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52. The measured dissociation rate constant (k_d) for the endonuclease-TAATTC complex at 0.08 M NaCl is ~0.7 sec^{-1}. At physiological salt (~0.2 M), k_d should be increased about 30-fold (*19*), whereas cleavage-rate constants are salt-independent (*14, 20*), so k_d/k_3 should be about 6×10^5.
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Cell Cycle Control of DNA Replication by a Homologue from Human Cells of the p34^{cdc2} Protein Kinase

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The regulation of DNA replication during the eukaryotic cell cycle was studied in a system where cell free replication of simian virus 40 (SV40) DNA was used as a model for chromosome replication. A factor, RF-S, was partially purified from human S phase cells based on its ability to activate DNA replication in extracts from G_1 cells. RF-S contained a human homologue of the *Schizosaccharomyces pombe* p34^{cdc2} kinase, and this kinase was necessary for RF-S activity. The limiting step in activation of the p34 kinase at the G_1 to S transition may be its association with a cyclin since addition of cyclin A to a G_1 extract was sufficient to start DNA replication. These observations suggest that the role of p34^{cdc2} in controlling the start of DNA synthesis has been conserved in evolution.

THE TIMING OF EVENTS DURING THE SOMATIC CELL CYCLE can be explained by the hypothesis that any one event does not begin until certain previous ones have been completed (*1*). Consequently, mutations that prevent particular cell cycle functions can cause cell cycle arrest (*2*). The arrest points often coincide with the time in the cell cycle when the affected gene products are required. Mutations in the *Saccharomyces cerevisiae CDC28* gene (*3*), or the *Schizosaccharomyces pombe cdc2* gene (*4*) both arrest the cell cycle at two points—just before the onset of DNA synthesis and just before entry into mitosis. In *S. cerevisiae*, the control point at the G_1-S boundary is called START and represents the major point at which cell growth and division are coordinated (*5*). In *S. pombe* the major coordination point for growth and division is at G_2-M, the second point in the cell cycle regulated by *cdc2*, although under certain conditions growth and division can also be coordinated at G_1-S (*6*). The *cdc2* and *CDC28* genes are homologous (*7*) and functionally interchangeable (*8*). They encode a 34-kD serine-threonine protein kinase, designated p34 (*9*).

The mitotic function of p34 has been evolutionarily conserved. Maturation promoting factor (MPF), initially isolated from vertebrate eggs, can induce meiotic maturation of G_2-arrested oocytes without new protein synthesis (*10*). MPF was later isolated from somatic cells of various species and shown to be active during mitosis, but inactive or latent during interphase (*11*). One component of human MPF is a 34-kD serine-threonine kinase that is 63 percent identical (amino acid sequence) to the *cdc2* protein kinase (p34^{cdc2}) of *S. pombe* (*12*). Furthermore, the human *cdc2* gene can rescue the mitotic function of *cdc2* in *S. pombe cdc2* mutants (*13*).

It is not known whether the G_1-S function of p34 occurs in higher eukaryotes. In mammalian cells, as in *S. cerevisiae*, the start of DNA synthesis reflects a commitment to complete the remainder of the cell division cycle (*14*). Hence, the focus of many regulatory signals that promote or inhibit cell duplication ultimately is on the transition from G_1 into S. Cells that cease proliferation usually exit during G_1 and enter a resting state called G_0 (*15*). The kinetics of

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passage through G_1 suggest that a single event during G_1 regulates commitment to DNA replication (*16*). This event, called the restriction point, may be analogous to the p34-dependent START transition in the yeast cell cycle. In fact, the human *cdc2* gene can substitute for the *S. pombe cdc2* gene in both its G_1-S and G_2-M roles (*13*). Experiments in which G_1 cells were fused to S phase cells suggested that the transition from G_1 into S phase occurs when a diffusible activator accumulates above a certain threshold (*17*). However, the relation between that factor and the p34 kinase remains unclear.

We investigated the mechanism that controls the start of DNA replication during the mammalian cell cycle using cell-free replication of simian virus 40 (SV40) DNA as a model for chromosome replication. Our experiments were based on two observations. First, cell-free replication of SV40 DNA is catalyzed entirely by cellular replication proteins (*18*), except for the action of the virally encoded T antigen, which participates in the initiation of DNA replication (*19, 20*). Second, we have shown earlier that replication in this system is cell cycle–dependent (*21*). Cellular extracts become active in supporting DNA synthesis from the SV40 origin as cells exit G_1 and enter the S phase of the cell cycle. We suggest that the activation of DNA replication in S phase extracts reflects the appearance at the G_1 to S transition of a limiting and essential cellular replication factor, called RF-S.

Purification of RF-S. We purified RF-S from MANCA cells—a human Burkitt lymphoma cell line (*22*)—by assaying the ability of protein fractions from S phase cells to activate DNA synthesis in extracts from G_1 cells. To prepare a synchronous population of G_1 cells, exponentially growing cells were first arrested in metaphase with nocodazole, then released for 1 to 2 hours from the mitotic block and newborn G_1 cells harvested by centrifugal elutriation (*21*). Similar results were obtained with G_1 cells prepared by elutriation of exponentially growing cells, but the synchrony of this population was not as good as with the technique described above. S phase extracts were prepared from cells harvested 4 hours after release from hydroxyurea induced synchronization at the G_1-S boundary. Measurements of [^{3}H]thymidine incorporation into cellular DNA in vivo indicated that this corresponded to the time of maximal DNA synthesis. The synchrony of this cell population was further supported by flow cytometry analysis of cellular DNA content, which showed that more than 90 percent of cells were in S phase, and measurement of the mitotic index, which demonstrated that more than 98 percent of the cells were in interphase.

RF-S was partially purified from soluble extracts of S phase cells by anion-exchange chromatography (DEAE cellulose), cation-exchange chromatography (Biorex 70), ammonium sulfate precipitation, and gel filtration (Superose-12). Gel filtration indicated that the molecular mass of RF-S was 250 kD. The extent of RF-S purification could not be measured directly since inhibitors of DNA synthesis co-fractionated with RF-S until the ammonium sulfate step. Therefore, we estimated the extent of RF-S purification by the specific activity of H1 kinase (Table 1), an activity that copurified with RF-S (see below). The large increase in specific activity of H1 kinase early in the purification could be attributed to both an increase in enzyme purity and removal of inhibitors of enzyme activity. We have also purified RF-S from untreated, exponentially growing cells to demonstrate that the presence of this factor was not a consequence of hydroxyurea-induced cell synchronization.

Replication was assayed by the addition of protein fractions to a G_1 cell extract and with subsequent measuring of the incorporation of [^{32}P]dCMP (deoxycytosine monophosphate) into DNA (Fig. 1A) and visualizing the replication products (Fig. 1C). The addition of protein fractions containing RF-S to G_1 extracts increased their replication activity to the same level as an extract from S phase cells. As in control reactions from S phase cells, activation of DNA synthesis in G_1 extracts by RF-S required both the SV40 origin of replication and large T antigen. RF-S had no detectable replication activity in the absence of G_1 extract.

We assayed fractions from the final stage of RF-S purification (Superose 12) for their ability to phosphorylate a peptide that is a substrate for the p34^{cdc2} kinase. This peptide, designated CSH103, contains the p34^{cdc2} phosphorylation site within SV40 large T antigen (*23*). A peak of peptide kinase activity copurified precisely with RF-S activity (Fig. 1A). Histone H1 kinase activity showed the same profile (*24*). Immunoblots of the same fractions with antiserum to a carboxyl-terminal peptide of human p34^{cdc2} showed a peak of this protein which coincided with the peak of kinase and RF-S activities (Fig. 1, B and C). Antiserum to the conserved PSTAIRE region of p34^{cdc2} (*13*) also recognized a 34-kD antigen in RF-S.

We used p13 Sepharose affinity chromatography to show that p34^{cdc2} was necessary for RF-S activity. The protein p13, which is the product of the *suc1* gene in *S. pombe*, binds p34^{cdc2} with high affinity (*25, 26*). Passage of RF-S over p13 Sepharose removed at least 80 percent of the CSH103 peptide kinase activity (Fig. 2A). This treatment also depleted the replication activity of RF-S (Fig. 2B). RF-S activity and CSH103 peptide kinase activity were unchanged after passage over a control column of Sepharose 4B (Fig. 2, A and B).

Addition of either highly purified recombinant clam cyclin A or sea urchin cyclin B (*27*) associated with xenopus p34 to human G_1 extracts activated DNA replication. Sea urchin cyclin B–xenopus p34 kinase and RF-S kinase were equally effective in activating DNA synthesis (Fig. 3). Some of our results also show that clam cyclin

Table 1. Purification of RF-S. Manca cells, grown as described (*21*), were synchronized in S phase with 2 mM hydroxyurea for 12 hours. Four hours after release, cells were collected (1200 rpm for 5 minutes), washed twice with cold tris-buffered saline (TBS) and resuspended in hyptonic buffer [20 mM Hepes-KOH (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.25 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF), leupeptin (0.5 μg/ml), and aprotinin (0.5 μg/ml)] at 4×10^8 cells/ml. Cells were incubated on ice for 10 minutes and lysed by vortexing. Extracts were adjusted to 150 mM NaCl, incubated for 30 minutes on ice, and centrifuged for 10 minutes at 10,000 rpm (Sorvall HB-4); the supernatant was then centrifuged at 100,000*g* for 1 hour. The final supernatant (S-100, 15 to 20 mg of protein per milliliter) was diluted to 50 mM NaCl in buffer A [25 mM Hepes-KOH (pH 8.0), 10 percent (v/v) glycerol, 0.25 mM EDTA, 0.25 mM EGTA, 0.25 mM DTT, 0.1 mM PMSF, leupeptin (0.5 μg/ml), aprotinin (0.5 μg/ml)] and applied to a 20-ml DEAE-cellulose column equilibrated in buffer A containing 50 mM NaCl. The column was washed with two volumes of loading buffer and eluted with buffer A containing 150 mM NaCl. Fractions containing the peak of protein were pooled, diluted to 50 mM NaCl with buffer A, and applied to a 5-ml Biorex 70 column. The column was washed with two volumes of buffer A containing 50 mM NaCl, and the protein was eluted with buffer A containing 300 mM NaCl. The peak of protein was pooled, precipitated with 40 percent NH_4SO_4, dissolved in 200 μl of buffer A containing 200 mM NaCl, and centrifuged at 250,000*g* for 20 minutes. The supernatant was applied to a Superose-12 column (flow rate, 0.4 ml/min); 0.5-ml fractions were collected, dialyzed in buffer A containing 50 mM NaCl, and 10 percent (w/v) sucrose, frozen in liquid nitrogen, and stored at −70°C. The activity of 1 μg of each fraction was measured by phosphorylation of histone H1 (*24*).

Procedure	Total protein (mg)	Total activity (cpm)	Recovery (%)	Specific activity (cpm/mg)
100,000*g*	379.3	2.8×10^5	2.8	755.7
DEAE cellulose	76.0	1.7×10^6	17.0	2.3×10^4
Biorex-70	22.6	1.0×10^7	100.0	4.6×10^5
$(NH_4)_2SO_4$	4.4	7.5×10^6	75.0	1.7×10^6
Superose 12	0.5	6.5×10^6	65.0	1.3×10^7

A–xenopus p34 complexes had replication activity equivalent to RF-S. These experiments confirmed that $p34^{cdc2}$ was necessary for activation of replication in G_1 extracts by RF-S. It remains possible that proteins associated with p34 within these complexes contributed to replication activity.

Activation of RF-S during the cell cycle. An important prediction of the hypothesis that RF-S activates DNA replication during the cell cycle is that RF-S would be absent from G_1 cells. We confirmed this by showing that fractionation of G_1 extracts by the same protocol used to obtain RF-S yielded material that was unable to activate replication in our standard RF-S assay.

Activation of the p34 kinase during the G_1 to S transition correlates with assembly of the p34 protein into a multi-protein complex. Cytosolic extracts of G_1 and S phase cells were fractionated directly by Superose 12 chromatography and assayed for p34 kinase activity toward peptide CSH103. No kinase activity was detected in extracts from newborn G_1 cells (Fig. 4A). However, a broad peak of kinase activity with an apparent molecular size of 200 to 300 kD was detected in S phase extracts, consistent with the previously determined apparent molecular size of RF-S. Analysis of the same fractions by immunoblotting with antiserum to p34 revealed that p34 was monomeric in G_1 cells but was present in at least one large complex in S phase cells (Fig. 4B). This complex corresponded to the peak of kinase activity. Monomeric p34 was selectively removed during the purification of RF-S from S phase extract (compare Fig. 1B and Fig. 4B).

To identify components of the p34 complex we analyzed Superose 12 fractions from our final step in the purification of RF-S by immunoblotting for the presence of cyclins A and B, two proteins that show cell cycle–dependent association with $p34^{cdc2}$ (*28*), as discussed below. Antiserum specific for cyclin A recognized a protein with an apparent molecular size of 60 kD (Fig. 5A), and antiserum specific for cyclin B recognized a protein with an apparent molecular size of 62 kD (Fig. 5B). These are the expected sizes of human cyclin A and cyclin B, respectively. Both cyclins were most abundant in the fractions containing RF-S and both cofractionated with $p34^{cdc2}$. We do not know whether association of p34 with cyclin A or B (or another unidentified cyclin) was responsible for RF-S activity.

Our observations showed that activation of the p34 kinase in S phase correlated with its association with cyclin proteins. This association may be a rate-limiting step in the activation of the p34 kinase at the G_1 to S transition since addition of recombinant clam cyclin A to a G_1 extract was sufficient to activate SV40 DNA replication (Fig. 6). This observation further supported the hypothesis that activation of the cyclin-associated p34 kinase was necessary for the onset of DNA synthesis.

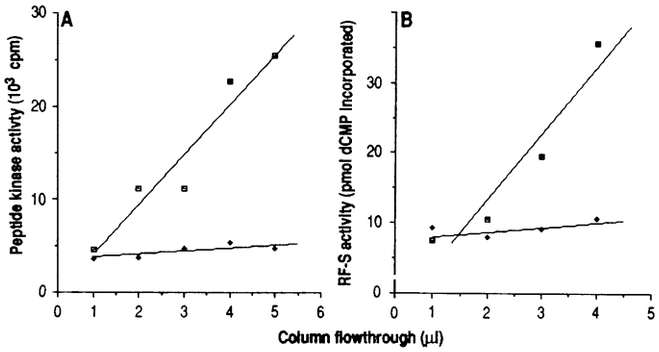

Fig. 2. Depletion of RF-S activity with p13-Sepharose. Kinase activity toward peptide CSH103 (**A**) and replication activity (**B**) after passage through Sepharose 4B (open symbols) or p13-Sepharose (closed symbols). RF-S from the ammonium sulfate stage of the purification (approximately 50 μg in 100 μl of buffer A containing 200 mM NaCl) was placed on a 200-μl Sepharose-4B column or a 200-μl column of p13 Sepharose (5 mg of p13 per milliliter of Sepharose) equilibrated in buffer A containing 200 mM NaCl. RF-S was allowed to penetrate and adsorb to the column for 30 minutes. Fractions were then collected, those containing the protein peak were pooled and diluted to 50 mM NaCl, and RF-S was concentrated by centrifugation in Centricon-30 filters (Amicon). Replication and kinase activities were assayed as described (Fig. 1, legend).

Activation of DNA replication by RF-S. The identification of cellular replication proteins that are activated by p34 will be necessary in order to understand the mechanism by which p34 initiates DNA synthesis. T antigen can be specifically phosphorylated on Thr^{124} by the p34–cyclin B complex (*29*). Phosphorylation of this site is essential for the binding of T antigen to the core replication origin (site II) and initiation of DNA replication. Because our assay depends on the viral T antigen, it was important

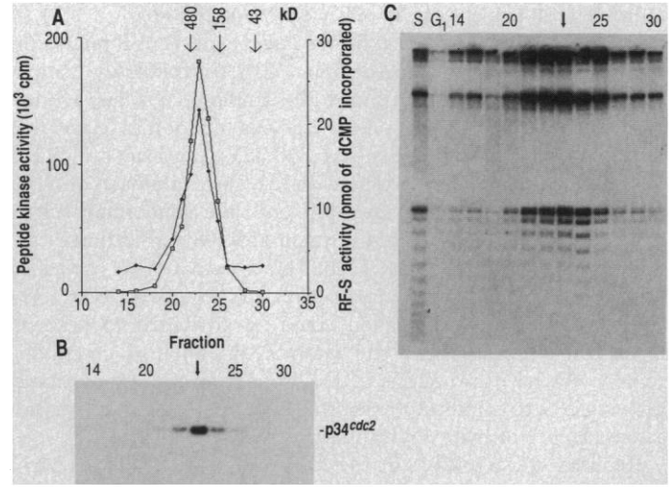

Fig. 1. Cofractionation of $p34^{cdc2}$ with RF-S. (**A**) Replication activity (closed symbols) and p34 kinase activity (open symbols) of Superose-12 fractions. Replication reactions with a plasmid (100 ng) containing the SV40 core origin of replication (nucleotides 5210 to 5231), 100 μg of S-100 cell extract, and 0.5 μg of SV40 large T antigen were incubated for 2.5 hours as described (*21*). Replication activity in G_1 extracts plus 6 μl of the indicated Superose-12 fractions are shown. The amount of DNA synthesis was quantitated by measuring incorporation of [^{32}P]dCMP into trichloroacetic acid precipitable material. T antigen was prepared by immunoaffinity chromatography from lysates of Ad5SV112-infected HeLa cells. For peptide kinase reactions, the Superose-12 fractions were incubated at 37°C for 10 minutes in a 20-ul, reaction mixture containing 25 mM Hepes-KOH (pH 7.5), 8 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 200 mM NaCl, 100 μM adenosine triphosphate (ATP), 5 μCi of [γ-^{32}P]ATP, and peptide CSH103 (500 μM). Bovine serum albumin (BSA) was then added (5 mg/ml, final) and protein was precipitated by addition of trichloroacetic acid (10 percent final). The supernatant was spotted on P-81 paper (Whatman); the paper was washed extensively in 10 mM phosphoric acid, and dried. The ^{32}P incorporated into peptide CSH103 was quantitated by scintillation counting. (**B**) Immunoblot of Superose-12 fractions analyzed with antiserum to the COOH-region of $p34^{cdc2}$. Superose-12 fractions were resolved by SDS-PAGE, transferred to nitrocellulose (BA85; Schleicher & Schuell) by semidry electroblotting, blocked for 30 minutes with TBS (tris-buffered saline) containing 0.05 percent Tween 20, and incubated overnight with antiserum to p34 (1:500). Blots were washed with TBS containing Tween, dried, and autoradiographed. (**C**) DNA replication products of reactions containing G_1 extracts supplemented with Superose -12 fractions used in (**A**). Also shown are control reactions with G_1 or S phase extracts. Replication products were visualized by autoradiography after electrophoresis through 1 percent agarose in TBE [89 mM tris-borate, 89 mM boric acid, 2 mM EDTA].

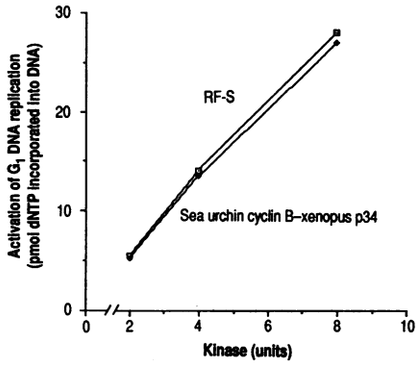

Fig. 3. Activation of DNA replication in G_1 extracts by addition of purified H1 kinase. Specific activity of H1 kinase (sea urchin cyclin B–xenopus p34, closed symbols) and RF-S (open symbols) were determined by measuring the stimulation of DNA replication (picomoles of dNTP's (deoxynucleotides) incorporated into DNA) per unit of CSH103 peptide kinase activity. One unit of kinase incorporated 10 pmole of PO_4^{3-} into CSH103 peptide in 10 minutes at 37°C.

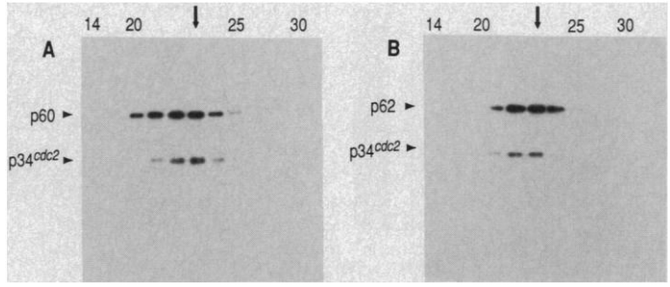

Fig. 5. Cylcins A and B are complexed with p34 in RF-S. Immunoblots of Superose 12 fractions analyzed with antiserum to the carboxyl-terminal region of $p34^{cdc2}$, and then with antiserum to cyclin A (**A**) or cyclin B (**B**). Polyclonal antiserum (*43*) to cyclin A was prepared in rabbits to a bacterially expressed fusion protein containing the carboxyl-terminal 46 amino acids of human cyclin A. Cyclin B polyclonal antiserum was raised in rabbits to bacterially expressed human cyclin B. Proteins corresponding to $p34^{cdc2}$, cyclin A (p60), and cyclin B (p62) are indicated.

to determine whether RF-S was stimulating DNA synthesis by increasing the activity of T antigen or, alternatively, that of a cellular replication factor.

We determined that RF-S was not activating DNA synthesis by altering the activity of T antigen. We demonstrated that the T antigen used in our experiments (Fig. 1, legend) was functionally active before incubation with RF-S by showing that it could bind to the core replication origin and that its binding activity was not increased by incubation with RF-S (Fig. 7A). T antigen (residues 1 to 259) produced in *Escherichia coli*, which was unphosphorylated at Thr^{124}, would only bind the core origin after incubation with RF-S.

Prior treatment of HeLa T antigen with RF-S also was not sufficient to activate DNA synthesis. By using RF-S that had been affinity-coupled to p13 Sepharose we were able to treat T antigen with approximately 1.5 times more RF-S kinase activity than would be necessary to activate G_1 DNA replication (*30*) and then purify the treated T antigen free from the RF-S kinase. This T antigen remained unable to activate G_1 replication while it remained fully active in supporting replication in extracts from S phase cells.

Since G_1 extracts contain cellular phosphatases, RF-S may have been required to maintain the preexisting phosphorylation state of T antigen. This is unlikely since the phosphorylation of T antigen was stable in G_1 extracts in the absence of RF-S (Fig. 7B). We treated *E. coli*–produced T antigen with p34 kinase immunoprecipitated from RF-S, removed the kinase by centrifugation, and then incubated the phosphorylated T antigen in a G_1 extract for up to 2 hours under conditions identical to those of the SV40 replication reactions. The phosphate added to bacterial T antigen by RF-S was stable in the G_1 extract. A control reaction performed without p34 antiserum (G6) showed that the phosphate present on T antigen after incubation in the G_1 extract was not added by cellular kinases present in the G_1 extract. Although we have not shown directly that RF-S phosphorylated T antigen at Thr^{124} in this experiment, we have found that the phosphorylation of *E. coli* T antigen by RF-S could be blocked by the addition of the CSH103 peptide, which corresponds to the Thr^{124} site of T antigen. Also, a mutation of Thr to Ala in T antigen at residue 124 eliminates all phosphorylation by p34 (*29*).

We concluded from these experiments that phosphorylation of SV40 T antigen by RF-S was not responsible for the activation of DNA replication in G_1 extracts. Therefore, it appears that at least one cellular protein must be phosphorylated by RF-S before DNA replication can begin. The essential cellular replication factor RF-A is one candidate.

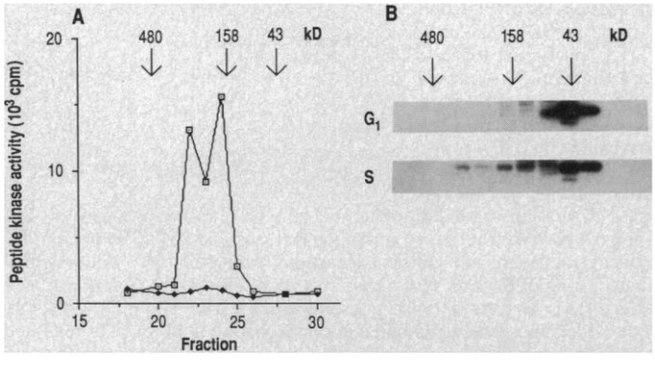

Fig. 4. Superose-12 chromatography of S-100 extracts from G_1 and S phase cells. (**A**) CSH103 peptide kinase activity in Superose-12 fractions from G_1 cells (closed symbols) and from S phase cells (open symbols). (**B**) Immunoblot analysis of $p34^{cdc2}$ in Superose-12 fractions from G_1 and S phase cells. An S-100 extract of G_1 or S phase cells (200 μl at 15 to 20 mg/ml) was adjusted to 200 mM NaCl and fractionated by fast performance liquid chromatography on a Superose-12 column in buffer A containing 200 mM NaCl as described (Fig. 1, legend). Immunoblots of Superose fractions of G_1 and S phase cell extracts were performed as described (Fig. 1, legend), except that blots were incubated with antiserum to the PSTAIRE domain of $p34^{cdc2}$ (*13*). Single letter amino acid abbreviations: P, Pro; S, Ser; T, Thr; A, Ala; I, Iso; R, Arg; E, Glu.

Cell cycle control of DNA replication. Kinetic analyses of SV40 DNA replication in vitro demonstrate that a presynthesis phase of approximately 15 minutes precedes the start of DNA synthesis. During this time a subset of replication proteins assemble into a nucleoprotein complex at the origin (*31*). Within this initiation complex the origin DNA becomes unwound thereby facilitating the association of the DNA synthetic enzymes with the replication origin (*19*). We have shown that this stage in DNA replication occurs inefficiently in extracts from G_1 cells and that this deficit can account for the inability of G_1 extracts to replicate DNA (*21*). In a purified system, three replication factors are required for the presynthesis initiation reaction: SV40 T antigen, RF-A, and topoisomerase I (*32*). Two of these factors, T antigen (*29*) and RF-A (*33*), are substrates of the p34 kinase.

Our data show that phosphorylation of T antigen is not sufficient to explain the activation of DNA replication by RF-S. RF-A is a cellular multiprotein complex that binds single-stranded DNA; it stabilizes the unwound DNA structure that forms during the presynthesis reaction and also participates in the elongation phase of DNA synthesis (*34*). RF-A is phosphorylated at the start of S phase in human and yeast cells (*35*). The RF-A kinase is probably $p34^{cdc2}$ since this kinase can phosphorylate RF-A in vitro at the sites phosphorylated in vivo (*33*). Thus, phosphorylation of RF-A may

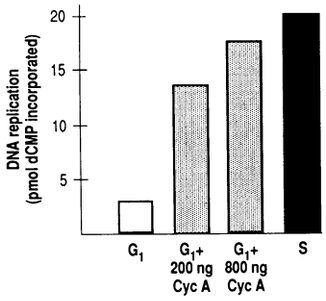

Fig. 6. Activation of DNA replication by cyclin A. Extracts of G_1, S, or G1 containing the indicated amount of recombinant clam cyclin A (*27*) were incubated under replication conditions for 20 minutes at 37°C. Replication was then started by the addition of T antigen and DNA, and the extent of DNA synthesis was quantified (Fig. 1).

contribute to the activation of DNA synthesis by RF-S by promoting the formation of the underwound initiation complex; however, it has not yet been shown directly that phosphorylation of RF-A stimulates single-stranded DNA binding or origin unwinding. T antigen, RF-A, and topoisomerase I are sufficient for the presynthesis reaction in purified systems. In more crude systems, such as the one used in our experiments, the requirements for the presynthesis reaction are less clear and other cellular factors may have important roles. We conclude that assembly of a functional presynthesis complex at the replication origin may be regulated by the $p34^{cdc2}$ kinase. In our cell-free assay this may require the activation of RF-A. At cellular chromosomal replication origins activation of RF-A and a specific T antigen-like initiator protein both might be required to start DNA synthesis.

Whereas our data suggest a direct role for the p34 kinase in activating DNA synthesis, they do not exclude the participation of the p34 protein in earlier events that result in commitment to S phase (for example, at the restriction point). In vivo multiple intra- and extracellular signals may impinge on the activation process of p34 at various points during progression from G_1 into S phase. Stepwise modifications of p34 (or a cyclin) could monitor essential cell cycle events throughout G_1 with the start of DNA replication being dependent on its activation as a kinase at the G_1-S boundary.

Regulation of p34. It is likely that activation of both the G_1-S and G_2-M forms of the p34 kinase requires the association of p34 with a cyclin subunit and, furthermore, that the two forms can be distinguished by their association with specific G_1 or G_2 cyclins. In support of this, cyclin B is necessary for the G_2-M activation of p34 in many species (*36*) including humans (*37*), and in *S. cerevisiae* a unique class of cyclins, the CLN proteins, is necessary for the activation of *CDC28* at START (*38*). Specific G_1 cyclins have not yet been identified in higher eukaryotic cells. Pardee and co-workers have suggested that passage of mammalian cells through the restriction point requires the synthesis during G_1 of a critical and labile 68-kD protein (*39*). More recently, other evidence suggested that cyclin A may take part in activating S phase in somatic cells because cyclin A is present early in S phase and the cyclin A–p34 complex has kinase activity during S phase, before the activation of the cyclin B–p34 complex (*40*). However, direct evidence that cyclin A plays a role in the activation of DNA replication in vivo is lacking.

Consistent with the hypothesis that cyclin accumulation leads to the activation of the p34 kinase in S phase, we found that p34 was present predominantly as a monomer in early G_1 cells and was assembled into a larger molecular size complex in S phase cells. Monomeric p34 could be found in G_1 and S phase but kinase activity was found only in the 250-kD complex from S phase cells. Addition of pure recombinant cyclin A was sufficient to activate DNA replication in a G_1 extract, suggesting that accumulation of a cyclin could be the rate-limiting step for activation of the p34 kinase at the G_1 to S transition. It is important to recognize that some aspects of cell cycle regulation may not be apparent in our in vitro assay and therefore we cannot conclude that cyclin accumulation would be sufficient to start S phase in vivo.

Our experiments did not directly address the question of which cyclin acts in vivo to activate the p34 kinase at the start of S phase. Immunoblotting experiments showed that both cyclin A and B were present in RF-S, and we observed that both recombinant clam cyclin A and sea urchin cyclin B when complexed with xenopus p34 had RF-S activity. It is paradoxical that two complexes of p34, one of which has been shown to induce mitosis in vivo (*36, 37*), could both start DNA synthesis in vitro. In vivo the cyclins may control the subcellular localization of p34. This function would not be apparent in our cell free assay. Also, since different cyclin-p34 complexes do not appear to have qualitatively different substrate specificities (*41*), there was little reason to expect that our assay would distinguish between different cyclins or cyclin-p34 complexes. Indeed some functions, such as promoting the activation of p34, could be shared by multiple different cyclins.

In summary, RF-S, a multiprotein complex from S phase cells that contains a homologue of the $p34^{cdc2}$ protein kinase, can activate DNA synthesis in extracts from G_1 cells. On the basis of our results we suggest that accumulation of a cyclin during G_1 leads to the activation of the p34 kinase at the start of S phase. DNA synthesis would begin when phosphorylation of specific replication proteins

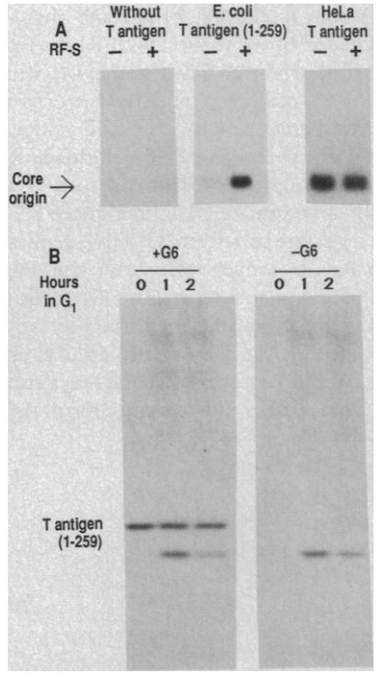

Fig. 7. (**A**) Effect of RF-S on binding of HeLa and *E. coli* T antigen to the SV40 replication origin. SV40 T antigen purified from either HeLa cells (see above), or from *E. coli* (*29*) was incubated with or without 1 μg of RF-S in 30 mM Hepes, pH 7.4, 7 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM ATP, 10 percent glycerol, BSA at 100 μg/ml in 25-μl total volume at 37°C for 30 minutes. Binding to the SV40 core origin (nucleotides 5210 to 138) (*42*) was monitored by immunoprecipitation of T antigen–DNA complexes essentially as described (*29*). The immune complexes were disrupted in 1 percent SDS, 25 mM EDTA and heated to 65°C for 15 minutes. The released DNA was separated on a 5 percent acrylamide gel (acrylamide:bis 20:1) and the extent of binding was determined by autoradiography. The extent of binding without T antigen is shown as a control. The T antigen purified from *E. coli* contained residues 1 to 259. This region of T antigen is necessary and sufficient for origin specific binding. Full-length T antigen (1 to 708) from HeLa cells was used. (**B**) (Left) *Escherichia coli* T antigen (residues 1 to 259) was phosphorylated with RF-S and the stability of the added phosphate determined upon incubation in G_1 extracts for 1 and 2 hours under replication conditions. RF-S was precipitated with antiserum to p34 carboxyl terminal (G6), washed, and mixed with 250 ng of *E. coli* T antigen for 20 minutes at 37°C in a 10-μl reaction mixture containing 20 μM ATP, 10 μCi of [γ-^{32}P]ATP, and insulin at 33 μg/ml (as carrier) in kinase buffer. The immune precipitate containing the p34 kinase was removed by centrifugation and the supernatant, containing the phosphorylated T antigen, was mixed with 150 μg of G_1 extract, 1 μg of HeLa T antigen, and sufficient deoxy- and ribonucleotides, phosphocreatine, and creatine phosphokinase to reconstruct replication conditions. Portions were removed at 1 and 2 hours, the samples were resolved by PAGE and visualized by autoradiography. (Right) Identical to above experiment except that the p34 antiserum (G6) was eliminated from the immunoprecipitation of RF-S.

by the p34^{cdc2} kinase promoted the formation of functional initiation complexes at chromosomal replication origins.

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