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Biochemistry

Replication *in vitro* of the 2-μm DNA plasmid of yeast

(cell-free extracts/cell cycle/growth control/*cdc* mutants/*Saccharomyces*)

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ABSTRACT **Cell-free extracts prepared from growing cells of the budding yeast *Saccharomyces* stimulated DNA synthesis directed by the supercoiled 2-μm yeast DNA plasmid. The major products of the reaction were open-circular daughter molecules possessing newly synthesized full-length linear DNA strands. Some of these were ligated and supertwisted by the extracts to yield a supercoiled DNA product. Both of the complementary DNA strands of the template were replicated. In addition, the extracts induced the appearance of Θ-forms of the plasmid DNA, which are presumed to be replicative intermediates. The results of experiments utilizing BrdUTP incorporation indicated that DNA repair did not contribute significantly to the overall reaction. Extracts prepared from the cell division cycle mutants *cdc7* and *cdc8*, held in culture at the nonpermissive temperature, possessed diminished activity. Because these mutants define a dependent sequence of events leading from the start of the cell cycle through G_1 to S phase, this result suggests that the activity that stimulates 2-μm DNA replication *in vitro* is subject to control in the yeast cell cycle.**

An understanding of the cell cycle in eukaryotes requires an analysis of the stimulatory signals and of the subsequent regulatory mechanisms involved in traversal of the G_1 phase and entry into the S phase. Essential to this analysis is a means of establishing the order of biochemical events leading to initiation of DNA synthesis, as well as assays that will allow isolation of the molecules responsible for these events. An important step in the realization of the first of these objectives was the isolation of temperature-sensitive yeast mutants defective in progression through the cell division cycle (1). Studies conducted with these mutants have provided an explanation of the orderly temporal sequence of events in terms of the dependence of each event on prior steps in the cycle (1).

The second objective, isolation of the molecules involved in each step, requires the development of suitable biochemical approaches. Our previous studies (2) showed that yeast extracts are active in the cell-free stimulation of DNA replication in nuclei isolated from resting cells of *Xenopus laevis;* this activity was found to be subject to control in the yeast cell cycle. Further support for these conclusions could be obtained through the use of a defined template for DNA replication *in vitro*. In addition, such a template would be particularly useful for routine assays during fractionation of the active components in the extracts and reconstitution of the system.

A good candidate for such a defined template is the 2-μm plasmid of yeast. This plasmid is a DNA element of unknown function that is present in 50–100 copies in the cytoplasm of many yeast strains. It occurs as a double-stranded, supercoiled molecule of approximately 6 kilobases, and its physical map has been determined (see, for example, ref. 3). The analysis of the replication of this plasmid *in vivo* indicates that it is under the same control as that of nuclear DNA (4, 5), and that it proceeds via a Θ-form replicative intermediate (4).

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In this paper, evidence is presented that cell-free extracts from growing yeast cells stimulate replication of 2-μm DNA. The results suggest that the expression of this stimulatory activity is under control of factors that also control the cell division cycle.

MATERIALS AND METHODS

Cells and Growth Conditions. *Saccharomyces cerevisiae* strains (2) and *Saccharomyces italicus* C-105 (provided by T. Petes of the University of Chicago) were used. For preparation of 2-μm plasmid DNA, cells were labeled with [8-^{3}H]adenine in a synthetic complete liquid medium (6) at 30°C. For preparation of cell-free extracts for DNA replication, cells were grown as described (2). Strain C-105 was cultured at 30°C, while A364A and the *cdc* mutants (temperature sensitive in the cell division cycle) were cultured at 23°C.

Preparation of 2-μm Plasmid DNA. Cells were harvested in late exponential phase and converted to spheroplasts as described (2). After washing, spheroplasts were resuspended in 0.6 M NaCl/100 mM EDTA (pH 8.5) at approximately 3×10^9 per ml, treated with 1% sodium dodecyl sulfate and proteinase K at 100 μg/ml for 3–5 hr at 37°C, and centrifuged for 30 min at 0°C at 25,000 × *g*. The DNA in the supernatant was sedimented through a sucrose gradient onto a CsCl shelf and treated with RNase A, and supercoiled DNA was prepared on a CsCl/ethidium bromide gradient. After extraction with isopropanol, the DNA was dialyzed and banded between 61% (wt/wt) and 39% (wt/wt) CsCl shelves. The DNA was dialyzed and precipitated with ethanol. Some preparations were further purified by rate zonal sedimentation in sucrose gradients. The supercoiled 2-μm DNA was resuspended in 20 mM Tris·HCl/2 mM EDTA (pH 7.5).

Preparation of Cell Extracts for DNA Replication. Exponential yeast cultures were harvested rapidly at room temperature, and the cells were resuspended in 10% sucrose/50 mM Tris·HCl (pH 8.2) at $3–6 \times 10^9$ per ml. The suspensions were frozen in liquid nitrogen, thawed at room temperature, and placed on ice. To these suspensions were added: 4 M KCl to a final concentration of 0.1 M, 100 mM spermidine (pH 7.8) to 5 mM, and Zymolyase 60,000 (Kirin Brewery) (5 mg/ml) to 0.5 mg/ml. After gentle mixing, the suspensions were incubated for 30 min. Brij-58 (5%) was added to a final concentration of 0.1%, the suspensions were incubated at 37°C for 1 min and then chilled and centrifuged at 2°C for 45 min at 165,000 × *g*. Supernatants were collected and centrifuged at 0°C for 10 min at 40,000 × *g* to remove the lipid pellicle.

Crude extracts were adjusted to pH 7.8 with solid Tris base, and solid ammonium sulfate was added to 50% saturation. Protein precipitates were resuspended in 25% (vol/vol) glycerol/50 mM Tris·HCl (pH 7.5)/1 mM dithiothreitol and ammonium sulfate was removed by centrifugation through Sephadex G-25. This fraction (≈80 mg of protein per ml) was stored on ice and used the same day.

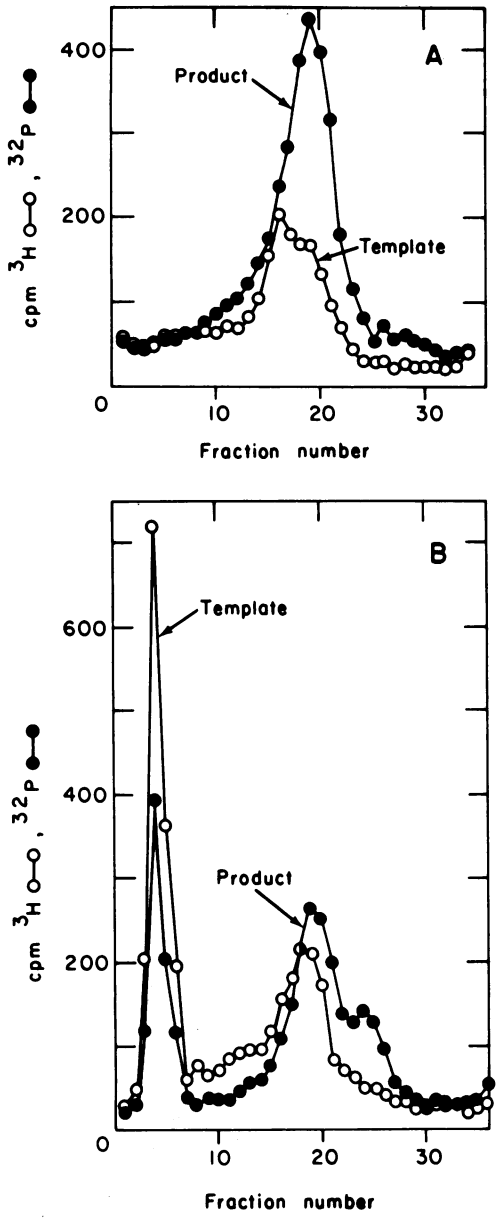

FIG. 1. Rate zonal sedimentation analysis of plasmid replication. Sedimentation is from right to left. The cpm of acid-precipitable material are plotted. (*A*) Neutral sucrose gradient, (*B*) alkaline sucrose gradient with CsCl shelf. Parallel gradients were run with ^3H-labeled bacteriophage f1 duplex circular DNA (a gift from K. Horiuchi of The Rockefeller University) to determine the positions of supercoils and open circles. Reaction mixtures that did not contain plasmid DNA were also analyzed in parallel, and the ^{32}P background near the top of the gradient has been subtracted in the figures.

Assay for 2-μm DNA Replication. Incubations were performed at 30°C for 30 min. The reaction mixture contained: 2 mM spermidine, 1 mM rATP, 100 μM each of rGTP, rUTP, rCTP, dATP, dGTP, and dCTP, 20 μM dTTP, 1 mM NAD, 1.25 mM phospho*enol*pyruvate, pyruvate kinase at one unit per ml, 10 mM $MgCl_2$, 12.5% (vol/vol) glycerol, 31 mM Tris·HCl (pH 7.5), 0.5 mM dithiothreitol, and 0.6 mM EDTA. The ammonium sulfate fraction described above was added to give a final protein concentration of approximately 25 mg/ml in the reaction mixture. Two-micrometer plasmid DNA was added (40–200 pmol of total nucleotides). [α-^{32}P]dTTP was present at specific activities of 100–500 cpm/pmol.

The reaction was stopped by the addition of an equal volume of 20 mM EDTA (pH 8.0)/2% Sarkosyl, and the samples were incubated for an additional 5 min at 30°C before further processing.

RESULTS

Extracts from Yeast Cells Stimulate Replication of 2-μm DNA. Incubation of purified 2-μm DNA supercoils with extracts prepared from exponentially growing yeast in the presence of Mg^{2+}, ATP, and the four dNTPs led to the incorporation of labeled dNTPs into acid-precipitable material. The reaction was essentially complete in 30 min at 30°C.

To determine the nature of the observed DNA synthesis, analyses of the newly synthesized DNA and the template were carried out in neutral (Fig. 1*A*) and alkaline (Fig. 1*B*) sucrose gradients. A preparation of [^3H]adenine-labeled 2-μm DNA that was free of RNA, as well as of contamination by nuclear and mitochondrial DNA, and that was comprised of 97% supercoiled DNA as judged by electron microscopy was employed as template. This template was incubated in the presence of [α-^{32}P]dTTP, with a yeast extract prepared from strain C-105. The reaction mixture was then analyzed by sedimentation in a neutral sucrose gradient. As shown in Fig. 1*A*, the newly synthesized DNA labeled with ^{32}P cosedimented with the ^3H-labeled template. Controls, in which the 2-μm DNA was absent from the reaction mixture demonstrated that the synthesis of the ^{32}P-labeled material cosedimenting with the plasmid was dependent on the presence of the plasmid and was not derived from the extract itself. The result illustrated in Fig. 1*A* indicates that the bulk of the product was present as open circles, although some newly synthesized supercoils sedimenting faster than open circles may have been present. The gradient profile also suggests that material was present that sedimented between the supercoil and open circle peaks; this sedimentation behavior is characteristic of replicative intermediates.

The interpretation of the analysis by neutral sucrose gradient sedimentation was extended by direct visualization of the DNA species in each of the individual gradient fractions covering inclusively the range from the supercoil peak to the open circle peak. A summary of this analysis is presented in Table 1, and a few representative molecules are shown in Fig. 2. In addition to the expected supercoils and open circles, replicative intermediates were also found. The percentage of replicative intermediates varied in different fractions of the gradient but was in the range of 8–18%. Although replicative intermediates at

Table 1. Electron microscopic analysis of the DNA molecules in a plasmid replication mixture

Molecules	Gradient fraction 16	17	18	19
Open circles	10	54	127	149
Supercoils	208	152	81	59
Linear DNA	2	4	5	9
O-Forms				
Early	14	22	44	42
Late	6	4	2	0
Unscorable molecules	2	1	3	4
% O-forms	8	11	18	16

The various DNA species present in the fractions spanning the supercoil to the open circle region of the gradient shown in Fig. 1*A* were scored as indicated. Early replicative intermediates are those in which less than 50% of the 2-μm plasmid was replicated, while the late intermediates are those in which greater than 50% was replicated. Samples were prepared as described for Fig. 2 and examined at ×25,800 magnification.

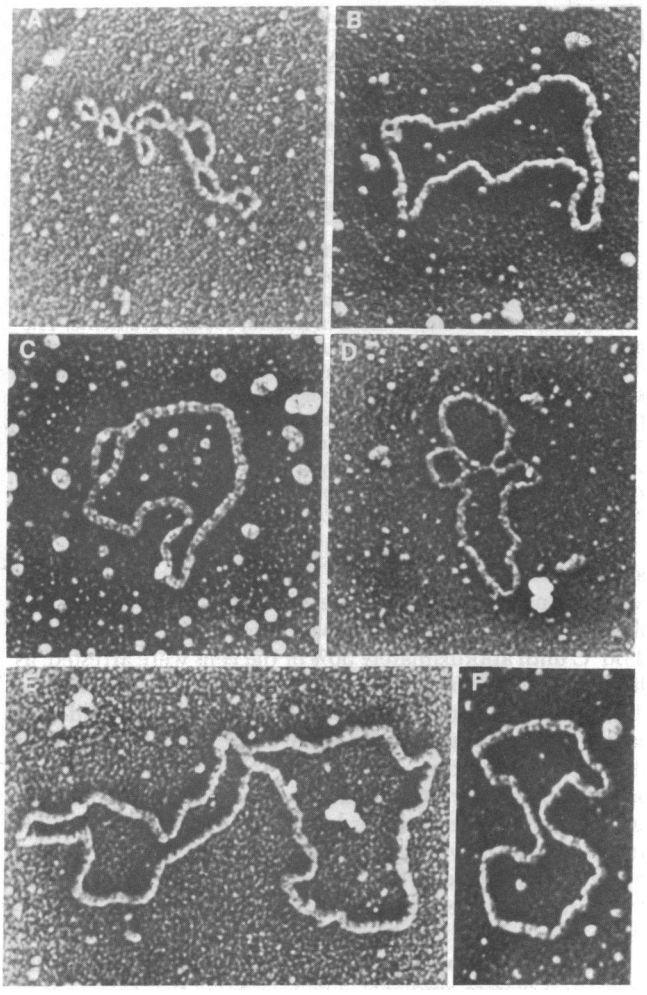

FIG. 2. Electron micrographs of replicating 2-μm DNA. Representative molecules found in the fractions described in Table 1 are shown. Samples were prepared by aqueous spreading (7) and rotary shadowed with Pt/Pd. (*A*) Supercoil, (*B–E*) replicative intermediates, (*F*) open circle. f1 DNA (see legend to Fig. 1) was used as a length standard. (×49,800.)

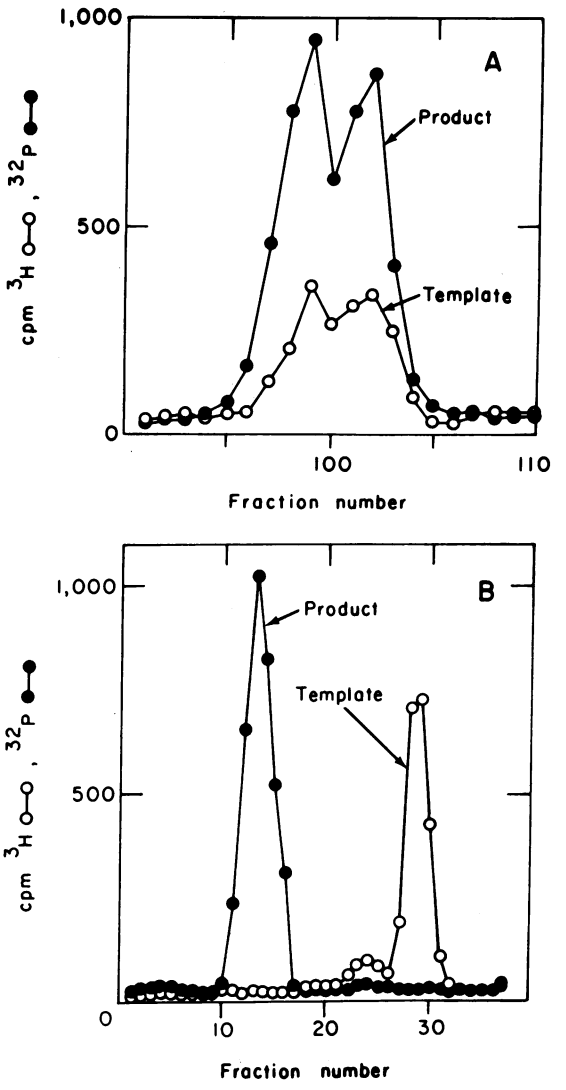

FIG. 3. Alkaline CsCl gradient analysis of plasmid replication. Density increases from right to left. The cpm of acid-precipitable material are plotted. (*A*) Separation of complementary DNA strands. In order to separate the strands of supercoils, the DNA in the plasmid replication mixture was subjected to limited depurination (0.1 M acetic acid, 22°C, 60 min) prior to treatment with alkali. The gradient contained 162 fractions; the region containing the separated, complementary DNA strands derived from supercoils, replicative intermediates, and open circles is shown. (*B*) BrdUTP density shift. Supercoiled and single-stranded, full-length circular and linear plasmid DNA was purified from a replication mixture in an alkaline sucrose gradient. The supercoiled DNA was subjected to limited depurination and was combined with the single-stranded DNA for sedimentation in alkaline CsCl. Separation of complementary strands is not evident because the fraction size is larger than in *A*. The small peak of DNA slightly denser than the bulk of the template DNA represents residual supercoiled DNA.

all stages of completion were detected, there was a preponderance of molecules with small to medium-sized replication "bubbles," suggesting that initiation is rate-limiting *in vitro* as it is *in vivo* (4). Finally, the 2-μm DNA plasmid appears to replicate via a Θ-form intermediate in the *in vitro* system, similar to its mode of replication *in vivo* (4).

These electron microscopic studies clearly demonstrated the formation of replicative intermediates in the reaction. To extend the analysis further, reaction mixtures were subjected to sedimentation in alkaline sucrose gradients (Fig. 1*B*). The bulk of the ^{32}P-labeled product sedimented more slowly than the ^{3}H-labeled, single-stranded, circular plasmid DNA found in the middle of the gradient, and was seen in the position of full-length linear DNA, confirming that the majority of the product consisted of open circles. It is also clear that the template DNA was not degraded. In addition to ^{32}P-labeled full-length linear DNA, some newly synthesized material sedimenting more slowly in alkali is apparent. This material is probably linear DNA shorter than the unit length and derived from replicative intermediates. Newly synthesized DNA was also found sedimenting rapidly in association with supercoiled DNA, suggesting that replication of some of the template proceeded to completion and that some of the daughter molecules were separated, ligated, and supertwisted.

The identification of replicative intermediates of the Θ-form in the reaction mixture suggests that both DNA strands of the 2-μm plasmid were replicated *in vitro*. To strengthen this conclusion, the product of replication was analyzed by isopycnic banding in alkaline CsCl gradients (Fig. 3*A*). A small separation of the complementary DNA strands of the plasmid was indicated by the presence of two peaks of ^{3}H label. Cobanding with them were peaks of newly synthesized ^{32}P-labeled DNA. This result supports the conclusion that both of the complementary DNA strands of the 2-μm plasmid were synthesized *in vitro*.

DNA Repair Does Not Contribute to the DNA Synthesis Observed with 2-μm DNA. The results presented thus far indicate that replication of 2-μm DNA is initiated *in vitro*. To determine whether some DNA repair synthesis was also occurring, a reaction was carried out in which BrdUTP replaced dTTP and the labeled nucleotide was dCTP. The BrdUTP at the 100 μM concentration required to give a significant density shift in CsCl gradients depressed total DNA synthesis to 1/3rd to 1/5th. In addition, the results of alkaline sucrose sedimentation indicated that, in contrast to the dTTP reaction, considerably more of the product was in linear DNA strands that were shorter than unit length. A large proportion of the material sedimented at the position corresponding to 2–4 S, suggesting that BrdUTP inhibits at or shortly after initiation.

Despite this inhibition, the products of replication in the presence of BrdUTP could be analyzed by isopycnic banding in alkaline CsCl gradients (Fig. 3*B*), provided that a purification procedure was used (see legend to Fig. 3*B*) to eliminate the majority of partially replicated molecules. The gradient pattern in Fig. 3*B* indicates that newly synthesized ^{32}P-labeled DNA that was density labeled with BrdUTP was separated from the light ^{3}H-labeled template. No contaminating ^{32}P label was apparent in the parental DNA peak and no contaminating ^{3}H label was apparent in the daughter DNA peak. This result indicates that DNA repair did not contribute significantly to the overall DNA synthesis observed in the system. The density shift in this experiment represents approximately 58% substitution by BrdUTP.

Plasmid replication *in vitro* is efficient. In the experiments discussed thus far (except for the BrdUTP density shift), 50–60% of the DNA was replicated as judged by incorporated ^{32}P label that cosedimented with the parental template. Input plasmid DNA did not appear to be degraded in the reaction mixture, whether DNA synthesis was allowed to proceed or not, and the recovery of template was essentially quantitative. Completion of the replication of the plasmid is also efficient, as evidenced by the relatively low percentage of replicative intermediates. Although the daughter molecules separate to yield open circle product, they are not efficiently ligated and twisted to yield supercoiled product. We have observed that the activity that promotes this final step in the reaction is largely precipitated with ammonium sulfate at 50–70% saturation. Unfortunately, this ammonium sulfate fraction also contains activities that depress the total DNA synthesis and further fractionation is therefore required. It should be noted that supercoiled 2-μm DNA is utilized preferentially *in vitro* as template: although open circles of 2-μm DNA are replicated, they are replicated with only about 20% of the efficiency of supercoil replication.

Extracts from *cdc* Mutants Arrested in G_1–S Phase Are Deficient in Plasmid Replicating Activity. Nuclear DNA replication in yeast requires that cells move through the G_1 phase of the cell cycle and enter S phase by completing the dependent sequence of events specified by *cdc* genes *28*, *4*, and *7*, in that order (8). Nuclear DNA replication also requires the function of the *cdc8* gene (9). Unlike nuclear DNA replication, mitochondrial DNA replication requires only completion of *cdc8*-mediated events, and not those specified by *cdc* genes *28*, *4*, and *7* (10). The replication of 2-μm DNA *in vivo* is under the same control as nuclear DNA replication, in that it is dependent on completion of each stage of the *cdc* 28 → 4 → 7 → 8 pathway (4, 5).

To determine whether this relationship holds true *in vitro*, we measured the stimulation of replication of 2-μm DNA supercoils by extracts prepared from various *cdc* mutants grown at the permissive temperature (23°C) or arrested at the nonpermissive temperature (36°C) (Table 2). Extracts from all the yeast strains grown at 23°C were capable of replicating the plasmid. However, extracts prepared from *cdc7* and *cdc8* held at 36°C until approximately 50% of the cells were arrested, as determined by display of the appropriate terminal phenotype (11), possessed diminished activity. Importantly, the wild-type parent strain possessed similar activity when the cells were held at either temperature. In addition, comparison of extracts prepared from *cdc10* showed that they possessed almost the same activity when the cells had been held at 36°C (to 70% arrest) as when they were maintained at 23°C. This *cdc* mutant is defective in cytokinesis and undergoes many rounds of DNA replication and nuclear division at the nonpermissive temperature (1, 11).

Table 2. Plasmid replicating activity in *cdc* mutants

Strain	pmol dNTPs incorporated 23°C	36°C
A364A	8.55 (100%)	8.15 (95%)
cdc7	8.03 (100%)	2.96 (37%)
cdc8	8.68 (100%)	3.47 (40%)
cdc10	7.18 (100%)	6.39 (89%)

Ammonium sulfate fractions of extracts prepared from *cdc* mutants and the parent strain A364A were compared *in vitro* for 2-μm plasmid replicating activity at 30°C for 30 min. Reaction mixtures were analyzed by rate zonal sedimentation in neutral sucrose gradients. The amount of DNA synthesis was calculated from the acid-precipitable ^{32}P cosedimenting with the template. Extracts were prepared from exponential cultures that had been held at either 23°C or 36°C for approximately 3 hr. In order to ensure full cell viability, the cultures were not held at 36°C for longer times. The degree of arrest of the *cdc* mutants at 36°C was approximately 50% for *cdc7* and *cdc8* cultures, and approximately 70% for *cdc10* cultures. Cells were suspended at the same concentrations for preparation of the extracts.

The reaction mixtures in these experiments were analyzed on neutral sucrose gradients, which revealed that there was no degradation of plasmid DNA in extracts from *cdc7* and *8* cells held at 36°C. Mixing experiments, in which extracts from cells grown at permissive temperature were combined in the assay with extracts from cells held at nonpermissive temperature, indicated that inhibitors of replication are not elaborated in *cdc7* and *8* cells arrested at the nonpermissive temperature.

These results suggest that the appearance of the activity responsible for plasmid replication *in vitro* is under the control of the *cdc* 28 → 4 → 7 → 8 pathway. We conclude that, for the features determined in this study, plasmid replication *in vitro* is a valid model of its replication *in vivo*.

DISCUSSION

The replication of the 2-μm DNA plasmid of yeast is stimulated by cell-free extracts prepared from growing yeast cells in the presence of the necessary low molecular weight cofactors and precursors. This conclusion is supported by the following observations: (*i*) the extracts induced the appearance of replicative intermediates of plasmid DNA (Figs. 1*A* and 2, Table 1); (*ii*) full-length plasmid DNA strands were synthesized (Fig. 1*B*); (*iii*) deoxynucleoside triphosphates were incorporated into complete supercoiled plasmid molecules (Fig. 1*B*); and (*iv*) both of the complementary DNA strands of the plasmid were synthesized (Fig. 3*A*). Furthermore, the contribution of repair in the overall DNA synthesis was effectively eliminated (Fig. 3*B*). Inasmuch as the plasmid DNA preparations used in these experiments were highly purified and were almost exclusively (97%) supercoils, these results strongly suggest that initiation of plasmid DNA replication occurs *in vitro*.

Although extracts from a variety of yeast strains are active in stimulating plasmid replication, extracts from *S. italicus* C-105 seem most effective. Extracts from *S. cerevisiae* strains are, on the average, 1/3rd as efficient in promoting plasmid replication. This difference may be related to the fact that *S. italicus* appears to possess more of the plasmid *in vivo* than does *S. cerevisiae*. The 2-μm DNA plasmid isolated from *S. italicus* was completely interchangeable with that isolated from *S. cerevisiae* A364A in the *in vitro* replication system.

Replication of the 2-μm plasmid appears to be under the same control as that of nuclear DNA *in vivo* (4, 5). Thus, the analysis of the activities responsible for plasmid replication and of the factors that induce their appearance provides a reasonable initial approach to the problem of determining the biochemical basis of the control of the cell division cycle in yeast. The fact that extracts prepared from *cdc7* and *8* cells that had been arrested at nonpermissive temperature are deficient in replication of the plasmid (Table 2) suggests that the relationship between nuclear and plasmid DNA replication is reflected *in vitro*, and that functioning of the *cdc 28* $\rightarrow$ *4* $\rightarrow$ *7* $\rightarrow$ *8* pathway *in vivo* is required for cells to develop replication activity as assayed *in vitro*. This, and the fact that the plasmid replicates via a Θ-form intermediate (Fig. 2) *in vitro* just as it does *in vivo* (4), suggests that the observed replication *in vitro* accurately reflects events occurring inside the cell.

It will be of interest to determine whether any of the *cdc* gene products are directly involved in plasmid replication *in vitro*. One candidate for such a protein is the *cdc9* product, which appears to be a DNA ligase (12). An outstanding question at this time is the location of the *in vitro* origin of replication of the plasmid and its comparison to the one used *in vivo*, which has not so far been located.

Inasmuch as the plasmid does not appear to reside *inside* the nucleus (13), the problem exists of how plasmid replication is subject to the same control as that of nuclear DNA. Although there are 50–100 copies of the plasmid inside the cell, it is inherited as if there were only a few copies present (14). A reasonable explanation is that the 2-μm plasmid may be attached to or interact with the nuclear plaque. Duplication of the plaque requires *cdc28* function and signals the start of the cell cycle in yeast (9). The association of the plasmid with the nuclear plaque could explain the dependence of plasmid replication on the *cdc 28* $\rightarrow$ *4* $\rightarrow$ *7* $\rightarrow$ *8* pathway. This hypothesis can be subjected to experimental test.

For the precise analysis of initiation events, the *in vitro* system for replication of 2-μm plasmid DNA described here is superior to our previous assay (2), at least for extracts from yeast. The development of this system opens up possibilities for the dissection and reconstitution of the machinery involved in initiation of nuclear DNA replication in this organism. This, in turn, provides an avenue for the analysis of the proximal controls involved in passage of a cell through the G_1/S boundary, and ultimately should lead to other assays useful in the study of the $G_0 \rightarrow G_1 \rightarrow$ S transition.

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