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In Vitro Cell Cycle Arrest Induced by Using Artificial DNA Templates

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In cell extracts of *Xenopus* eggs which oscillate between S and M phases of the cell cycle, the onset of mitosis is blocked by the presence of incompletely replicated DNA. In this report, we show that several artificial DNA templates (M13 single-stranded DNA and double-stranded plasmid DNA) can trigger this feedback pathway, which inhibits mitosis. Single-stranded M13 DNA is much more effective than double-stranded plasmid DNA at inhibiting the onset of mitosis. Furthermore, we have shown that low levels of M13 single-stranded DNA and high levels of double-stranded plasmid DNA can elevate the tyrosine kinase activity responsible for phosphorylating $p34^{cdc2}$, thereby inactivating maturation-promoting factor and inhibiting entry into mitosis. This constitutes a simplified system with which to study the signal transduction pathway from the DNA template to the tyrosine kinase responsible for inhibiting $p34^{cdc2}$ activity.

The typical eukaryotic cell cycle consists of distinct phases, G_1, S (DNA replication), G_2, and M (mitosis), which occur in a defined order. There is considerable evidence for the existence of feedback mechanisms which prevent cell cycle progression when DNA replication or mitosis are not completed correctly (for review, see reference 16). The existence of a negative feedback mechanism which couples the onset of mitosis to the completion of DNA replication was suggested by the observation that interruption of DNA replication by conditional mutation of genes required for progression through the S phase or treatment with drugs which arrest DNA replication (hydroxyurea or aphidicolin) prevents onset of mitosis. The cell cycle arrest prior to mitosis induced by these drugs can be overridden by the addition of caffeine (33, 34), indicating that all of the cellular components necessary for executing mitosis are functional but are kept inoperative by regulatory factors. Mutations that disrupt feedback control have been identified in several organisms. For example, at the restrictive temperature, the temperature-sensitive BimE7 mutant of *Aspergillus nidulans* induces chromosome condensation and mitotic spindle formation even in cells treated with hydroxyurea or blocked in S or G_2 by mutation of other cell cycle control genes (30). Similarly, BHK cells mutant in the *RCC1* gene will undergo premature mitosis at the nonpermissive temperature (28, 29).

Like incomplete DNA replication, DNA damage prevents entry into mitosis. Damage of DNA with ionizing radiation or alkylating agents results in an elongation of the G_2 phase of the cell cycle, allowing for the repair of DNA damage prior to onset of mitosis (16). The product of the *rad9* gene of *Saccharomyces cerevisiae* is a likely component of this DNA damage feedback pathway, since the normal cell cycle delay in response to DNA damage does not occur in this mutant, though the repair processes themselves are not deficient (39).

Entry into mitosis in eukaryotic cells is controlled by maturation-promoting factor (MPF) (reviewed in reference 21), which is composed of a serine/threonine kinase (the product of the *cdc2* gene) and a regulatory subunit (cyclin B) (6, 7, 35). The cyclin component of MPF oscillates in abundance through the cell cycle, peaking at the G_2/M transition and being degraded abruptly at the metaphase/anaphase transition (10, 25, 26). In the absence of cyclin protein, $p34^{cdc2}$ is inactive in promoting entry into mitosis. Upon binding of cyclin, $p34^{cdc2}$ is held in an inactive state by phosphorylation on both tyrosine and threonine residues present in the ATP-binding site (8, 13, 20, 37).

Activation of the *cdc2*-cyclin complex involves the dephosphorylation of the tyrosine and threonine residues in the ATP-binding site of $p34^{cdc2}$ and phosphorylation of a different threonine residue (20, 37). Genetic evidence from *Schizosaccharomyces pombe* and subsequent biochemical analyses in several systems have implicated a number of gene products in the control of phosphorylation and dephosphorylation of $p34^{cdc2}$. In particular, the *cdc25* gene product is, at least in part, responsible for the tyrosine dephosphorylation of $p34^{cdc2}$, while the *mik1* and *wee1* gene products are kinases which negatively regulate *cdc2* activity via tyrosine phosphorylation (15, 31, 32, 38). The *wee1* kinase has been shown to be capable of both serine/threonine and tyrosine phosphorylation (11). Genetic analysis has shown that these gene products are excellent potential targets of inhibitory signals emanating from incompletely replicated DNA. Recently, Enoch and Nurse have shown that overexpression of *cdc25* of *S. pombe* can override a hydroxyurea arrest (9). *S. pombe* cells mutant in both the *mik1* and *wee1* genes can also enter mitosis in the presence of hydroxyurea (23). Thus, mutations that result in $p34^{cdc2}$ tyrosine dephosphorylation can override the negative feedback signal from incomplete DNA replication, suggesting that this feedback signal may operate through p34 phosphorylation.

The genetic observations made with *S. pombe* have been confirmed and extended at a biochemical level by using *Xenopus* egg extracts which can oscillate between S and M phases in vitro (5, 26). These extracts can decondense added sperm chromatin, form nuclei around the DNA templates, replicate the DNA, and enter mitosis (as judged by chromosome condensation and nuclear envelope breakdown). These extracts have a cell cycle characteristic of the early embryo, oscillating between S and M phases with no appreciable G_1 or G_2 intervals. When few nuclei are present in these extracts, addition of aphidicolin does not prevent progression through mitosis, as is the case with the early

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embryonic cell cycle (5). The feedback system which prevents entry into mitosis prior to completion of DNA replication can function in these cycling extracts upon addition of a threshold number of nuclei (greater than 2,000 nuclei per μl of extract) (5). This effect is presumably due to depletion of components in the extract which are required for complete replication, thus leaving incompletely replicated DNA to propagate a feedback signal. Aphidicolin inhibits replication of added DNA (though replication complexes remain intact [17]) and therefore significantly lowers the threshold number of nuclei required to arrest this in vitro cell cycle (to <300 nuclei per μl) (5). Extracts arrested in this manner accumulate p34-cyclin B complexes which are inactive due to tyrosine phosphorylation of p34 (36).

When nuclei and aphidicolin are used to arrest the cycling extract prior to mitosis, addition of *cdc25* protein can override the arrest (18). Similarly, addition of caffeine or okadaic acid can circumvent the feedback control (5, 36). As recently shown by Smythe and Newport, the tyrosine kinase responsible for phosphorylating $p34^{cdc2}$ on tyrosine in *Xenopus* extracts (possibly *wee1* or *mik1*) is activated in the presence of nuclei and aphidicolin, suggesting that the feedback system works, at least in part, by increasing the rate of tyrosine phosphorylation of $p34^{cdc2}$, thereby inactivating MPF (36). This tyrosine kinase activation is suppressed by addition of caffeine or okadaic acid (36).

The suppression of $p34^{cdc2}$ activity by incompletely replicated DNA can be viewed as a signal transduction pathway from the DNA template to the kinases and phosphatases modulating $p34^{cdc2}$ activity. While the molecular participants in the reception of the signal from DNA blocked in replication (*cdc25*, *wee1*, and *mik1*) are being characterized biochemically, the nature of the signal generator is not very clear. It is possible that the cell in some way detects DNA that has not yet been replicated and generates a signal to inhibit mitosis until no unreplicated DNA remains. Alternatively, some configuration of a replicating DNA template may be detected at the nucleic acid level. The presence of an active replication complex at a replication fork could also generate a signal, indicating that replication had not yet been completed.

The complexity of sperm chromatin makes it a difficult template to use for studying the generation of the signal at the DNA level. Using the *Xenopus* cycling extract, we have been able to generate feedback signals from replicating M13 single-stranded DNA and from double-stranded plasmid DNA. Moreover, we find that these simple templates can activate the tyrosine kinase(s) responsible for phosphorylating $p34^{cdc2}$. Using these templates, we have shown that unreplicated DNA itself is not likely to be responsible for generating the feedback signal. Rather, the signal is likely to be propagated by actively replicating DNA. The assay system reported here constitutes a first step in the development of a purified system which will allow dissection of the signalling pathway from replicating DNA to the enzymes which modulate $p34^{cdc2}$ activity.

MATERIALS AND METHODS

Preparation of cycling extracts. Cycling extracts were prepared as described by Murray and Kirschner (26). Extracts were incubated at room temperature following addition of DNA (M13 single-stranded DNA from U.S. Biochemical, double-stranded plasmid prepared by cesium chloride gradient centrifugation, or sperm chromatin prepared as described by Lohka and Masui [22]). At 10-min intervals, 1-μl aliquots of extract containing sperm chromatin were diluted 1:1 with 10 μg of Hoechst 33258 per ml in 37% formaldehyde and examined by phase-contrast and fluorescence microscopy. Progress through the cell cycle was monitored by observation of nuclear formation and breakdown and by sperm chromatin condensation and decondensation. For visual observation of samples containing M13 or double-stranded DNA, nuclei were preformed by incubation of sperm chromatin in the cycling extract for 25 min at 2,000 nuclei per μl and then diluted 10-fold into fresh extract with DNA.

Where indicated, caffeine (0.5 mM) or aphidicolin (0.05 mg/ml) was added at the beginning of the incubation. Cyclin protein prepared as described by Solomon et al. (37) was added to the extract 60 min after the start of the room temperature incubation.

Replication assays. For continuous labeling of DNA, extracts and DNA were incubated at room temperature in the presence of 0.1 μCi of [^{32}P]dCTP (3,000 Ci/mmol) per μl. Samples of 10 μl were withdrawn into 10 μl of sample buffer containing 80 mM Tris HCl (pH 8.0), 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% sodium dodecyl sulfate (SDS), and 0.2% bromphenol blue. For pulse-labeling, 10-μl aliquots of unlabeled extract containing DNA were added to 1 μCi of [α-^{32}P]dCTP and incubated for 20 min prior to addition of sample buffer. Both continuously labeled and pulse-labeled samples were further treated with 1 mg of proteinase K per ml for 1 h at 37°C prior to agarose gel electrophoresis on 1% agarose gels. Dried gels were exposed to X-ray film at −70°C with intensifying screens.

Histone H1 kinase assays. For assaying histone H1 kinase activity, 2-μl aliquots of extract were withdrawn at 10-min intervals into 2 μl of EB buffer (80 mM sodium glycerophosphate [pH 7.3], 20 mM EGTA, 15 mM $MgCl_2$), frozen immediately in liquid nitrogen, and stored at −70°C. Thawed samples were diluted 60-fold in EB buffer and were then further diluted 1:1 with 5 μl of H1 assay cocktail (40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.3], 10 mM sodium EGTA, 20 mM $MgCl_2$, 0.2 mg of histone H1 per ml, 0.2 mM ATP, 5 μCi of [γ-^{32}P]ATP, 10 μM cyclic AMP-dependent protein kinase inhibitor peptide [Sigma]). After 10 min of incubation at room temperature, 7.5 μl of each sample was spotted onto a 1-cm square of phosphocellulose paper, washed twice for 20 min each time in 1% phosphoric acid, rinsed in 95% ethanol, air dried, and scintillation counted. For H1 kinase assays analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), reactions were stopped by addition of 10 μl of SDS sample buffer (125 mM Tris [pH 6.8], 2% SDS, 50% glycerol, 5% β-mercaptoethanol, 0.001% bromphenol blue). Samples were boiled and electrophoresed on 10% gels. The dried gels were exposed to X-ray film at −70°C with intensifying screens.

Tyrosine kinase assays. To assay the rate of tyrosine phosphorylation of *cdc2* protein, 90-μl aliquots of extract containing various amounts of DNA were incubated for 40 min at room temperature. The tyrosine phosphatase activity in these reactions was stopped by addition of sodium orthovanadate to 0.5 mM, and glutathione *S*-transferase–sea urchin cyclin B (GST-cyclin) fusion protein (37) was added for 10 min. The extract was then diluted 1:1 with buffer I (80 mM β-glycerophosphate, 5 mM EDTA, 2 mM sodium orthovanadate, 0.1% Nonidet P-40, 0.5 M NaCl) prior to freezing in liquid nitrogen and storage at −70°C. Samples were thawed and incubated with 0.5 volume of glutathione agarose for 20 min at 4°C with continuous agitation. The glutathione beads

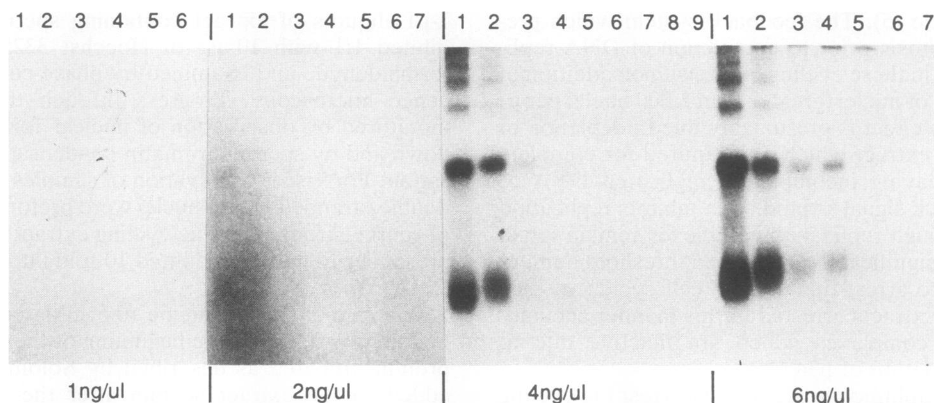

FIG. 1. Replication of M13 single-stranded DNA. Extracts containing the indicated concentrations of single-stranded DNA were placed at room temperature. Aliquots were withdrawn and pulse-labeled for 20 min with [α-^{32}P]dCTP for the following times after the start of the room temperature incubation: lanes 1, 0 to 20 min; lanes 2, 20 to 40 min; lanes 3, 40 to 60 min; lanes 4, 60 to 80 min; lanes 5, 80 to 100 min; lanes 6, 100 to 120 min; lanes 7, 120 to 140 min; lane 8, 140 to 160 min; lane 9, 160 to 180 min. Samples were digested with proteinase K, run on 1% agarose gels, and autoradiographed.

were then washed once in buffer I, twice in buffer II (80 mM β-glycerophosphate, 5 mM EDTA, 2 mM sodium orthovanadate), and once in buffer III (10 mM HEPES [pH 7.5], 1 mM dithiothreitol, 2 mM sodium orthovanadate). Beads were boiled in sample buffer (5 mM EDTA, 5 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 10% SDS, 80 mM Tris [pH 6.8], 10% glycerol, 0.1 M dithiothreitol) and pelleted. Forty microliters of each sample was electrophoresed on an SDS–10% polyacrylamide gel, transferred electrophoretically to nitrocellulose, and probed with an antiphosphotyrosine antibody (a gift from J. Wang) and ^{125}I-protein A.

RESULTS

Replication and feedback by M13 single-stranded DNA. When sperm chromatin is added to cycling extracts of *Xenopus* eggs, intact nuclei are formed and the chromatin decondenses and replicates. After approximately 40 to 60 min in S phase, the chromatin then recondenses and the nuclear envelopes break down. Following mitosis, the nuclear envelopes re-form and another round of DNA replication ensues. If high numbers of nuclei are present or if DNA replication is blocked by addition of aphidicolin, MPF activation and entry into mitosis are prevented.

As a first step toward characterizing the requirements for generating a feedback signal, we assayed the ability of various artificial DNA templates to replicate and to prevent the onset of mitosis in cycling extracts. We added increasing amounts of M13 single-stranded DNA into cycling extracts and monitored incorporation of [α-^{32}P]dCTP into newly synthesized DNA (Fig. 1). When 1 ng of M13 DNA per μl was pulse-labeled, little if any ^{32}P incorporation was observed. Some ^{32}P-labeled DNA was detectable in the 2-ng/μl sample, while 4- and 6-ng/μl samples replicated well. The nonlinearity in the incorporation suggests that there may be some negatively acting factor which must be titrated by the added DNA before replication can occur. This nonlinearity in the efficiency of replication, however, is not consistent from extract to extract.

Visible nuclei do not form around M13 DNA. Therefore, to monitor visually the timing of mitosis in these samples, it was necessary to include sperm nuclei as indicators. We used low numbers of sperm nuclei which would not substantially affect cell cycle progression. The nuclei were preformed in a cycling extract at high concentrations (1 to 2,000 nuclei per μl, 3 pg of DNA per sperm nucleus) and diluted 20-fold into fresh extract containing the M13 DNA in order to avoid any inhibition of nuclear formation by the added naked DNA templates.

The M13 DNA was able to inhibit entry into mitosis. The ability of the added DNA to inhibit entry into mitosis correlated well with its ability to replicate rather than with the absolute amount of DNA present in the extract. Thus, 1 and 2 ng of M13 DNA per μl had no effect on the cycling time of the extract (time until entry into mitosis); the time of mitosis was 85 min. However, the interphase period did lengthen with the addition of 4 ng of DNA per μl (110 min), and the cell cycle was arrested prior to mitosis at 6 ng of DNA per μl (>130 min).

Histone H1 kinase activation. As shown by Dasso and Newport (5), unreplicated sperm chromatin blocks entry into mitosis by preventing activation of MPF. Histone H1 kinase activity can be used as a marker for MPF activation, since the growth-associated histone H1 kinase activity tightly correlates with MPF activity (2, 7, 14, 19). To determine whether single-stranded DNA also blocked histone H1 kinase activation or was inhibiting nuclear breakdown and chromatin condensation at some other level, the following experiment was done. Cycling extracts containing various amounts of M13 DNA were incubated at room temperature; and 2-μl aliquots were withdrawn at 10-min intervals, frozen, and later assayed for histone H1 kinase activity. With low numbers of indicator sperm nuclei and no added DNA, the histone H1 kinase activity peaked abruptly and dropped to nearly undetectable levels before rising again in the second cell cycle. The peak of H1 kinase activity at 40 min (Fig. 2, no M13 DNA added) corresponded well with the timing of mitosis observed microscopically. The sensitivity of the cycling extract to addition of single-stranded DNA varies somewhat from extract to extract. In this experiment, M13 DNA at 2 ng/μl delayed the onset of mitosis by approximately 40 min, as indicated by visual observation. Likewise, the peak of H1 kinase activation was delayed. At higher DNA concentrations, the visible interphase period was further lengthened, and the H1 kinase activation was concomitantly later (data not shown).

Although the delay of mitosis caused by the M13 DNA

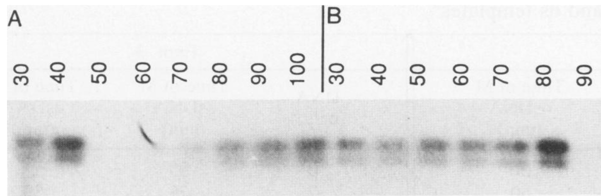

FIG. 2. Histone H1 kinase assays. Cycling extracts containing no DNA (A) or 2 ng of single-stranded DNA per μl (B) were incubated at room temperature. Aliquots of 2 μl were withdrawn at the indicated times (minutes) following the start of the incubation and frozen in liquid nitrogen. Samples were assayed for histone H1 kinase activity, and the reactions were stopped by addition of SDS sample buffer. Samples were resolved by SDS-PAGE and exposed to X-ray film in the presence of an intensifying screen at −70°C.

seemed to mimic the feedback regulation observed with sperm nuclei, it remained possible that the M13 DNA was nonspecifically inactivating the mitotic machinery. In the cycling extract, there is an excess of free *cdc2* protein which can be converted into an active pool of MPF and histone H1 kinase by the addition of exogenous cyclin protein. GST-cyclin fusion protein produced in bacteria (37) can induce mitosis when added to a *Xenopus* egg extract even in the presence of unreplicated DNA. In other words, it behaves in a dominant manner, overriding the feedback system. Addition of GST-cyclin at 60 min to the extract arrested with M13 produced a peak in H1 kinase activity sufficient to drive the extract into mitosis (Fig. 3), indicating that the newly formed cyclin-*cdc2* complex was not subject to inhibition by the M13 DNA and confirming that the machinery necessary for activating the H1 kinase and executing mitosis was intact.

It has been shown that the feedback of unreplicated DNA on mitosis can also be overridden by the addition of caffeine

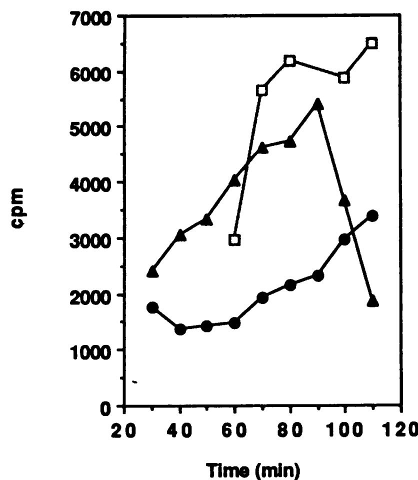

FIG. 3. Cyclin and caffeine override of feedback. M13 DNA (6 ng/μl) was added to the cycling extract to arrest the extract prior to mitosis. To one aliquot, caffeine was added at the start of the room temperature incubation; to another, cyclin was added at 60 min following the start of incubation. Aliquots of 2 μl were withdrawn at 10-min intervals. Each sample was assayed for histone H1 kinase activity. The reactions were stopped by spotting onto phosphocellulose filters. Filters were washed in 1% phosphoric acid to remove unincorporated ^{32}P. The washed filters were counted in a scintillation counter. The counts per minute at each time point in the presence of caffeine (▲), cyclin (□), and DNA alone (●) are shown.

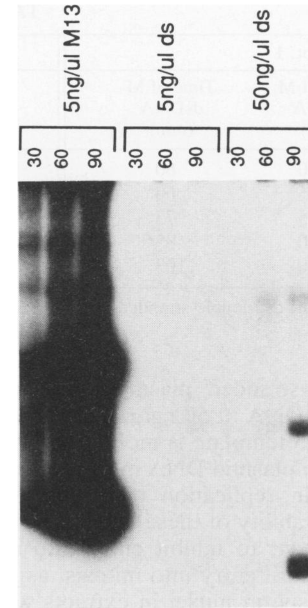

FIG. 4. Replication of single- and double-stranded DNAs in the cycling extract. Aliquots of extract containing the indicated concentrations of M13 single-stranded DNA (ss) or double-stranded plasmid DNA (ds) were continuously labeled with [α-^{32}P]dCTP as for Fig. 1B for 30, 60, or 90 min. Following proteinase K treatment, replication products were visualized by agarose gel electrophoresis and autoradiography.

(33, 34). To further demonstrate that the mitotic machinery was still functional in the presence of single-stranded DNA, we added caffeine to extracts containing M13 DNA (6 ng/μl) and assayed samples for histone H1 kinase activity (Fig. 3). Addition of caffeine at the beginning of the incubation overrode the M13-induced feedback, as evidenced by the peak in histone H1 kinase activity at 90 min. Visual monitoring of parallel samples showed this rise in histone H1 kinase activity to be sufficient to induce mitosis. The histone H1 kinase activity of the 6-ng/μl sample without caffeine gradually increased over time but did not peak during the course of the experiment (120 min). Indicator nuclei, present at low levels in parallel samples, never entered mitosis. This finding demonstrates that the feedback induced by M13 is susceptible to caffeine override in the same way as is feedback generated in vitro by sperm nuclei and in tissue culture cells by hydroxyurea treatment.

Double-stranded plasmid templates. Unlike sperm chromatin, single-stranded DNA does not require nuclear structure for replication in *Xenopus* egg extracts (1, 24). Indeed, M13 replicates perfectly well in extracts which are further fractionated to remove membranes (data not shown). Conversely, double-stranded plasmid DNA, like sperm nuclei, requires nuclear structure for efficient replication (3, 27). While double-stranded plasmids can serve as templates for nuclear assembly in vitro (3), this process is considerably less rapid and efficient than nuclear formation on sperm chromatin templates, resulting in relatively poor replication of plasmids in the egg extract. If unreplicated DNA itself were monitored by a DNA feedback system, plasmid DNA might be expected to trigger feedback as effectively as an equivalent amount of M13 or sperm chromatin DNA, even in the absence of efficient replication.

Figure 4 shows the replication of single-stranded DNA at

TABLE 1. Feedback by ss and ds templates[a]

Expt. 1			Expt. 2			Expt. 3		
DNA (ng/μl	Time of M ssDNA (min)	Time of M dsDNA (min)	DNA (ng/μl)	Time of M ssDNA (min)	Time of M dsDNA (min)	DNA (ng/μl)	Time of M ssDNA (min)	Time of M dsDNA (min)
0	60	60	0	60	60	0	55	55
2	95	60	2	70	60	2	75	55
4	95	73	4	80	60	4	85	55
8	110	83	8	110	70	8	105	65
16	>120	103	16	>120	70	16	120	

[a] ss, single stranded; ds, double stranded.

5 ng/μl, double-stranded plasmid DNA at 5 ng/μl, and double-stranded DNA at 50 ng/μl. It is clear that the replication of the M13 template is more than 10-fold better than replication of the plasmid DNA in the extract. Significantly, this difference in replication efficiency is reflected in a difference in the ability of these templates, upon addition to the cycling extract, to inhibit entry into mitosis. Table 1 shows the time until entry into mitosis, as judged by monitoring indicator sperm nuclei in extracts with various concentrations of single-stranded M13 or double-stranded plasmid DNA. While the efficiency of feedback varies somewhat from extract to extract, single-stranded DNA is consistently more effective than double-stranded plasmid DNA, completely inhibiting progression of the cell cycle at concentrations of DNA which do not even slow the cell cycle when the added template is double stranded.

Representative histone H1 kinase assays from several different experiments are shown in Fig. 5. Single-stranded DNA at 2 ng/μl lengthened interphase by 35 min in comparison with samples containing no added DNA, while the same concentration of double-stranded plasmid DNA had no effect on the peak of histone H1 kinase activity or on entry into mitosis (Fig. 5A). In a different cycling extract, the peaks of histone H1 kinase activity in extracts containing no added DNA and in extracts containing 4 ng of plasmid DNA per μl were coincident, while the peak was delayed by 30 min in samples with the same concentration of single-stranded M13 DNA (Fig. 5B). Similarly, double-stranded plasmid DNA at 16 ng/μl lengthened the interphase by only 15 min, while the same concentration of single-stranded DNA inhibited H1 kinase activation sufficiently to block entry into mitosis for the duration of the experiment (120 min) (Fig. 5C). The relative ineffectiveness of double-stranded plasmid DNA in generating the feedback signal suggests either that some feature of replicating DNA, rather than unreplicated DNA per se, generates the signal which inhibits entry into mitosis or that single-stranded DNA itself, unlike double-stranded plasmid DNA, is a potent inhibitor of entry into mitosis.

Tyrosine phosphorylation of *cdc2*. Activation of the *cdc2* kinase component of MPF can be blocked by tyrosine phosphorylation of *cdc2*. Smythe and Newport have re-

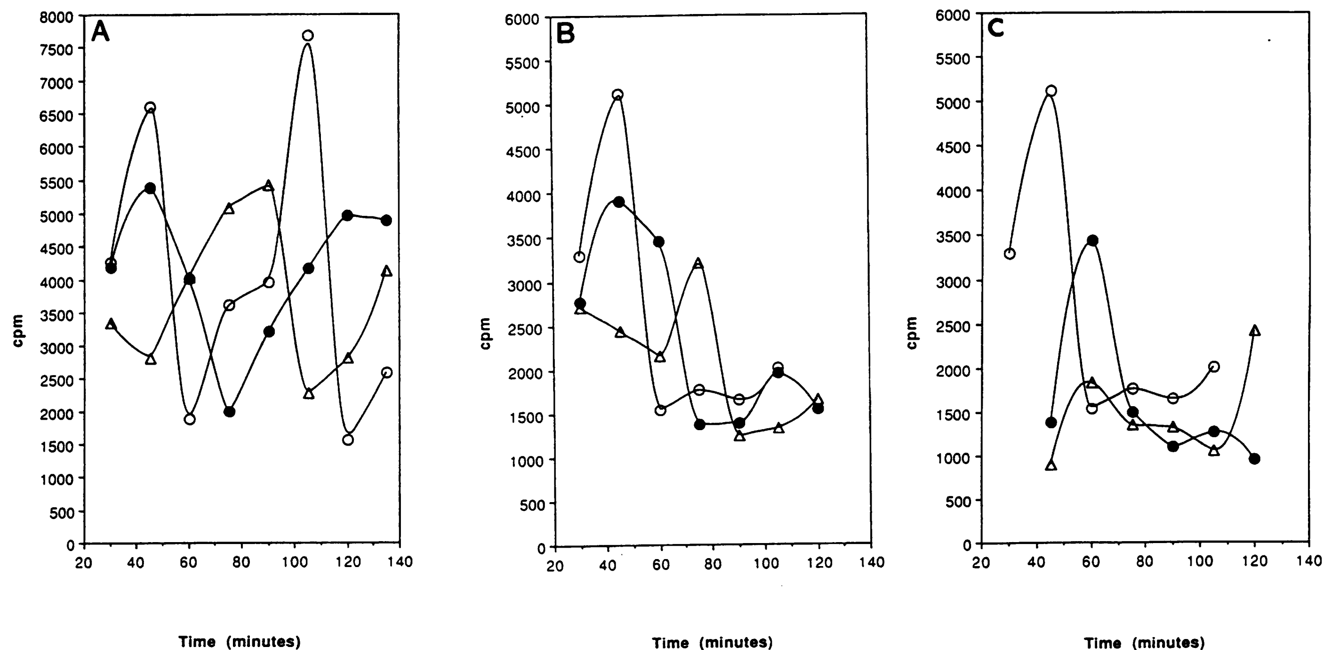

FIG. 5. Histone H1 kinase assays of extracts containing single-stranded or double-stranded templates. Aliquots of cycling extract containing 2 ng of DNA per μl (A), 4 ng of DNA per μl (B), and 16 ng of DNA per μl (C) were withdrawn at 10-min intervals and assayed for histone H1 kinase activity as described in the legend to Fig. 3. Panels B and C are from the same cycling extract; panel A is from an extract prepared at a different time. Shown are data for no DNA (○), double-stranded plasmid DNA (●), and single-stranded M13 DNA (△).

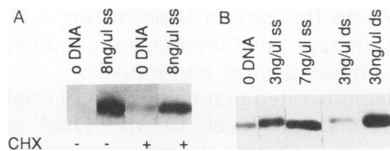

FIG. 6. Assays of the tyrosine kinase which phosphorylates p34^{cdc2}. (A) Recombinant GST-cyclin was added in the presence of sodium orthovanadate to aliquots of cycling extract containing 0 or 8 ng of single-stranded M13 DNA (ss) per μl in the presence or absence of cycloheximide (CHX). The addition of sodium orthovanadate inhibits tyrosine phosphatase activity, preventing the induction of mitosis by the GST-cyclin. After 10 min, samples were frozen in liquid nitrogen. Thawed samples were incubated with glutathione agarose. The agarose beads were washed as indicated in Materials and Methods, boiled in sample buffer, and electrophoresed on SDS-polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, filters were probed with antiphosphotyrosine antibody. (B) Samples containing the indicated amounts of single-stranded M13 DNA (ss) or double-stranded plasmid DNA (ds) were processed as for panel A.

cently shown that a tyrosine kinase which phosphorylates *cdc2* is activated in the presence of replication-arrested sperm DNA (sperm chromatin plus aphidicolin), thereby leading to inhibition of MPF activity and arrest of the cell cycle (36). This tyrosine kinase can be assayed by blocking tyrosine phosphatase activity with sodium orthovanadate and then adding an excess of GST-cyclin protein to the extract. This cyclin protein complexes with free *cdc2* in the extract (which is not phosphorylated on tyrosine prior to complex formation), providing a fresh substrate pool for the tyrosine kinase. These reactions are stopped 10 min after addition of the cyclin, allowing us to measure the rates of *cdc2* tyrosine phosphorylation since the reaction does not go to completion in this short time (36). The GST-cyclin complexes are then purified on glutathione Sepharose, resolved by SDS-PAGE, and immunoblotted with antiphosphotyrosine antibody.

Rates of p34^{cdc2} tyrosine phosphorylation were determined in the absence and presence of sufficient M13 DNA to bring about cell cycle arrest. We added 8 ng of M13 DNA per μl to the cycling extract (to arrest the cell cycle) and measured the rate of tyrosine phosphorylation of *cdc2* complexed to GST-cyclin. As shown in Fig. 6A, the rate of tyrosine phosphorylation of *cdc2* was fivefold greater in the presence of 8 ng of M13 DNA per μl than in the absence of added DNA. Interestingly, this increase in tyrosine kinase activity occurred in the presence of cycloheximide, demonstrating that protein synthesis is not required for kinase activation (Fig. 6A). In a separate experiment, either single-stranded or double-stranded DNA at 3 ng/μl, single-stranded DNA at 7 ng/μl, or double-stranded DNA at 30 ng/μl was added to aliquots of cycling extract, and the *cdc2*-directed tyrosine kinase activity was measured (Fig. 6). As expected from the data presented above, M13 DNA, which was more effective than double-stranded plasmid DNA at inhibiting H1 kinase activation, also induced more tyrosine kinase activity.

DISCUSSION

During the cell cycle, DNA replication and initiation of mitosis are linked by a feedback system. By maintaining MPF kinase in an inactive state, this feedback system ensures that the initiation of mitosis does not occur until DNA synthesis is complete. In this report, we have demonstrated that the feedback system which blocks entry into mitosis during S phase can be activated by simple, well-defined DNA templates. Specifically, we have shown that addition of either single- or double-stranded plasmid DNA to cycling *Xenopus* egg extracts affects the time at which mitosis initiates. This effect is dose dependent; at low concentrations, mitosis is delayed, and at higher concentrations, it is blocked.

By measuring histone H1 kinase activity, we have shown that addition of the M13 or plasmid DNA to an extract inhibits activation of MPF. This observation strongly indicates that these small DNA templates activate the feedback pathway which normally keeps MPF inactive during S phase. In support of this view, we have shown that the mitotic delay caused by addition of the DNA templates is reversed by caffeine, a drug known to inactivate the feedback pathway. Moreover, we have shown that the rate of phosphorylation of the *cdc2* subunit of MPF, a modification known to inactivate MPF, is stimulated fivefold by addition of plasmid DNA. Together, these results demonstrate that single-stranded M13 DNA and double-stranded plasmid DNA, like nuclear DNA, inhibit mitosis in a cycling extract by stimulating the feedback pathway which inactivates MPF via tyrosine phosphorylation of p34^{cdc2}.

Although it is clear that MPF is a primary downstream target regulated by the feedback system, very little is known about how DNA generates the feedback signal. We have shown that single-stranded DNA templates, which do not appear to form nuclei, activate the feedback system. Therefore, it appears unlikely that complex nuclear structures or nuclear transport is directly involved in generating the signal that activates the feedback pathway. Rather, our results argue that this signal is generated by proteins which either can recognize unreplicated DNA or, alternatively, can directly recognize DNA which is actively being replicated. For example, the signal-generating protein(s) could bind along the length of the DNA at the beginning of S phase. The subsequent displacement of this protein by passage of the replication complex could provide a simple mechanism for attenuating the feedback signal. Such a process should, in principle, provide a reliable estimate of when replication was complete. Alternatively, the signal-transducing protein could be a part of the replication complex or bind to a DNA substrate generated by the complex. In this case, as long as replication were ongoing, mitosis would be suppressed.

In the first model described above, the signal which activates the feedback pathway is generated in the absence of replication complexes. By contrast, in the second model, the formation of replication complexes is essential for generating a signal. Previously, we have shown that sperm nuclei will arrest the in vitro cell cycle completely when present at greater than 2,000 nuclei per μl extract (5). In our experiments, double-stranded plasmid DNA did not arrest the cell cycle at levels as high as 16 ng of DNA per μl, the equivalent of more than 5,000 sperm nuclei per μl of DNA. This finding strongly suggests that unreplicated DNA itself is not the substrate that activates the feedback pathway which inhibits mitosis. The efficiencies of single-stranded and double-stranded plasmid DNAs to act as templates for DNA replication in cycling extracts differ markedly. Single-stranded templates are replicated much more efficiently than are double-stranded templates. Similarly, single-stranded templates are considerably more efficient at generating a feedback signal (Fig. 5; Table 1). The correlation between replication efficiency and signal generation further suggests

that the feedback pathway is activated by proteins involved in DNA replication or by DNA structures formed during replication rather than by proteins which bind to unreplicated DNA.

In *Xenopus* embryos, the replication complex proceeds at a rate of between 500 and 1,000 bp/min (4). Therefore, during the 15-min embryonic S phase, replication complexes would have to form approximately every 7,500 to 15,000 bp of DNA. With these figures, there would be approximately equal numbers of active replication complexes on equal masses of M13 template and sperm nuclei. Approximately 6 ng of each of these templates per μl is required to arrest the cell cycle in vitro. This conclusion is consistent with the idea that active replication complexes themselves may generate the feedback signal during replication.

An alternative mechanism for activation of the feedback system would involve single-stranded DNA. During DNA replication, single-stranded-DNA-binding proteins stabilize single-stranded DNA at the replication fork. Proteins which recognize and bind to these replication-dependent single-stranded regions could generate the feedback signal which inhibits mitosis during replication. It has been estimated, from analogy to prokaryotic replication systems, that the amount of single-stranded DNA generated at a eukaryotic replication fork is not likely to exceed 500 to 1,000 bp of DNA. If the feedback system is activated by the single-stranded DNA present at replication forks, then addition of M13 single-stranded DNA could be activating feedback directly. In this case, feedback could be uncoupled from DNA replication by the addition of single-stranded DNA unable to replicate in the extract. We have attempted to determine whether this is the case by adding synthetic oligonucleotides 57 bp in length to cycling extracts. These oligonucleotides did not replicate and did not inhibit the cell cycle. However, the added oligonucleotide was not sufficiently stable in the extract to allow us to draw any firm conclusions from these experiments. We have also added modified oligonucleotides (16-mers with phosphoramidate linkages) which are stable in extracts to try to activate feedback independently of DNA replication. In this case, feedback was not inhibited at oligonucleotide concentrations up to 80 ng per μl of extract. Although this observation might indicate that the activation of feedback requires DNA replication, it may also be possible that a 16-bp single-stranded oligonucleotide is not a suitable substrate for the feedback proteins. A number of other experiments designed to determine whether activation of feedback is independent of replication have yielded equally ambiguous results. Therefore, a clear answer to this question will have to await further characterization of the signalling system itself.

Like the replication feedback pathway, the repair feedback pathway prevents cells from entering mitosis until damaged DNA is repaired (see the introduction and reference 16). Given the similarity in the substrates recognized by these two systems, replicating DNA and damaged DNA, it is quite likely that they have some features in common. Indeed, because DNA replication creates substrates which are likely to be recognized as DNA damage (single-stranded DNA, unligated fragments, and mismatched base pairs) it is probable that the DNA-binding proteins which activate the repair pathway are active during both S and G_2 phases. From this point of view, the repair pathway, although activated by different substrates, may be an extension of the replication pathway; i.e., the replication pathway ensures that replication is completed, while the repair pathway ensures that it is completed properly. It is possible that addition of single-stranded DNA to the extract is activating a DNA damage detection system (e.g., that involving the *rad9* gene product) and thereby inhibiting entry into mitosis.

We have demonstrated that high levels of double-stranded plasmid DNA and low levels of M13 DNA added to the cycling extract can increase the rate of tyrosine phosphorylation of $p34^{cdc2}$. It is of interest that the level of H1 kinase activity at mitosis in the absence of DNA was higher than that attained in the presence of DNA even when the DNA was not able to prevent entry into mitosis (e.g., 8 ng of double-stranded DNA per μl or 2 ng of single-stranded DNA per μl) (Fig. 5). Our data show that with higher amounts of added DNA, the activation of the tyrosine kinase which suppresses histone H1 kinase activity is greater (Fig. 6). When the tyrosine kinase(s) is only partially activated, only a subpopulation of the cyclin-*cdc2* complexes is suppressed. If the remaining active complexes supply sufficient MPF activity, the sample enters mitosis despite some activation of the feedback pathway.

Our ability to activate tyrosine kinase activity by using DNA templates opens up the possibility of dissecting the signal transduction pathway leading from the template to the tyrosine kinase. It should be possible to activate the tyrosine kinase(s) with the artificial templates and further fractionate the extracts for intermediates in the pathway. It should also be possible to remove the templates from the extracts to examine bound proteins. It is possible that *cdc2* interacts with regulatory kinases and phosphatases while bound directly to the DNA template. Indeed, Fotedar and Roberts have recently shown direct binding of $p34^{cdc2}$ to simian virus 40 templates replicating in vitro (12). Kinases and phosphatases regulating *cdc2* activity might be bound directly to the DNA template, using DNA as a cofactor for activation. Future work aimed at addressing these questions will be greatly simplified by the ability to generate feedback by using simple artificial DNA templates.

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REFERENCES

1. **Almouzni, G., and M. Mechali.** 1988. Assembly of spaced chromatin promoted by DNA synthesis in extracts from Xenopus eggs. EMBO J. **7:**665–672.
2. **Arion, D., L. Meijer, L. Brizuela, and D. Beach.** 1988. cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell **55:**371–378.
3. **Blow, J., and R. A. Laskey.** 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. Cell **47:**577–587.
4. **Callan, H. G.** 1972. Replication of DNA in the chromosomes of eucaryotes. Proc. R. Soc. London Ser. B **181:**19–41.
5. **Dasso, M., and J. W. Newport.** 1990. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. Cell **61:**811–823.
6. **Draetta, G., F. Luca, J. Westendorf, J. Ruderman, and D. Beach.** 1989. cdc2 protein kinase is complexed with cyclin A and B: evidence for proteolytic inactivation of MPF. Cell **56:**829–838.
7. **Dunphy, W. G., L. Brizuela, D. Beach, and J. W. Newport.** 1988. The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell **54:**423–431.
8. **Dunphy, W. G., and J. W. Newport.** 1989. Fission yeast p13

blocks mitotic activation and tyrosine dephosphorylation of the Xenopus cdc2 protein kinase. Cell **58**:181–191.

9. **Enoch, T., and P. Nurse.** 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell **60**:665–673.
10. **Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt.** 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell **33**:383–396.
11. **Featherstone, C., and P. Russell.** 1991. Fission yeast $p107^{wee1}$ mitotic inhibitor is a protein tyrosine/serine kinase. Nature (London) **349**:808–811.
12. **Fotedar, R., and J. M. Roberts.** Cell cycle dependent association of $p34^{cdc2}$ with replicating DNA. Cold Spring Harbor Symp. Quant. Biol., in press.
13. **Gautier, J., T. Matsukawa, P. Nurse, and J. Maller.** 1989. Dephosphorylation and activation of Xenopus p34 protein kinase during the cell cycle. Nature (London) **339**:626–629.
14. **Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller.** 1988. Purified maturation promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene $cdc2^{+}$. Cell **54**:433–439.
15. **Gould, K. L., and P. Nurse.** 1989. Tyrosine phosphorylation of the fission yeast $cdc2^{+}$ protein kinase regulates entry into mitosis. Nature (London) **342**:39–45.
16. **Hartwell, L., and T. Weinert.** 1989. Checkpoints: controls that ensure the order of cell cycle events. Science **246**:629–634.
17. **Hutchinson, C., and I. Kill.** 1989. Changes in the nuclear distribution of DNA polymerase alpha and PCNA/cyclin during the progress of the cell cycle in a cell-free extract. J. Cell Sci. **93**:605–613.
18. **Kumagai, A., and W. G. Dunphy.** 1991. The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. Cell **64**:903–914.
19. **Labbe, J. C., A. Picard, G. Peaucellier, J. Cavadore, P. Nurse, and M. Doree.** 1989. Purification of MPF from starfish: identification as the H1 histone kinase $p34^{cdc2}$ and a possible mechanism for its periodic activation. Cell **57**:253–263.
20. **Lee, T. H., M. J. Solomon, M. C. Mumby, and M. W. Kirschner.** 1991. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell **64**:415–423.
21. **Lewin, B.** 1990. Driving the cell cycle: M-phase kinase, its partners and substrates. Cell **61**:743–752.
22. **Lohka, M., and Y. Masui.** 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. Science **220**:719–721.
23. **Lundgren, K., N. Walworth, R. Booher, M. Dempski, M. Kirschner, and D. Beach.** 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64**:1111–1122.
24. **Mechali, M., and R. Harland.** 1982. DNA synthesis in a cell-free system from Xenopus eggs: priming and elongation on single-stranded DNA in vitro. Cell **30**:93–101.
25. **Minshull, J., J. Blow, and T. Hunt.** 1989. Translation of cyclin mRNA is necessary for extracts of activated Xenopus eggs to enter mitosis. Cell **56**:947–956.
26. **Murray, A. W., and M. W. Kirschner.** 1989. Cyclin synthesis drives the early embryonic cell cycle. Nature (London) **339**:287–292.
27. **Newport, J.** 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell **48**:205–217.
28. **Nishimoto, T., E. Eilen, and C. Basilico.** 1978. Premature chromosome condensation in a ts DNA- mutant of BHK cells. Cell **15**:475–483.
29. **Ohtsubo, M. R. Kai, N. Foruno, T. Sekiguchi, M. Sekiguchi, H. Hayashida, K. Kuima, T. Miyata, S. Fukushige, T. Murotsu, K. Matsubara, and T. Nishimoto.** 1987. Isolation and characterization of the active cDNA of the human cell cycle gene (RCC1) involved in the regulation of onset of chromosome condensation. Genes Dev. **1**:585–593.
30. **Osmani, S. A., D. B. Engle, J. H. Doonan, and N. R. Morris.** 1988. Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. Cell **52**:241–251.
31. **Russell, P., and P. Nurse.** 1986. $cdc25^{+}$ functions as an inducer in the mitotic control of fission yeast. Cell **45**:145–153.
32. **Russell, P., and P. Nurse.** 1987. Negative regulation of mitosis by $wee1^{+}$, a gene encoding a protein kinase homolog. Cell **49**:559–567.
33. **Schlegel, R., and A. Pardee.** 1986. Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science **232**:1264–1266.
34. **Schlegel, R., and A. Pardee.** 1987. Periodic mitotic events induced in the absence of DNA replication. Proc. Natl. Acad. Sci. USA **84**:9025–9029.
35. **Simanis, V., and P. Nurse.** 1986. The cell cycle control gene $cdc2^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell **45**:261–268.
36. **Smythe, C., and J. W. Newport.** 1991. Coupling of mitosis to the completion of S-phase in Xenopus occurs via modulation of the tyrosine kinase that phosphorylates p34cdc2. Cell **68**:787–797.
37. **Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner.** 1990. Cyclin activation of $p34^{cdc2}$. Cell **63**:1013–1024.
38. **Strausfeld, U., J. C. Labbe, D. Fesquet, J. C. Cavadore, A. Picard, K. Sadhu, P. Russell, and M. Doree.** 1991. Dephosphorylation and activation of a $p34^{cdc2}$/cyclin complex in vitro by human cdc25 protein. Nature (London) **351**:242–245.
39. **Weinert, R., and L. Hartwell.** 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science **241**:317–322.