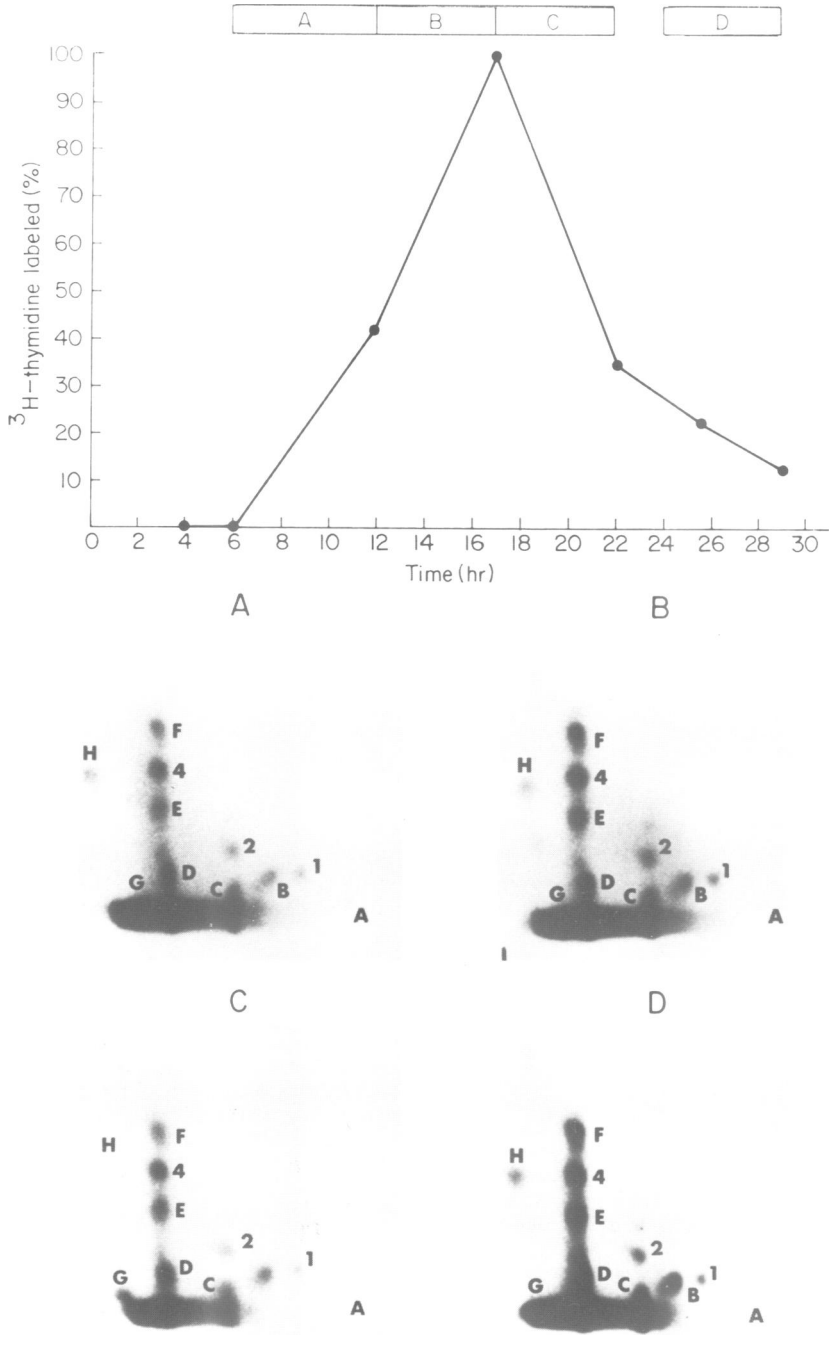

Fig. 2. Phosphopeptide map of ^{32}P-labeled RPA p34 across the cell cycle. In an experiment similar to that shown in Figure 1, cultures were labeled with ^{32}P during the time-periods A−D indicated by the bars above the graph, or pulse-labeled for 1 h periods with [^{3}H]thymidine at the points shown. Chymotryptic phosphopeptide maps of immunoprecipitated and gel-purified RPA p34 from each of these periods is shown below. Electrophoresis is in the horizontal direction with the anode to the left. TLC is from the bottom to the top. The origin is below and to the left. Nomenclature of peptides is explained in Figure 5.

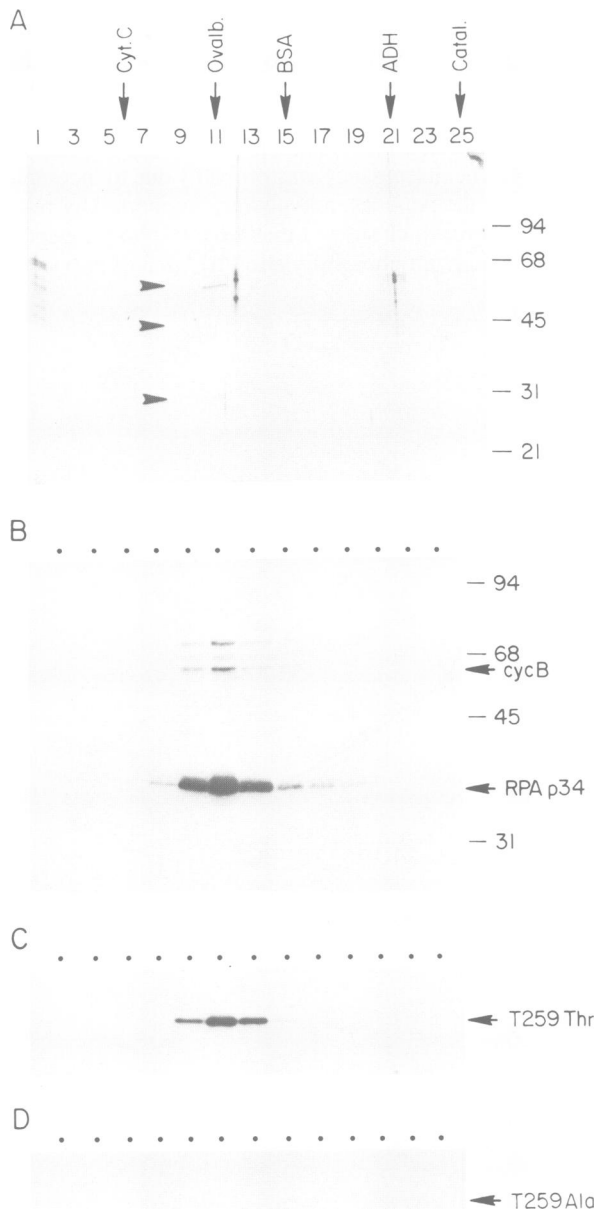

Fig. 3. Glycerol gradient fractionation of the purified RPA kinase. (**A**) Silver stain of 10 μl of the indicated fractions from the glycerol gradient. Sedimentation positions of indicated molecular weight marker proteins are indicated with arrows. To the right of the gel, molecular weight markers for the electrophoresis are shown. Arrowheads point to protein bands of 62 kDa, 45 kDa and 31 kDa (doublet) present in the peak kinase fractions. (**B**) 5 μl of the indicated fractions were used to phosphorylate 200 ng of RPA. The reaction products were separated by SDS–PAGE, transferred to nitrocellulose and autoradiographed. Arrows point to phosphorylated RPA p34 subunit and cyclin B. (**C**) Same as (B) except that the substrate was 160 ng of a bacterially produced N-terminal fragment of SV40 T Ag (T259T; McVey *et al.*, 1989). (**D**) Same as (C) except that the T Ag fragment produced had alanine instead of threonine at the position corresponding to amino acid 124 (T259A; McVey *et al.*, 1989).

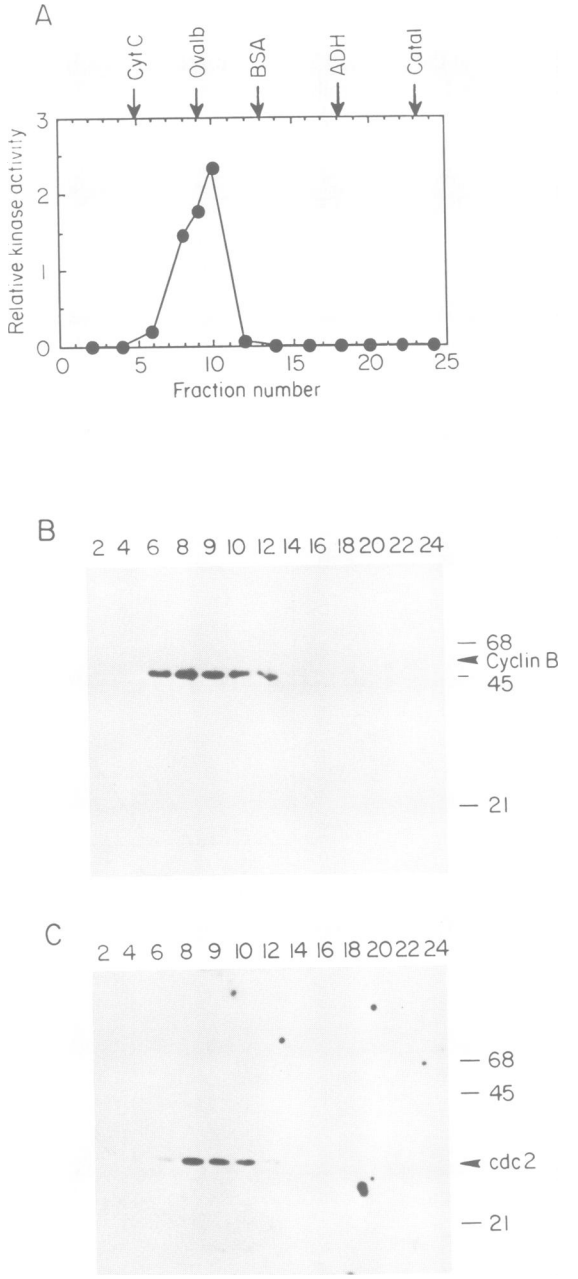

Fig. 4. Identification of RPA kinase as cyclin–*cdc2* complex. (**A**) Graphical representation of RPA kinase activity in the glycerol gradient fractions. The relative kinase activity was determined by soft laser scanning densitometry of the RPA p34 band in an autoradiogram similar to that shown in Figure 3B. (**B**) 10 μl of the indicated fractions were analyzed by gel electrophoresis and transferred to nitrocellulose. The blot was probed with a polyclonal anti-human cyclin B antibody followed by [^{125}I]protein A. (**C**) Same as (B) except the antibody used was G8 which recognizes human *cdc2* protein.

the most. Phosphoamino acid analysis of RPA phosphorylated *in vitro* indicate that only phosphoserine was present, in agreement with what is observed with RPA phosphorylated *in vivo* (data not shown; Din *et al.*, 1990). Therefore, cyclin B–*cdc2* phosphorylated RPA on some of the peptides that were phosphorylated in the cell.

Phosphorylation of RPA by cyclin A–cdc2 complex

Like cyclin B, cyclin A can also associate with *cdc2* kinase, and an additional *cdc2*-like kinase called *cdk2*, to form an active kinase complex (Giordano *et al.*, 1989; Pines and Hunter, 1990; Tsai *et al.*, 1991). This kinase is activated earlier in the cell cycle than cyclin B–*cdc2* (R.Marraccino, E.Firpo and J.Roberts, personal communication; Giordano *et al.*, 1989; unpublished results), and we examined if this too could phosphorylate RPA. During the purification of RPA kinase, a 60 kDa protein recognized by a monoclonal

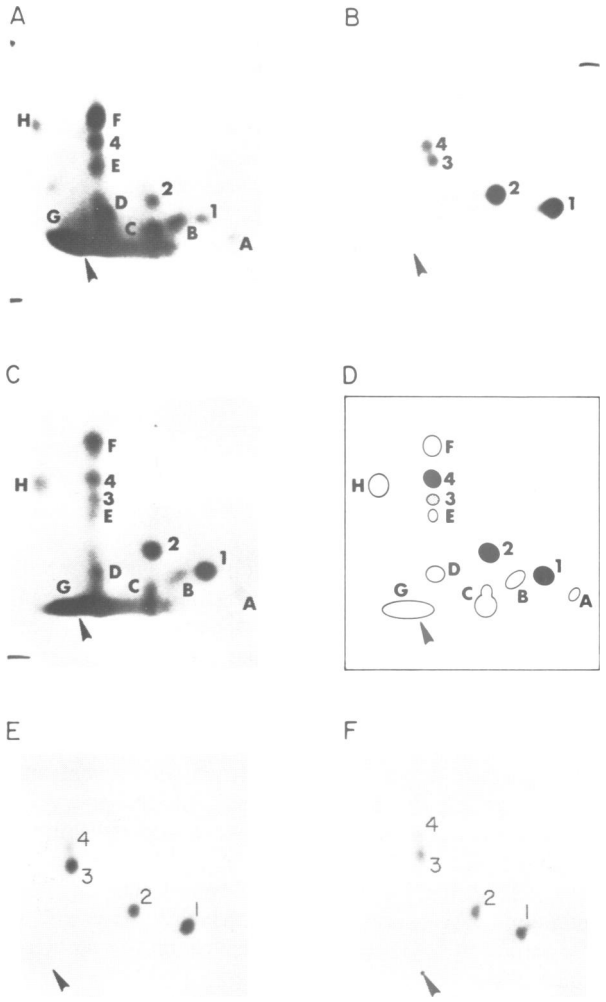

Fig. 5. Chymotryptic phosphopeptide maps of RPA phosphorylated (**A**) *in vivo* or (**B**) *in vitro* with pure cyclin B−*cdc2* kinase, (**C**) a mixture of the two, (**D**) a diagram summarizing the results, (**E**) RPA phosphorylated by protein kinases present in a cyclin A immunoprecipitate and (**F**) a mixture of (E) and (B). The orientation of the maps is the same as in Figure 2 and the arrow shows the origin. Phosphopeptides in (B) were numbered 1−4. Phosphopeptides unique to (A) were lettered A−H. In (D), the black peptides are a subset of those *in vivo* believed to be phosphorylated by *cdc2* kinase.

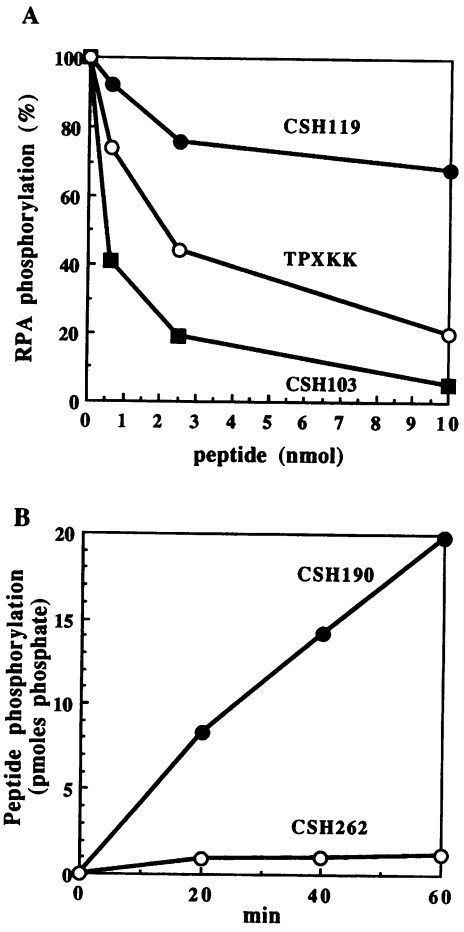

Fig. 6. (**A**) Competitive inhibition of RPA kinase activity with *cdc2* kinase substrate peptides. Each reaction contained 270 ng RPA (2.3 pmol), 0.125 μl of the Mono S RPA kinase pool and the indicated amounts of the various peptides in 10 μl kinase buffer. The amount of phosphorylation of the RPA 34 kDa subunit was determined and is shown as a percentage of the control without added peptide. (**B**) Cyclin B−*cdc2* kinase phosphorylates CSH190 but not CSH262. Each reaction contained 1 nmol peptide, 1 μl *cdc2* kinase, 500 μM ATP and 10 μCi [γ-^{32}P]ATP. A fraction of the kinase reaction was spotted onto P81 paper at each time point and the picomoles of phosphate incorporated into the peptide were determined (Marshak *et al.*, 1991).

antibody against cyclin A (C160) eluted from the Mono S column a few fractions before the peak of RPA kinase activity (data not shown). Thus in the course of the extensive purification, cyclin A may have been dissociated from the catalytic *cdc2* or *cdk2* p31 polypeptide. Nevertheless, using an anti-cyclin A antibody (C160) to immunoprecipitate the cyclin A−*cdc2* and cyclin A−*cdk2* kinases, we determined that these complexes also phosphorylated RPA p34. The phosphopeptide map of RPA p34 phosphorylated by the cyclin A associated kinases was identical to that of RPA phosphorylated by the purified cyclin B−*cdc2* kinase (Figure 5B, E and F).

Serine residues in RPA p34 that are essential for phosphorylation by cdc2 kinase are essential for phosphorylation in vivo

The preferred substrate for *cdc2* kinase is a serine or threonine followed by a proline (Kemp and Pearson, 1990), and examination of the sequence of the RPA p34 cDNA suggested that only two candidate serines were present at positions 23 and 29 (Erdile *et al.*, 1990). To test the role of these serines in RPA phosphorylation, we performed the following experiments. A synthetic peptide containing amino acids 2−42 of RPA p34 (CSH190) was phosphorylated *in vitro* by the purified cyclin B−*cdc2* kinase (Figure 6B). Phosphopeptide maps after digestion with chymotrypsin and trypsin showed that all the peptides on RPA p34 that were phosphorylated by *cdc2* kinase were present in CSH190 (not shown). Another synthetic peptide identical to CSH190 except for the substitution of the two candidate serines with alanines (CSH262), was not phosphorylated by *cdc2* kinase (Figure 6B).

To confirm that these putative sites for phosphorylation by *cdc2* kinase were required for phosphorylation *in vivo*, identical serine to alanine mutations (S23A and S29A) were made in a cDNA clone of human RPA p34. A eukaryotic expression plasmid carrying wild-type or mutant cDNA was transfected into mouse NIH3T3 cells and stable cell lines selected which expressed either the wild-type (RP-3, RP-9)

or mutant (23−9, 23−11, 23−12) human RPA p34. Expression of the transfected human RPA p34 was followed using a monoclonal antibody made against the human protein (p34-20). This antibody did not recognize endogenous mouse RPA p34 (not shown) even though it is very similar in primary structure to the human protein (Nakagawa *et al.*, 1991). In different cell lines, the wild-type human RPA p34 was found to be phosphorylated, while the mutant human RPA p34 was not (Figure 7). The immunoblot shown on the left demonstrated that similar quantities of wild-type and mutant RPA p34 were being compared in cell lines RP-3, 23−9 and 23−12 or in cell lines RP-9 and 23−11. In contrast, the autoradiogram of the blot shown on the right demonstrated that the wild-type protein expressed in the RP cell lines was phosphorylated, whereas the mutant protein in the 23 cell lines was not. Therefore, the serines on RPA p34 that were necessary for phosphorylation by *cdc2* kinase were also necessary for phosphorylation in the cell. Further characterization of these cell lines and the properties of wild-type and mutant RPA p34 is in progress.

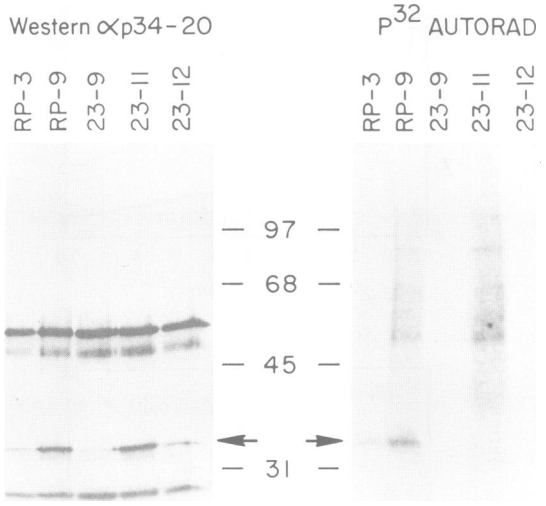

Fig. 7. Human RPA p34 is not phosphorylated in NIH3T3 cells when mutated at Ser23 and Ser29. Cell lines expressing wild-type RPA p34 (RP−) or mutated RPA p34 (23−) were labeled for 4 h with [^{32}P]orthophosphate and cell extracts were immunoprecipitated with monoclonal antibody p34-20. After gel electrophoresis, the immunoprecipitates were transferred to nitrocellulose and immunoblotted with p34-20 antibody using the horseradish peroxidase developing system (**left**). The arrow points to human RPA p34 which is absent in untransfected NIH 3T3 cells (not shown). All the other bands are immunoglobulin heavy and light chains. The panel on the **right** shows an autoradiogram of the same blot showing the extent of phosphorylation of the human RPA p34 protein. Note that only the RP cell lines contain phosphorylated RPA p34.

Purified cdc2 kinase stimulates DNA replication in G_1 S100 extracts

DNA replication (S100) extracts from cells synchronized in G_1 were less active than extracts prepared from cells in S phase (Roberts and D'Urso, 1988). Addition of RF-S, a partially purified protein fraction that contained *cdc2* kinase activity, stimulated DNA replication in G_1 extracts (D'Urso *et al.*, 1990). Furthermore, S100 extracts from G_1 cells did not contain significant *cdc2* kinase activity (D'Urso *et al.*, 1990; data not shown). Having purified *cdc2* kinase much more extensively by virtue of its ability to phosphorylate a cellular replication factor, RPA, we tested its ability to stimulate DNA replication. When the highly purified *cdc2* kinase was titrated into an *in vitro* DNA replication system containing T Ag expressed from a baculovirus vector and S100 extract from G_1 cells, DNA replication was stimulated (Figure 8A).

We checked that the stimulation was due to kinase activity rather than to unforeseen non-catalytic interactions between the kinase or the cyclin with replication proteins, or to some

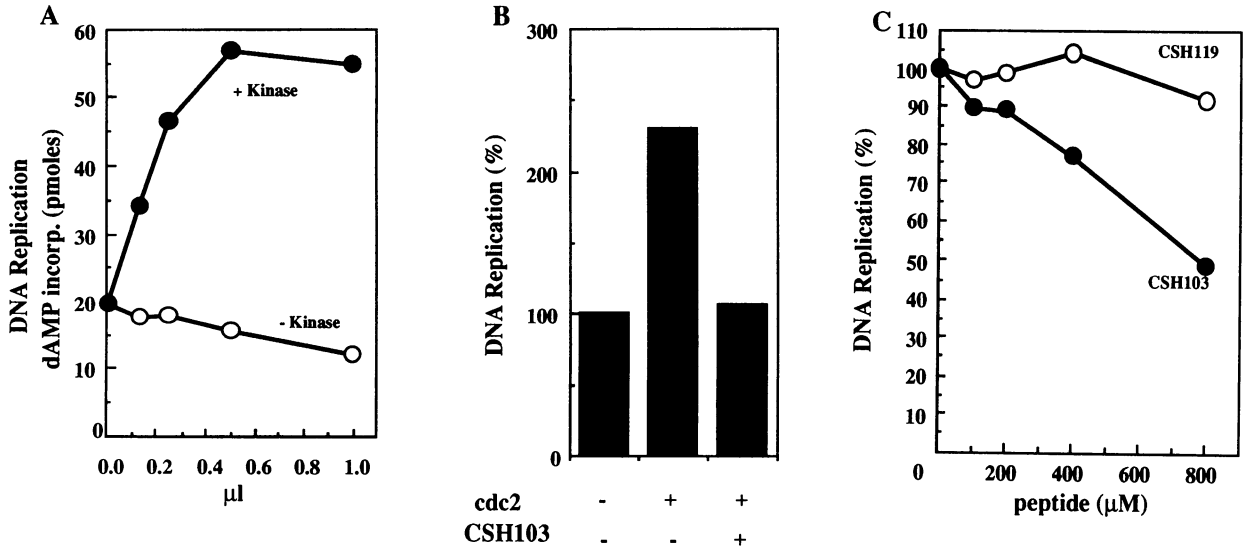

Fig. 8. Effect of *cdc2* kinase on DNA replication in G_1 S100 extracts. (**A**) The indicated amount of kinase (+ kinase) or kinase storage buffer (− kinase) was added to the *in vitro* DNA replication reaction and the amount of radiolabeled dAMP incorporated into the replication products was measured. 1 μl of kinase phosphorylated 80 pmol CSH103 in 1 h in a standard kinase reaction. (**B**) 1 mM of CSH103, a competitive inhibitor of *cdc2* kinase, inhibited the stimulation of replication seen upon addition of 0.5 μl of *cdc2* kinase. 100% is the amount of replication seen with 0.5 μl control buffer (13 pmol dAMP incorporated). (**C**) CSH103 inhibited a replication reaction containing *cdc2* kinase, but CSH 119 failed to do so. 100% is the standard replication reaction with G_1 S100 extract and 0.5 μl *cdc2* kinase (9.1 pmol dAMP incorporated).

other co-purifying biochemical activity. The stimulation was prevented by CSH103, a competitive inhibitor of *cdc2* kinase (Figure 8B). Furthermore, CSH119, a related peptide which was not a substrate for the kinase, failed to inhibit the *cdc2* kinase stimulated replication reaction (Figure 8C). No effect was seen upon addition of either peptide into a G_1 S100 replication reaction without added *cdc2* kinase (not shown). Analysis of the replication products on native and alkaline agarose gels showed that the products were normal in the absence or presence of *cdc2* kinase. Therefore, the stimulation of DNA replication was due to the catalytic activity of *cdc2* kinase on the DNA replication machinery.

Stage of DNA replication stimulated by cdc2 kinase

Stimulation of DNA replication could be at the initiation and/or elongation stages. The initiation step involves ori recognition by T Ag, followed by unwinding of the ori in co-operation with RPA. It has previously been demonstrated that phosphorylation of T Ag on Thr124 by *cdc2* kinase is required for T Ag recognition of site II within the SV40 ori and for DNA replication (McVey *et al.*, 1989). T Ag produced by the baculovirus vector, however, is maximally phosphorylated on Thr124 (Hoss *et al.*, 1990), so addition of *cdc2* kinase was not expected to alter its activity. This was confirmed by directly measuring the ability of baculovirus T Ag to bind to site II DNA in the presence of G_1 S100 extract using an immunoprecipitation–DNA binding assay (Figure 9). *cdc2* kinase did not stimulate binding of T Ag to site II DNA and therefore the stimulation of DNA replication was not due to an effect at this stage. Similar results have been reported previously (D'Urso *et al.*, 1990). Furthermore, the G_1 S100 did not inhibit the ability of T Ag to bind to site II DNA (Figure 9).

The first step in DNA replication that involves RPA is unwinding of duplex DNA at the origin of replication. If the stimulation of DNA replication by *cdc2* kinase was due to the phosphorylation of RPA, one might expect a stimulation at this stage. Origin unwinding activity was measured using a covalently closed plasmid containing the ori and studying the formation of a highly underwound DNA molecule (form U) in the presence of purified T Ag, RPA and topoisomerase I (Figure 10A; Dean *et al.*, 1987; Wold *et al.*, 1987; Tsurimoto *et al.*, 1989). An alternative method (Goetz *et al.*, 1988) that used linear duplex DNA, T Ag, RPA and no topoisomerase I gave similar results (not shown).

When pure RPA, T Ag and topoisomerase I were used in the unwinding reaction, the addition of purified *cdc2* kinase produced only a weak stimulation of unwinding (Figure 10A). Interestingly, addition of an S100 extract from G_1 phase cells completely blocked the low ori unwinding activity by RPA and T Ag (Figure 10A). When purified *cdc2* kinase was then added to the reaction, not only was the inhibition reversed, but ori unwinding was stimulated (Figure 10A and B). Quantification of the results with a phosphorimaging device showed that the extent of stimulation was comparable to the stimulation of DNA replication. Therefore, the stimulation of DNA replication observed upon addition of *cdc2* kinase is mostly at the level of ori unwinding, a result consistent with RPA phosphorylation playing a regulatory role in the G_1–S transition.

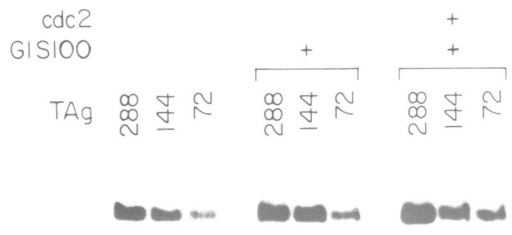

Fig. 9. DNA binding assay with T Ag. Each reaction contained the indicated amounts of T Ag (ng). The amount of site II DNA bound to T Ag was analyzed by immunoprecipitation, gel electrophoresis and autoradiography.

Discussion

Cyclin–cdc2 kinases and entry into S phase

RPA is required in a very early stage of initiation of DNA replication *in vitro* and there is evidence from studies in yeast suggesting a requirement during chromosomal DNA replication. Our finding that the human *cdc2* kinase phosphorylated RPA in a cell cycle dependent manner suggested that the *cdc2* kinase could have a direct effect on the DNA replication machinery of the cell. Though the sites

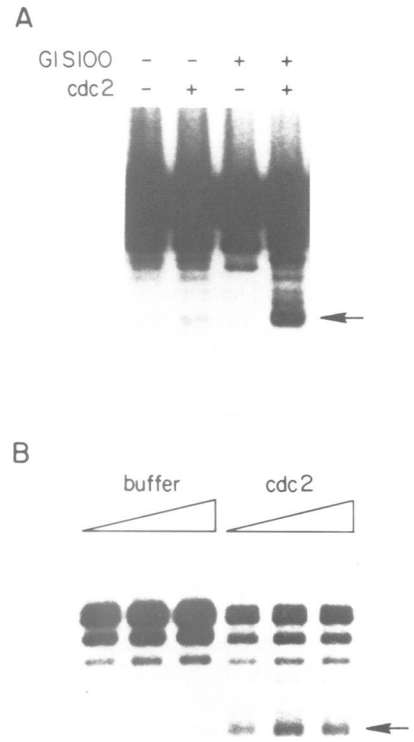

Fig. 10. Effect of *cdc2* kinase on unwinding at ori. The extensively underwound form of the plasmid, form U, is indicated with an arrow and is the product of the unwinding reaction. The other more slowly migrating topoisomers are present in the substrate radiolabeled plasmid. (**A**) Effect of purified cyclin B–*cdc2* kinase in the absence or presence of G_1 S100 extract. (**B**) Effect of increasing amounts of *cdc2* kinase (0.25, 0.5 and 1.0 μl) on unwinding in the presence of G_1 S100. The reactions without kinase contained an equal volume of kinase storage buffer. Following the unwinding reaction, DNA products were isolated, separated on an agarose gel and autoradiographed.

on RPA p34 phosphorylated by *cdc2* were only a subset of the sites phosphorylated *in vivo*, it is of interest that mutation of this subset abolished all RPA phosphorylation in cells. This suggests that phosphorylation by *cdc2* was required for all the other phosphorylations. It is unlikely that the mutations completely disrupted the structure of RPA p34, because the mutagenized protein could still associate with the RPA p70 and p13 subunits specifically (our unpublished results).

The second half of this paper demonstrates that *cdc2* kinase stimulated DNA replication in G_1 extracts, and did so selectively at the first step where RPA was required, namely the unwinding of the ori. Furthermore, the onset of DNA replication *in vitro* is associated with the concurrent phosphorylation of RPA p34 (S.-U.Din and B.Stillman, unpublished results). Taken together, the results support the hypothesis that *cdc2* kinase directly activates the cellular DNA replication machinery, at least partly by phosphorylating RPA p34.

In view of the onset of RPA phosphorylation at the G_1-S transition, it was surprising that cyclin B–*cdc2* kinase was purified as an RPA kinase. The mitotic form of *cdc2* kinase was purified probably because it is by far the dominant activity of the cyclin–*cdc2* family of kinases in an extract from asynchronously growing cells. Alternatively, the other members of the family may be inactivated, or the cyclin B–*cdc2* kinase activated, during the purification. Phosphopeptide maps of RPA p34 labeled at distinct stages across the cell cycle demonstrated that the peptides phosphorylated at the G_1-S transition include those phosphorylated by cyclin B–*cdc2*, a kinase that has been shown to be maximally activated later in the cell cycle at the G_2-M transition (Pines and Hunter, 1990). However, cyclin A and its associated kinase are activated at the G_1-S transition (Pagano *et al.*, 1992a; J.Roberts, R.Marraccino and E.Firpo, personal communication) and could phosphorylate RPA on the same peptides as cyclin B–*cdc2*. Indeed, it has recently been reported that cdk2 immunoprecipitates can phosphorylate RPA p34 (Elledge *et al.*, 1992). The mutagenesis studies showed that the sites on RPA that were phosphorylated by cyclin B–*cdc2* were required for phosphorylation *in vivo*. Therefore, RPA was phosphorylated at the G_1-S transition by a kinase that had the same specificity as the cyclin–*cdc2* family, implicating a member of the family in the phosphorylation. In fact, the RPA kinase at the G_1-S transition could be cyclin A and its associated kinases.

In view of the recent discovery of cyclins C, D and E (Koff *et al.*, 1991; Leopold and O'Farrell, 1991; Lew *et al.*, 1991; Matsushime *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991) and a large family of *cdc2*-like kinases (Paris *et al.*, 1991; Tsai *et al.*, 1991), we suspect that several members of the family are responsible for the phosphorylation of RPA at a given point in the cell cycle. It will be interesting to determine the time of maximal activity of a cell cycle regulated kinase and ascribe its function to that time, e.g. cyclin B–*cdc2* is maximally activated at mitosis, and so it functions only in mitosis. This may be misleading, especially when one compares the absolute levels of activity of cyclin B–*cdc2* and cyclin A associated kinases in S phase cells. In our hands, and those of others (Giordano *et al.*, 1989; Pagano *et al.*, 1992b), the absolute level of activity of cyclin B–*cdc2* in hydroxyurea blocked cells is as much as one-third that of cyclin A associated kinases. Such comparisons are also likely to be affected by the antibodies used. The same caveat applies to arguments based on subcellular localization of a given cyclin, because such studies reflect relative levels of the protein in two different compartments (Pines and Hunter, 1991). Again, a small amount of a very active kinase in a nuclear compartment may have a significant role in RPA phosphorylation. Therefore, it may not be possible to know with absolute certainty which member of a large family of kinases phosphorylates a substrate protein in a particular cell compartment at a given point in the cell cycle. Our results suggest that one or more members of the cyclin–*cdc2* family phosphorylates RPA and activates DNA replication.

Despite such problems of potential redundancy, there exist a number of reports that support the possibility that cyclin A and a *cdc2*-related kinase are required for entry into S phase. Addition of recombinant cyclin A to a G_1 S100 extract stimulated its ability to support DNA replication (D'Urso *et al.*, 1990). Microinjection of anti-cyclin A antibody, or plasmid expressing antisense cyclin A mRNA, into G_1 phase cells delayed or prevented the entry of cells into S phase (Girard *et al.*, 1991; Pagano *et al.*, 1992b). Likewise, there are reports implicating *cdc2* kinase or a related kinase in G_1-S transition. Depletion of *cdc2* kinases from *Xenopus* egg extracts using agarose beads containing the *S.pombe* suc1 protein resulted in the extracts failing to support DNA replication (Blow and Nurse, 1990). Similar immunodepletion experiments have revealed the requirement of *cdk2* kinase for activation of DNA replication (Fang and Newport, 1991). Antisense oligonucleotides to *cdc2* kinase prevented entry of mitogen stimulated T cells into S phase (Furukawa *et al.*, 1990). A number of these reports, of course, must be qualified by the requirement of events such as nuclear assembly, transcription of genes encoding replication proteins etc., which must be executed before the onset of DNA replication in egg extracts or intact cells. A cyclin–*cdc2* kinase may be required for these other events. This paper directly implicates the DNA replication machinery as a substrate for the *cdc2* kinase, and identifies a specific stage in DNA replication that is affected.

These recent reports, however, contradict other studies. In the *Drosophila* embryonic cell cycle, depletion of maternal cyclin A in embryos deficient in the cyclin A gene resulted in G_2 arrest (Lehner *et al.*, 1991). In *S.cerevisiae* and *S.pombe*, genetic studies have shown that *CDC28/cdc2* is required late in G_1 but not for the onset of S phase (Nurse and Bissett, 1981; Pringle and Hartwell, 1981). We argue that these differences are due either to the special requirements of an embryonic cell cycle in *Drosophila*, or to the persistence of residual *cdc2* kinase activity from START to the onset of DNA replication in yeast, or to as yet undiscovered redundancies in the activities of *cdc2*-like kinases and cyclins.

Additional factor(s) in G_1 S100 extracts

Roberts and D'Urso (1988) showed that a factor present in extracts from cells in S phase stimulated ori unwinding when added to extracts from G_1 phase cells. They also demonstrated that cyclin A or a partially purified fraction (RF-S) could be added to the crude G_1 extract to stimulate DNA replication (D'Urso *et al.*, 1990). Using a more purified system we have discovered an additional factor(s), present in the crude G_1 extract, that is required to observe

stimulation of ori unwinding by cyclin−*cdc2*. The extract contained an inhibitor of unwinding which did not act on the ability of T Ag to bind to site II DNA. The effect of this inhibitor was overcome by the addition of *cdc2* kinase. The kinase did not activate the baculovirus-produced T Ag directly. However, the kinase phosphorylated the other component of the unwinding machinery, namely RPA. The simplest hypothesis would be that the G_1 S100 extract contained a modulatory or inhibitory factor which prevented RPA from co-operating with T Ag to unwind the ori in an uncontrolled manner. Addition of *cdc2* kinase activated ori unwinding, possibly by phosphorylating RPA and/or the inhibitor itself. The inhibition and relief of inhibition could occur in a protein−protein complex on the DNA. The G_1 S100 extract could also contain a co-activator of the unwinding stimulatory activity of *cdc2* kinase. Clearly, many different models could be suggested and future experiments will aim to determine which of these is correct. Because RPA is a three subunit complex that has not been reconstituted from purified subunits, it is not yet possible to test directly whether the phosphorylation deficient mutant of RPA p34 is defective in DNA replication *in vitro*. Once cDNA clones for all three subunits become available, such reconstitution experiments will be attempted.

Although our results suggest that the phosphorylation of RPA contributes to activation of S phase, we do not imply that this is the only activating event. As for the induction of mitosis, it is likely that activation of DNA replication is much more complicated than the phosphorylation of a single replication factor by a single protein kinase.

S phase promoting factor

If cells are arrested at the beginning of S phase using an inhibitor of DNA synthesis and then released from the block, the time taken to complete DNA replication is considerably shorter than the time required by an untreated cell to complete DNA synthesis (Carnevali and Mariotti, 1977) and there is an increase in the number of origins of replication that are utilized (Taylor, 1977). These results imply that an activator of DNA replication accumulates in cells that are committed to cell division, but are blocked pharmacologically in the S phase. Similarly, cell fusion studies have revealed that a diffusible S phase promoting factor (SPF) is present in S phase cells which can induce G_1 nuclei to enter DNA synthesis prematurely (Rao and Johnson, 1970; Rao *et al.*, 1977). Finally, Pardee and co-workers have shown that low doses of cycloheximide block proliferating cells at a restriction point late in G_1, suggesting that a labile activator has to accumulate beyond a threshold level in order for cells to enter S phase (Rossow *et al.*, 1979).

What could this SPF and its substrate be? One possibility is that SPF acts on the transcriptional apparatus, chromatin or other cellular processes which facilitate the onset of DNA replication. Alternatively, SPF could activate the DNA replication apparatus directly. The finding that a cyclin−*cdc2* kinase, whose activity increases at the G_1−S transition, phosphorylates a cellular factor required for the initiation of DNA replication and activates ori unwinding and DNA replication, supports the latter possibility. In this model, the cyclin−*cdc2* kinase is itself a component of SPF, the gradual accumulation of SPF activity in S phase is due to the synthesis of a cyclin protein which activates the kinase, and SPF directly activates the DNA replication factors. In the future we hope to identify other components of SPF as well as other substrates of the DNA replication machinery that are activated by SPF.

Materials and methods

Cells and antibodies

293 cells derived from human embryonic kidney cells by transformation with adenovirus E1A and E1B proteins and HeLa cells derived from a human cervical epithelial carcinoma were grown as described previously (Stillman and Gluzman, 1985). The anti-RPA antibody p34-20 is a mouse monoclonal antibody that reacts with human RPA p34, but not with mouse RPA p34 (Din *et al.*, 1990; S.-U.Din and B.Stillman, in preparation). G8 is a polyclonal antibody raised against the *S.pombe cdc2* protein (Draetta *et al.*, 1987) and G6 is a polyclonal antibody raised against the C-terminal six amino acids of the human *cdc2* protein (Draetta and Beach, 1988). The polyclonal antibodies against human cyclin A and cyclin B were a gift from J.Pines (Pines and Hunter, 1989, 1990), that against cyclin B2 was from S.Reed (unpublished) and that against PSTAIRE from A.Koff. C160 is a mouse monoclonal antibody raised against a 60 kDa cyclin A protein that associates with adenovirus E1A protein (Harlow *et al.*, 1986; Whyte *et al.*, 1988; Giordano *et al.*, 1989; Pines and Hunter, 1990).

Assay for RPA kinase

RPA was purified from 293 cells according to published procedures (Tsurimoto and Stillman, 1989a). The kinase reaction was carried out for 20 min at 30°C using 50 ng of purified RPA in 50−100 μl kinase buffer (50 mM Tris−HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 5−50 μM ATP containing 5 μCi [γ-^{32}P]ATP). RPA was immunoprecipitated with 50 μl of mAb p34-20 (hybridoma culture supernatant), 30 μl of a 1:3 protein A−Sepharose slurry and 0.3 μl of rabbit anti-mouse immunoglobulin antibody. The immunoprecipitate was separated by electrophoresis on a 15% SDS−polyacrylamide gel.

Immunoblots, immunoprecipitations and immunoprecipitation−kinase reactions

Transfers of protein to nitrocellulose were done on an ABN semi-dry transfer apparatus according to the manufacturer's recommendations. The blots were washed with 0.1% Triton in PBS, blocked with 3% (w/v) ovalbumin, probed with a 1:100 dilution of the primary antibody and [^{125}I]protein A or HRP-conjugated rabbit anti-mouse antibody. All immunoprecipitations were done in the presence of an excess of antibody. For the immunoprecipitation−kinase reactions, 20 μl of kinase buffer (same as above) was added to the protein A−Sepharose pellet, along with RPA at 20 ng/μl. After incubation at 30°C for 20 min, the products were resolved by SDS−PAGE and transferred to nitrocellulose (to remove free ATP), and the phosphorylated substrate was visualized by autoradiography.

Purification of RPA kinase

S100 extract was prepared from 64 l of exponentially growing human 293 cells (5×10^5) cells/ml by hypotonic lysis (Li and Kelly, 1984; Stillman and Gluzman, 1985). 2.3 g of protein was loaded onto a phosphocellulose column (320 ml, 5 cm×16.5 cm) in buffer A (25 mM Tris−HCl pH 7.5, 1 mM Na_2EDTA, 10% v/v glycerol, 0.01% v/v NP40. 0.1 mM PMSF, 1 mM DTT and 5 mM sodium metabisulfite) with 0.2 M NaCl. The flowthrough (PC I) and fractions eluted with NaCl concentrations of 0.33 M (PC IIA), 0.66 M (PC IIC) and 1 M (PC IID) were collected (Tsurimoto and Stillman, 1989a) and assayed for RPA kinase activity. The majority (>90%) of the RPA kinase activity was present in PC IIA, with a small fraction found in PC I.

The PC IIA pool was diluted to 0.1 M NaCl and applied onto a Blue 4 agarose column (30 ml, 2.5 cm×8.5 cm). All the RPA kinase activity bound to the column which was washed with 5 vol of the same buffer and eluted with 150 ml buffer A containing 1 M NaCl. The fractions containing the kinase activity were pooled and concentrated by dialysis for 8 h against buffer A without NP40 but with 0.1 M NaCl and 25% w/v sucrose. This was then applied to a Sephacryl S-300 column (280 ml, 2.5 cm×59 cm) in buffer B (same as buffer A except pH was 8.0) containing 0.2 M NaCl. The RPA kinase activity eluted as a single peak just after the major protein peak. The fractions containing the kinase activity were pooled, diluted 1:4 in buffer B and loaded onto a Mono Q HR5/5 column (Pharmacia). All the activity bound to the column, which was then washed with 5 ml of buffer B containing 0.05 M NaCl and eluted with a 12 ml NaCl gradient (50−400 mM NaCl) in buffer B. The kinase activity eluted as a single peak at 175 mM. The pool from the Mono Q column was diluted 1:5 with buffer

C (20 mM potassium phosphate buffer, pH 7.0, 0.5 mM Na_2EDTA, 0.1 mM Na_2EGTA, 20% v/v glycerol, 0.01% NP40, 10 mM sodium bisulfite, 0.1 mM PMSF, 1 mM DTT) and applied onto a heparin–Sepharose column (1.5 ml, 0.75 cm×2.5 cm). The column was washed with 10 ml buffer C containing 100 mM NaCl and eluted with a 20 ml NaCl gradient (100–500 mM NaCl) in buffer C. The kinase activity eluted as a single peak at 225 mM. Fractions containing the kinase were pooled and diluted 1:6 in buffer D (25 mM potassium phosphate buffer pH 7.0, 1 mM Na_2EDTA, 10% v/v glycerol, 0.01% NP40, 10 mM sodium bisulfite, 0.1% PMSF and 1 mM DTT). This was then applied onto a Mono S HR 5/5 column (Pharmacia) which was washed with 5 ml buffer D containing 50 mM NaCl and eluted with a 7.5 ml NaCl gradient (50–500 mM NaCl) in buffer D. The RPA kinase activity eluted as a single peak at 210 mM. Most of the functional studies were done with this fraction. 100 μl of the Mono S pool was loaded onto a 5 ml glycerol gradient (10–40% v/v) in buffer A containing 150 mM NaCl. The gradient was run in a Beckman SW50.1 rotor at 45 000 r.p.m. for 24 h. 150 μl fractions were collected and assayed for RPA kinase activity.

All steps were carried out at 4°C. For long term storage, the active fractions were divided into aliquots, snap frozen in dry ice/ethanol and stored at −70°C.

Phosphopeptide and phosphoamino acid analysis

The phosphate labeled RPA was immunoprecipitated from cell extracts and analyzed by SDS–PAGE, the proteins were transferred to nitrocellulose and the p34 band was visualized by autoradiography. Nitrocellulose containing the RPA p34 was cut out, treated with PVP 360 (Aebersold *et al.*, 1987), washed extensively with distilled water and digested with 20–40 μg of chymotrypsin or trypsin in 400 μl of 50 mM ammonium bicarbonate at 37°C for 16 h. All the counts were released from the nitrocellulose. Phosphopeptides present in the supernatant were analyzed according to Pines and Hunter (1989) except that electrophoresis was at 80 V/cm for 15 min and TLC was for 6–8 h. The same batch of chymotrypsin was used for all the phosphopeptide maps shown in this paper, so that they can be compared with one another. However, we now have reason to believe that the chymotrypsin was contaminated with trypsin, so the phosphopeptide maps are really chymotrypsin + partial trypsin maps. This does not alter any of the conclusions of the paper. Phosphoamino acid analysis of the supernatant was done by electrophoresis at pH 3.5 in glacial acetic acid/pyridine/water, 50:5:945 (v/v) (Hunter and Sefton, 1980).

Cell synchronization

4 l of HeLa cells at 5×10^5 cells/ml were blocked in M phase with nocodazole at 40 ng/ml for 19 h. The cells were released from nocodazole block by harvesting, washing and re-inoculating into fresh medium. After 2 h, cells were harvested and subjected to centrifugal elutriation (Giordano *et al.*, 1989). The first two fractions, containing 50% of the cells, were pooled and plated at 2×10^6 cells/ml in 10% FCS containing DEM. At the indicated times the cells were labeled with 2 mCi of ^{32}P per 100 mm plate in phosphate-free DEM for 1 h and harvested. The cells were lysed by boiling in 1% SDS, diluted to RIPA buffer conditions and immunoprecipitated with the anti-RPA monoclonal antibody p34-20. The immunoprecipitates were separated by PAGE and the amount of phosphorylation of RPA p34 was quantified using a Molecular Dynamics phosphorimaging device. Parallel plates were labeled with 10 μCi of [^{3}H]thymidine for 1 h. These were fixed and overlayed with Kodak emulsion. After exposure for two days, the plates were developed and the percentage of cells that were labeled with thymidine was counted. To make the G_1 S100 extract, 15×10^8 cells from the elutriation were lysed in 2 ml hypotonic buffer using standard conditions (Stillman and Gluzman, 1985). The protein concentration in the extract was 7.25 mg/ml.

Peptides

TPXKK peptide was a gift from Eric Nigg (Peter *et al.*, 1990). CSH103 has the sequence H-ADAQHATPPKKKRKVEDPKDF-OH. The peptide was modeled on the SV40 T Ag *cdc2* phosphorylation site (Thr124) except that the serines corresponding to T Ag residues 120 and 123 were changed to alanine to avoid phosphorylation by other protein serine kinases (Marshak *et al.*, 1991). CSH119 has the same sequence except that the threonine which is phosphorylated by *cdc2* kinase is replaced with alanine (D.Marshak, unpublished). Both these peptides were a gift from Dr Dan Marshak, Cold Spring Harbor Laboratory. CSH190 contains amino acids 2–42 of human RPA p34 (Erdile *et al.*, 1990): Ac-WNSGFESYGSSSYGGAGGYTQSP-GGFGSPAPSQAEKKSRAR-$CONH_2$. CSH262 has the same sequence except that the underlined serines are changed to alanines. All peptide kinase assays were done as described by Marshak *et al.* (1991).

Mutant human RPA p34

A cDNA clone of human RPA p34 was obtained from L.Erdile and T.Kelly (Erdile *et al.*, 1990). The 900 bp *Eco*RI–*Asp*718 fragment from phRPA32 was transferred to pBS, KS+ and appropriate oligonucleotides were used to make the S23A and S29A mutations that correspond to the serine to alanine changes in CSH262. A *Bam*HI–*Asp*718 fragment from wild-type and mutant plasmids was cloned into the eukaryotic expression vector pMEXNEO (Martin-Zanca *et al.*, 1989) such that RPA p34 is expressed under the control of a murine sarcoma virus LTR. NIH 3T3 mouse fibroblasts were transfected by the calcium phosphate method with the MEXNEO derivatives, and cells stably expressing the transfected plasmids were selected by G418 selection (400 μg/ml). Individual colonies were cloned and cell lines created.

Functional studies of the effect of cdc2 kinase

SV40 DNA replication was carried out as described by Stillman and Gluzman (1985). 150 ng of pSVO10, a pUC18 based plasmid containing the whole SV40 genome, was replicated in a 25 μl reaction containing 72 ng of T Ag and 54 μg of G_1 S100 extract. The ori unwinding reaction (form U production) was carried out in 20 μl unwinding buffer (30 mM HEPES pH 8.0, 7 mM $MgCl_2$, 4 mM ATP, 0.5 mM DTT, 0.1 mg/ml BSA, 40 mM creatine phosphate and 0.4 μl creatine phosphokinase) at 37°C for 2 h. Each reaction used 50–60 ng of radiolabeled pSVO11 (which has the *Hin*dIII–*Sph*I fragment of SV40 DNA spanning the ori cloned into pUC18), 1 μg of carrier DNA (pUC119 digested with *Sau*3AI), 288 ng of T Ag, 270 ng of RPA, 54 ng of purified calf thymus DNA topisomerase I (Tsurimoto *et al.*, 1989) and 54 μg of G_1 S100 extract (the last only where indicated). The reaction was stopped by adding EDTA to 20 mM and SDS to 0.1% (w/v) and digesting with 20 μg pronase at 37°C for 1 h. The products were extracted with phenol–chloroform and analyzed on a 0.8% agarose gel in TBE buffer (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA).

For the T Ag mediated immunoprecipitation–DNA binding assay (McKay, 1981), the indicated amount of T Ag was incubated in 20 μl of unwinding buffer containing 200 mM KCl and 10% (v/v) glycerol, along with 2 ng of radiolabeled *Hin*dIII–*Eco*RI fragment of pSVO14 (SV40 5209–128 cloned in the polylinker of pUC18) and 500 ng of pUC119 cut with *Sau*3AI. 54 μg of G_1 S100 extract and 1 μl of *cdc2* kinase were added as indicated. Following incubation at 37°C for 60 min, the T Ag was immunoprecipitated with an excess of the anti-T Ag antibody, pAB 419 (Harlow *et al.*, 1981) and the DNA present in the precipitate was analyzed by gel electrophoresis (McVey *et al.*, 1989).

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References

Aebersold,R.H., Leavitt,J., Saavedra,R.A., Hood,L.E. and Kent,S.B.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6970–6974.

Blow,J.J. and Nurse,P. (1990) *Cell*, **62**, 855–862.

Borowiec,J.A., Dean,F.B., Bullock,P.A. and Hurwitz,J. (1990) *Cell*, **60**, 181–184.

Brill,S.J. and Stillman,B. (1989) *Nature*, **342**, 92–95.

Brill,S.J. and Stillman,B. (1991) *Genes Dev.*, **5**, 1589–1600.

Carnevali,F. and Mariotti,D. (1977) *Chromosoma*, **63**, 33–37.

Challberg,M.D. and Kelly,T.J. (1989) *Annu. Rev. Biochem.*, **58**, 671–717.

Collins,K.L. and Kelly,T.J. (1991) *Mol. Cell. Biol.*, **11**, 2108–2115.

Dean,F.B., Bullock,P., Murakami,Y., Wobbe,C.R., Weissbach,L. and Hurwitz,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 16–20.

Din,S.-U., Brill,S.J., Fairman,M.P. and Stillman,B. (1990) *Genes Dev.*, **4**, 968–977.

Dornreiter,I., Erdile,L.F., Gilbert,I.U., von Winkler,D., Kelly,T.J. and Fanning,E. (1992) *EMBO J.*, **11**, 769–776.

Draetta,G. and Beach,D. (1988) *Cell*, **54**, 17–26.

Draetta,G., Brizuela,L., Potashkin,J. and Beach,D. (1987) *Cell*, **50**, 319–325.

D'Urso,G., Marraccino,R.L., Marshak,D.R. and Roberts,J.M. (1990) *Science*, **250**, 786–791.
Elledge, S.J., Richman,R., Hall,F.L., Williams,R.T., Lodgson,N. and Harper,J.W. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2907–2911.
Erdile,L.F., Wold,M.S. and Kelly,T.J. (1990) *J. Biol. Chem.*, **265**, 3177–3182.
Erdile,L.F., Heyer,W.-D., Kolodner,R. and Kelly,T.J. (1991) *J. Biol. Chem.*, **266**, 12090–12098.
Fairman,M.P. and Stillman,B. (1988) *EMBO J.*, **7**, 1211–1218.
Fang,F. and Newport,J.W. (1991) *Cell*, **66**, 731–742.
Furukawa,Y., Piwnica-Worms,H., Ernst,T.J., Kanakura,Y. and Griffin,J.D. (1990) *Science*, **250**, 805–808.
Giordano,A., Whyte,P., Harlow,E., Franza,B.R., Beach,D. and Draetta,G. (1989) *Cell*, **58**, 981–990.
Girard,F., Strausfeld,U., Fernandez,A. and Lamb,N.J.C. (1991) *Cell*, **67**, 1169–1179.
Goetz,G.S., Dean,F.B., Hurwitz,J. and Matson,S.W. (1988) *J. Biol. Chem.*, **263**, 383–392.
Harlow,E., Crawford,L.V., Pim,D.C. and Williamson,N.M. (1981) *J. Virol.*, **39**, 861–869.
Harlow,E., Whyte,P., Franza,B.R. and Schley,C. (1986) *Mol. Cell. Biol.*, **6**, 1579–1589.
Heyer,W.-D., Rao,M.R.S., Erdile,L.F., Kelly,T.J. and Kolodner,R.D. (1990) *EMBO J.*, **9**, 2321–2329.
Hoss,A., Moarefi,I., Scheidtmann,K.-H., Cisek,L.J., Corden,J.L., Dornreiter,I., Arthur,A.K. and Fanning,E. (1990) *J. Virol.*, **64**, 4799–4807.
Hunter,T. and Sefton,B.M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1311–1315.
Ishimi,Y., Claude,A., Bullock,P. and Hurwitz,J. (1988) *J. Biol. Chem.*, **263**, 19723–19733.
Kemp,B.E. and Pearson,R.B. (1990) *Trends Biochem. Sci.*, **15**, 342–346
Kenny,M.K., Lee,S-H. and Hurwitz,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9757–9761.
Kenny,M.K., Schlegel,U., Furneaux,H. and Hurwitz,J. (1990) *J. Biol. Chem.*, **265**, 7693–7700.
Koff,A., Cross,F., Fisher,A., Schumacher,J., Leguellec,K., Philippe,M. and Roberts,J.M. (1991) *Cell*, **66**, 1217–1228.
Lehner,C., Yakubovich,N. and O'Farrell,P.H. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 465–475.
Leopold,P. and O'Farrell,P.H. (1991) *Cell*, **66**, 1207–1216.
Lew,D.J., Dulic,V. and Reed,S.I. (1991) *Cell*, **66**, 1197–1206.
Li,J.J. and Kelly,T.J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6973–6977.
Marshak,D.R., Vandenberg,M.T., Bae,Y.-S. and Yu,I.J. (1991) *J. Cell. Biochem.*, **45**, 391–400.
Martin-Zanca,D., Oskam,R., Mitra,G., Copeland,T. and Barbacid,M. (1989) *Mol. Cell. Biol.*, **9**, 24–33.
Matsumoto,T., Eki,T. and Hurwitz,J. (1991) *Proc. Natl. Acad. Sci. USA*, **87**, 9712–9716.
Matsushime,H., Roussel,M.F. and Ashmun,R.A. (1991) *Cell*, **65**, 701–713.
McKay,R.O.G. (1981) *J. Mol. Biol.*, **145**, 471–488.
McVey,D., Brizuela,L., Mohr,I., Marshak,D.R., Gluzman,Y. and Beach,D. (1989) *Nature*, **341**, 503–507.
Melendy,T.M. and Stillman,B. (1991) *J. Biol. Chem.*, **266**, 1942–1949.
Motokura,T., Bloom,T., Goo-Kim,H., Juppner,H., Ruderman,J.V., Kronenberg,H.M. and Arnold,A. (1991) *Nature*, **350**, 512–515.
Nakagawa,M., Tsukada,S., Soma,T., Shimizu,Y., Miyake,S., Iwamatsu,A. and Sugiyama,H. (1991) *Nucleic Acids Res.*, **19**, 4292.
Nurse,P. and Bissett,Y. (1981) *Nature*, **292**, 558–560.
Pagano,M., Draetta,G. and Jansen-Durr,P. (1992a) *Science*, **255**, 1144–1147.
Pagano,M., Pepperkok,R., Verde,F., Ansorge,W. and Draetta,G. (1992b) *EMBO J.*, **11**, 961–971.
Paris,J., Le Guellec,R., Couturier,A., Le Guellec,K., Omilli,F., Camonis,J., MacNeill,S. and Philippe,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1039–1043.
Peter,M., Nakagawa,J., Doree,M., Labbe,J.C. and Nigg,E.A. (1990) *Cell*, **60**, 791–801.
Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.
Pines,J. and Hunter,T. (1990) *Nature*, **346**, 760–763.
Pines,J. and Hunter,T. (1991) *J. Cell. Biol.*, **115**, 1–17.
Pringle,R. and Hartwell,H. (1981) In Strathern,J.N., Jones,E.W. and Broach,J.E. (eds), *The Molecular Biology of the Yeast Saccharomyces, Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 97–142.
Rao,P.N. and Johnson,R.T. (1970) *Nature*, **225**, 159–164.
Rao,P.N., Sunkara,P.S. and Wilson,B.A. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 2869–2873.
Roberts,J.M. and D'Urso,G. (1988) *Science*, **241**, 1486–1489.
Rossow,P.W., Riddle,V.G.H. and Pardee,A.B. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4446–4450.
Stillman,B. (1989) *Annu. Rev. Cell. Biol.*, **5**, 197–245.
Stillman,B.W. and Gluzman,Y. (1985) *Mol. Cell. Biol.*, **5**, 2051–2060.
Taylor,J.H. (1977) *Chromosoma*, **62**, 291–300.
Tsai,L.-H., Harlow,E. and Meyerson,M. (1991) *Nature*, **353**, 174–177.
Tsurimoto,T. and Stillman,B. (1989a) *EMBO J.*, **8**, 3883–3889.
Tsurimoto,T. and Stillman,B. (1989b) *Mol. Cell. Biol.*, **9**, 609–619.
Tsurimoto,T. and Stillman,B. (1991a) *J. Biol. Chem.*, **266**, 1950–1960.
Tsurimoto,T. and Stillman,B. (1991b) *J. Biol. Chem.*, **266**, 1961–1968.
Tsurimoto,T., Fairman,M.P. and Stillman,B. (1989) *Mol. Cell. Biol.*, **9**, 3839–3849.
Tsurimoto,T., Melendy,T. and Stillman,B. (1990) *Nature*, **346**, 534–539.
Weinberg,D.H., Collins,K.L., Simancek,P., Russo,A., Wold,M.S., Virshup,D.M. and Kelly,T.J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8692–8696.
Whyte,P., Buchkovich,K.J., Horowitz,J.M., Friend,S.H., Raybuck,M., Weinberg,R.A. and Harlow,E. (1988) *Nature*, **334**, 124–125.
Wobbe,C.R., Dean,F., Weissbach,L. and Hurwitz,J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5710–5714.
Wold,M.S. and Kelly,T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2523–2527.
Wold,M.S., Li,J.J. and Kelly,T.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3643–3647.
Wold,M.S., Weinberg,D.H., Virshup,D.M., Li,J.J. and Kelly,T.J. (1989) *J. Biol. Chem.*, **264**, 2801–2809.
Xiong,Y., Connolly,T., Futcher,B. and Beach, D. (1991) *Cell*, **65**, 691–699.

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