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Biochemistry

Activity of yeast extracts in cell-free stimulation of DNA replication

(DNA synthesis *in vitro*/growth control/cell cycle/*cdc* mutants)

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ABSTRACT **Extracts of the cytoplasm of disrupted spheroplasts of *Saccharomyces cerevisiae* (bakers' yeast) stimulated DNA synthesis in a cell-free system consisting of nuclei from spleen cells of the frog *Xenopus laevis*. The stimulation required Mg^{++}, ATP, and the deoxynucleoside triphosphates, was saturated by an excess of nuclei or extract, and had kinetics resembling those obtained previously with extracts from mammalian and avian cells. After addition of the yeast extract, replication "eyes" were formed in the DNA from the nucleochromatin of the frog, suggesting that the extract stimulated the initiation of DNA replication. The activity was susceptible to heat, was nondialyzable, and was abrogated by tryptic digestion.**

Temperature-sensitive mutants of the cell division cycle (*cdc* mutants *4, 7, 8,* and *28*) grown at permissive temperature (23°) yielded extracts that were capable of stimulating DNA replication. When the cells were incubated for one generation at the nonpermissive temperature (36°), their extracts showed very low or no activity. All of these mutants are deficient in events of the dependent pathway leading to initiation of DNA synthesis in the yeast cell cycle. A *ts* mutant, *cdc10,* deficient in the separate pathway for cytokinesis, showed little or no loss of activity at the nonpermissive temperature. These data indicate that the "initiation" activity, as assayed *in vitro,* is subject to control in the yeast cell cycle, and its appearance may be one of the terminal events in the pathway leading to DNA synthesis. The finding that extracts from yeast cells can stimulate DNA synthesis in nucleochromatin from frog cells, and the fact that the *cdc* mutants *4, 7, 8,* and *28* describe a dependent pathway terminating in development of "initiation" activity, are in accord with the hypothesis that the function of proteins in the dependent pathways of the cell cycle is conserved during evolution.

An understanding of the biochemistry of the cell cycle in eukaryotes requires a molecular analysis of the nature of the initial signals and the subsequent controls of a highly ordered sequence of events resulting in DNA synthesis and cell division. It is particularly important to understand the regulation of the initial events commiting a cell to the entry from a resting stage to the G_1 phase of the cycle. This requires: (*i*) a means of establishing the order of biochemical events that are essential for the initiation of DNA synthesis; and (*ii*) assays that will allow isolation of the molecules responsible.

A particularly significant step towards realizing the first of these objectives has been taken by Hartwell and his colleagues (1). Using a series of temperature-sensitive yeast mutants, they have provided an explanation for the orderly temporal sequence of events in terms of the dependence of each event on prior events in the cycle.

The second objective, isolation of the molecules involved in each step, requires the development of suitable biochemical approaches. We have recently described a cell-free assay for the onset of DNA synthesis (2). Using this assay, we have shown that cytoplasmic factors from proliferating but not from resting cells of various kinds can induce the onset of DNA replication in isolated nuclei of cells from adult frog spleens and livers. Moreover, we provided evidence that the appearance of the active factors is under control and can be influenced by density-dependent inhibition, by mitogens, and by nonpermissive conditions in cells transformed by temperature-sensitive viral mutants.

In the present paper, we describe the application of the "initiation" assay to an analysis of the factors of wild-type and selected cell division cycle (*cdc*) mutants of yeast (1, 3). This may be useful in the isolation and characterization of the proteins involved in initiating DNA replication.

Abbreviation: *cdc*, temperature-sensitive mutants of the yeast cell division cycle (1).

MATERIALS AND METHODS

[*methyl*-^{3}H]dTTP (50 Ci/mmol) was from Schwarz/Mann, nucleotides were from P-L Biochemicals, and glusulase from Endo Laboratories (Garden City, N.Y.)

Yeast Strains and Cultures. *Saccharomyces cerevisiae* (bakers' yeast) was from Fleischmann (New York, N.Y.) and was used in the experiments described in Figs. 1 and 2, and in Tables 1 and 2. The temperature-sensitive cell division cycle mutants of *S. cerevisiae, cdc4-1, cdc7-1, cdc8-1, cdc10-1,* and *cdc28-1,* as well as the parent strain A364A were from the Yeast Genetic Stock Center (University of California, Berkeley, Calif.). Yeast cells (Fleischmann's Yeast) were grown in 2% peptone–1% yeast extract–2% glucose (Difco) on a shaker at 30°. The other strains (A364A and the *cdc* mutants) were grown in the same medium at 23°, the permissive temperature.

Preparation of Cell Extracts and Nuclei. Exponential yeast cultures were harvested and the cells converted to spheroplasts according to the method of Kuo and Lampen (4). The spheroplasts (3×10^9 per ml) were suspended in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) (pH 8.5)–5 mM KCl–0.5 mM $MgCl_2$–0.1 mM (ethylene dinitrilo)tetraacetate (EDTA)–0.5 mM dithiothreitol, and extracts were prepared as described (2). This procedure disrupted the spheroplasts, leaving many intact but swollen nuclei. The extracts consisted mainly of cytoplasmic material, although the presence of some nuclear material cannot be excluded. Nuclei were prepared from frog spleen cells (*Xenopus laevis,* NASCO, Fort Atkinson, Wisc.) as before (2). These cells are composed mainly of resting cells that do not replicate their DNA (2).

Other Methods. The assay for chromosomal DNA synthesis has been described (2) and was performed at 30°. Samples were prepared for electron microscopy (2) and the DNA was visualized by the aqueous spreading technique of Davis *et al.* (5) with uranyl acetate staining and no shadowing. Duplex circular DNA of bacteriophage f1 (a gift of Dr. Peter Model of The Rockefeller University) was used as a 2-μm length standard.

RESULTS

Extracts of *S. cerevisiae* stimulate cell-free DNA synthesis

As shown in Fig. 1A, incubation of extracts from wild-type yeasts with nuclei of adult frog spleen cells, in the presence of

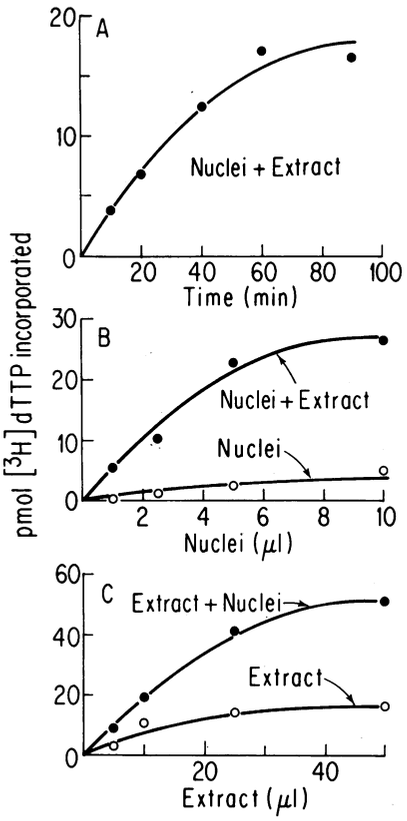

FIG. 1. Kinetics of [^{3}H]dTTP incorporation and the dependence on added nuclei and cell extract. (A) Time course: 4.8×10^5 nuclei were incubated with 25 μl of extract. (B) Nuclear dependence: Varying amounts of suspensions of nuclei (6×10^7 per ml) were incubated with 25 μl of extract. (C) Cell extract dependence: 5×10^5 nuclei were incubated with varying amounts of extract. Incubation in (B) and (C) was for 60 min. The values for a blank, as well as the extract and nuclear backgrounds, have been subtracted in (A). In (B), the blank and the extract backgrounds have been subtracted, and in (C) the blank and the nuclear backgrounds have been subtracted.

Mg^{++}, ATP, and the four deoxynucleoside triphosphates, resulted in the incorporation of [^{3}H]dTTP into acid-insoluble material. The reaction was linear for about 40 min and was complete by 90 min. This reaction resembled that previously obtained using extracts of cells from mammalian and avian sources (2) in respect both to kinetics and to the dependence of [^{3}H]dTTP incorporation on added nuclei and cell extract (Fig. 1B and C). A given amount of extract could be saturated by nuclei, and a fixed number of nuclei was saturated by excess yeast extract. The extent of incorporation stimulated by the extract depended upon the amount of DNA added in the form of nuclei.

Evidence for initiation of DNA replication and nature of active factors

The DNA synthesis required Mg^{++} and the deoxynucleoside triphosphates, and in particular, it was ATP-dependent (Table 1). Thus, as in our studies with tissue culture cells (2), yeast extracts appeared to promote true DNA replication and not simply repair. Further evidence for this conclusion was provided by the appearance of replication "eyes" in DNA molecules isolated from a reaction mixture after a 60-min incubation of nuclei with yeast extract (Fig. 2). Very few replication "eyes" were seen in the control sample (0.6%, three "eyes" in 500 molecules), but in the sample exposed to yeast extract the frequency of molecules possessing eyes was 8.0% (40 "eyes" in 500 molecules). The total length of DNA examined in the control and the extract-stimulated reaction was equivalent, and the DNA examined was not derived from the cell extract.

Table 1. Dependence of [^{3}H]dTTP incorporation on Mg^{++}, ATP, and deoxynucleoside triphosphates

Conditions	pmol of [^{3}H]dTTP incorporated
Experiment 1	
—	19.3
+ EDTA (10 mM)	0
−dATP, −dGTP, −dCTP, +0.06 μM dTTP	0.06
Experiment 2	
—	18.8
−ATP (glycerol kinase, 30 min, 30°)	2.5
+ATP (glycerol kinase mock)	20.0

Nuclei (5×10^5) were incubated with 25 μl of yeast extract for 60 min. Reagents were treated with glycerol kinase prior to the assay. The values for a blank, as well as the extract and nuclear backgrounds, have been subtracted.

A χ^2 value of 33.2 (at one degree of freedom) indicates that it is extremely unlikely ($P \ll 0.005$) that the difference between the control and the extract-treated sample was due to chance alone. The main example shown in Fig. 2 was a very rare one in the sense that the "eye" was found in a particularly long DNA molecule. The average size of the "eyes" was 0.08 μm (SD = 0.05 μm). These data on the induction of "eyes" in the DNA of the frog by the yeast extract suggest that initiation of DNA replication is occurring. This provisional conclusion is supported by the fact that the nuclei were derived from resting cells that are not in the process of replicating their DNA (2). Furthermore, as shown below, only extracts from cells in the process of traversing the cell cycle were active; this was in contrast to the inactive extracts from cells arrested prior to the onset of DNA synthesis.

At least one of the "initiation" factors from mammalian cells

Table 2. Characterization of the activity in yeast extract

Treatment of extract	pmol of [^{3}H]dTTP incorporated
Experiment 1	
—	17.0
60°, 15 min	0
Dialysis, 3 hr	13.7
Experiment 2	
—	15.7
Trypsin (150 μg/ml), 30 min, 30°	0
Soybean inhibitor (300 μg/ml)	17.2
Trypsin + soybean inhibitor	17.0
Experiment 3	
—	23.8
Retained by Amicon XM50 filter	20.6

Assays were performed for 60 min. In Experiment 1, nuclei (4.8×10^5) were incubated with 25 μl of yeast extract. In Experiment 2, nuclei (5.1×10^5) were incubated with 25 μl of yeast extract. In Experiment 3, nuclei (5×10^5) were incubated with 30 μl of yeast extract. The extract was treated prior to the assay. It was concentrated 5-fold on an Amicon XM50 filter, and then readjusted to the initial volume. The values for a blank, as well as the extract and nuclear backgrounds, have been subtracted.

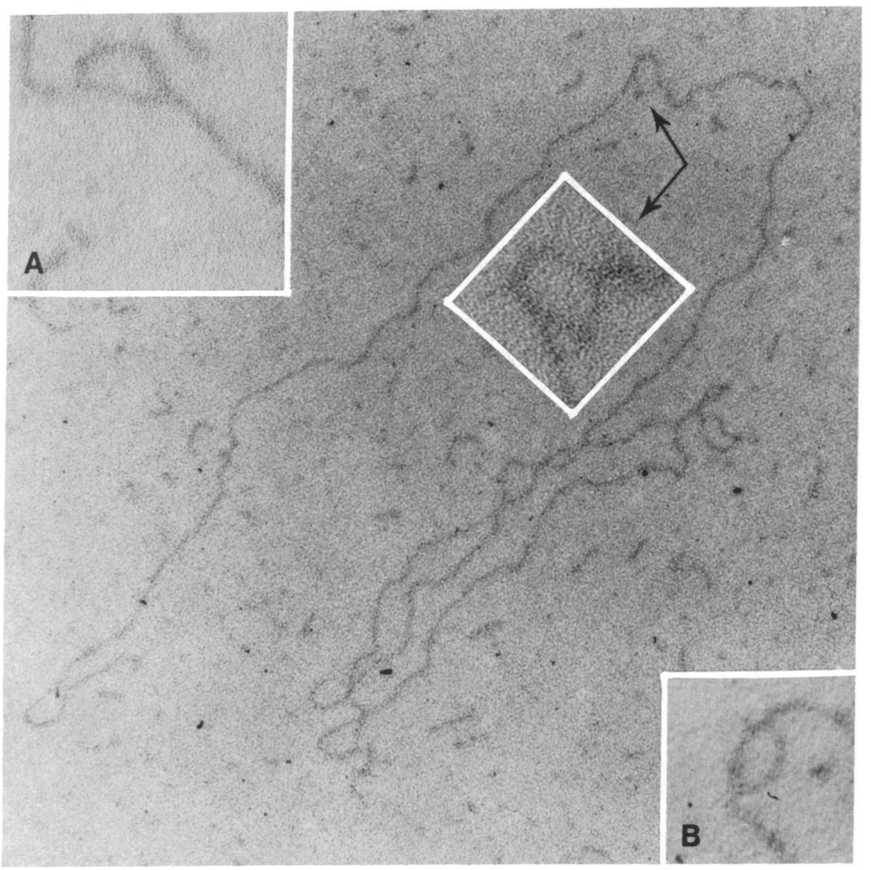

FIG. 2. Replication "eyes". Nuclei (5×10^6) were incubated with or without 100 μl of yeast extract for 60 min in the assay for chromosomal DNA synthesis. The arrows indicate an "eye" in the DNA molecule (magnification 61,800 ×) and an inset in which the "eye" is shown at higher magnification (173,000 ×). In (A) and (B) "eyes" from two other molecules are shown at the same magnification.

has been shown to be protein in nature (2). As shown in Table 2, the active factors in extracts from wild-type yeast also appeared to be proteins, as indicated by their irreversible inactivation by elevated temperatures, their retention in dialysis bags, and their susceptibility to specific proteolytic enzymes. In addition, retention of the activity by Amicon XM50 filters suggests that the active components had molecular weights in excess of 50,000.

"Initiation" activity in extracts from *cdc* mutants

In view of the functional resemblance of the factors from wild-type yeast to those obtained from proliferating cells of higher organisms, it was of particular interest to examine the yeast *cdc* mutants. The presence of activity in extracts of selected mutants grown at permissive temperatures, and its absence when the cells are incubated at nonpermissive temperatures, are important in substantiating the suggestion that the assay is testing for factors involved in the control of DNA replication.

The mutants *cdc28*, *cdc4*, *cdc7*, and *cdc8* were chosen for this purpose. The first is associated with the "start" of the yeast cell division cycle, the second two are related to events leading to the initiation of a round of DNA replication, and the last is concerned with propagation of DNA synthesis (1, 6, 7). As shown in Table 3, extracts from each of these mutants were active in the assay when prepared from cells grown at the permissive temperature. Exposure of the cells to the nonpermissive temperature for one generation abolished activity in extracts subsequently prepared from these cells. This was not due to loss of cell viability, as indicated by colony-forming ability of the cells exposed to the nonpermissive temperature. After incubation at the nonpermissive temperature, greater than 90% of the cells displayed the phenotype (3) characteristic of the mutant at termination of cell cycle development. Extracts prepared from cultures exhibiting a lower level of arrest at the

Table 3. *cdc* mutants arrested at the nonpermissive temperature lack "initiation" activity

Strain	Cells cultured at: 23°	36°
	(units/ml)	
A364A	0.67	0.62
*cdc*28	0.22	0
*cdc*4	0.30	0.03
*cdc*7	0.41	0.04
*cdc*8	0.54	0
*cdc*10	0.61	0.49

Exponential cultures grown at the permissive temperature (23°) were divided into two portions. Half the culture was used for preparing extracts. The other half was incubated at the nonpermissive temperature (36°) for 3 hr. The cells were monitored for their terminal phenotype as an indication of the degree of arrest of the culture (>90% in this experiment). Cells were converted to spheroplasts at 23° and 36° for cultures at permissive and nonpermissive conditions, respectively. Extracts were then prepared as described in *Materials and Methods* at 3×10^9 spheroplasts/ml, and were titrated for "initiation" activity at 30° with 5×10^5 nuclei. 1 unit = 1 nmol of dTTP incorporated in 60 min at 30°.

nonpermissive temperature displayed substantial levels of activity. Mixing experiments indicated that the lack of activity in extracts from cells incubated at the nonpermissive temperature was not due to the presence of inhibitors in these extracts.

These results with *cdc28, 4, 7,* and *8* should be contrasted with those obtained with the parent strain A364A and with *cdc10* (Table 3). Extracts prepared from these latter strains exhibited similar levels of activity whether the cells were incubated at the permissive or nonpermissive temperatures. The mutant *cdc10* is defective in cytokinesis; nevertheless, cell cycle development continues for several rounds, resulting in multinucleated structures (1, 3).

The analysis of the *cdc* mutants with the *in vitro* assay for stimulation of DNA replication indicates that the activity is subject to control in the dependent pathway of events leading to the onset of DNA synthesis in the yeast cell cycle. This analysis would also tend to place the appearance of the activity near the G_1/S boundary of the cell cycle, because *cdc28* is related to G_1 and *cdc4, 7,* and *8* appear to be related either to G_1 or the S phase (1, 6, 7).

DISCUSSION

The basic observation in these studies is that yeast extracts can stimulate dTTP incorporation in isolated frog spleen nuclei from resting cells. This stimulation closely resembles that observed using extracts of tissue culture cells (2), and the evidence (Table 1, Figs. 1 and 2) strongly suggests that DNA replication is occurring. Moreover, the presence of a large increase in the frequency of replication "eyes" prompts the hypothesis that the extracts stimulate initiation of DNA replication, an interpretation supported by the facts that the nuclei were derived from resting cells (2) and that extracts from cells arrested prior to the onset of DNA replication lacked activity (Table 3). As noted before with extracts from higher cells (2), the size of the "eyes" suggests that they might accommodate only a few 4S DNA fragments. Definite proof that the stimulation seen is concerned with DNA initiation must await clarification of the mechanism of initiation and detailed studies of the state of the DNA in the frog nuclei.

The resemblance of the reaction stimulated by yeast extracts to that observed with extracts from higher cells (2) gives some support to the idea (1) that at least some portions of the cell cycle controls related to the onset of DNA synthesis are conserved during evolution. That *cdc* mutants of yeast also function in the "initiation" assay using frog nuclei is potentially significant for the biochemical analysis of the cell cycle in higher organisms in which so complete a set of *cdc* mutants is unlikely to be obtained.

The lack of activity in extracts from *cdc28, 4, 7,* and *8* cells at the nonpermissive temperature (Table 3) indicates that the proteins affected are subject to control in the dependent pathway of events leading to DNA synthesis in the yeast cell cycle. The question arises as to the precise function of the *cdc28, 4, 7,* and *8* gene products, and their physiological role in their respective phenotypes. An answer to this question clearly would provide clues to the control mechanisms operating in the G_1 phase of the cell cycle. These gene products could be directly involved in DNA synthesis, could activate suitable genes for other proteins, or could activate polymerases or other proteins concerned with replication by either direct or indirect means. Moreover, they could act either at the level of the cytoplasm or the nucleus.

In any case, since the requirements for protein synthesis necessary for entry into S phase are complete at the time the *cdc7* gene product appears to function (7), the "initiation" activity must be present by that time, albeit in an inactive form. Thus, it is tempting to speculate that at least the *cdc7* gene product may be one of the factors assayed in the cell-free system, or alternatively that this protein converts the "initiation" factors to an active form. If either of these alternatives is true, it would suggest that control of cell cycle development in the G_1 phase is achieved by interaction with or induction of proteins involved in initiation of DNA replication. Commitment of cells to entry into G_1 from a resting stage might occur at or very close to the same processes. The finding that extracts from *cdc28* grown at the nonpermissive temperature are inactive particularly suggests a close coupling of early control events to the production of key factors for DNA replication.

Fractionation of the active components in the yeast extract, coupled with further utilization of the *cdc* mutants in this and in other assays, should clarify the connection between commitment and initiation events. In particular, the possibility of testing the heat-sensitivity of the extracts themselves and of complementing the various *cdc* mutants in the *in vitro* assay opens up the prospect of reducing certain features of cell cycle control to molecular terms.

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