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Isolation of yeast DNA replication mutants in permeabilized cells

(*in vitro* replication/cell-division cycle/*cdc2* mutant/*cdc16* mutant/*Saccharomyces cerevisiae*)

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ABSTRACT **A random population of temperature-sensitive mutants was screened by assaying for defects in DNA synthesis in a permeabilized yeast DNA replication system. Twenty mutants defective in *in vitro* DNA synthesis have been isolated. In this paper we describe eight of these mutants. Seven of them fall into three complementation groups—*cdc2*, *cdc8*, and *cdc16*—involved in the control of the cell-division cycle. Because synthesis *in vitro* represents propagation of replication forks active *in vivo* at the time of permeabilization, our finding that *cdc2* and *cdc16* mutants can incorporate dTMP into DNA in such permeabilized cells at 23°C but not at 37°C supports the conclusion that these two mutations directly affect DNA synthesis at replication forks. Such an involvement was previously suggested by *in vivo* analysis for *CDC2* but was less clear for *CDC16*. Finally, the usefulness of our screening procedure is demonstrated by the isolation of replication mutants in previously undescribed complementation groups. One strain shows a serious defect in *in vivo* DNA synthesis but normal RNA synthesis.**

Attention has focused recently on the microbial eukaryote, *Saccharomyces cerevisiae*, in which both genetic and biochemical approaches to the study of gene expression are possible. Our interest in the yeast system is related to the ability to take a combined genetic and biochemical approach to the study of DNA replication. Specifically, we would like to isolate and characterize the proteins required for DNA replication in yeast by complementation of DNA replication mutants in a cell-free *in vitro* DNA replication system such as has recently been described (1–4).

Genetic analysis of yeast DNA replication began with the isolation and characterization of a number of temperature-sensitive mutants having defects in cell division (5). Of these, several were found to be deficient in DNA synthesis (*cdc28*, *cdc4*, *cdc7*, *cdc2*, *cdc6*, *cdc8*, *cdc21*, and *cdc9*) (5–11). The products of *CDC28* and *CDC4* are believed to have execution points in the cell cycle before the actual onset of DNA synthesis, and *CDC7* seems to act at the time of entry into S phase (7, 9). *CDC21* has been shown to be defective in the synthesis of dTMP (12). Thus only *CDC2*, *CDC6*, *CDC8*, and *CDC9* appear to be directly involved in DNA synthesis. *CDC2* must function to complete DNA synthesis, and *CDC6* seems to be involved in initiation (8). *CDC8* has been shown to be involved in elongation and is required for mitochondrial DNA synthesis (7, 13). Recently, the CDC8 protein was purified and shown to bind to single-stranded DNA (14, 15). *cdc9* mutants synthesize DNA at the nonpermissive temperature, but the DNA is of low molecular weight. *cdc9* strains contain no DNA ligase, and therefore *CDC9* may be the structural gene for ligase (11).

It is likely that a considerable number of additional genes are involved. Johnston and Thomas (16) have isolated seven temperature-sensitive mutants that show a reproducible decrease in DNA synthesis at the nonpermissive temperature, but specific defects have not been elucidated. Dumas *et al.* (17) have described a screening procedure for identifying mutants defective in incorporation of [^{3}H]uracil into DNA *in vivo*. They have defined 60 complementation groups, many of which are likely to play direct roles in DNA synthesis. We have adopted a different approach that consists of screening a randomly mutagenized population of conditionally lethal yeast strains for defects in replication *in vitro*. The *in vitro* assay uses yeast cells made permeable to nucleoside triphosphates with the detergent Brij 58 (15, 18, 19). Such a screening procedure has the advantage that every one of the mutants identified probably has a lesion in a gene directly involved in DNA synthesis. Twenty mutants have been identified. Three fall in previously identified *cdc* complementation groups, but 14 have not been previously identified. Here we report results pertaining to *CDC2* and to *CDC16*, cell cycle mutants whose *in vivo* phenotype did not previously allow one to conclude whether or not these mutants had a specific defect in DNA synthesis.

MATERIALS AND METHODS

Strains. The parent strain A364a (*a ade1 ura1 gal1 tyr1 his7 lys2*) and a strain derived from A364, strain 198, *cdc8-1* were from L. H. Hartwell (University of Washington) (20). Strain *cdc7* was derived from A364 and was from John Scott (University of Illinois). The 400 haploid temperature-sensitive mutants, which do not form colonies at 36°C but do form colonies at 23°C, were derived from A364a by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine by L. H. Hartwell (5, 20) and were provided by Fred Sherman (University of Rochester). Strain D273-11a (*α ade1 his1 trp2*) was from the Cold Spring Harbor Laboratory collection. Strains of *α* mating type for complementation studies, obtained by crossing each mutant with strain SRG05-1 (*α trp1-1 met8-1 ile-1 ilv-2*) were from Steve Reed (University of California, Santa Barbara). Segregants of *α* mating type were identified using standard test strains.

Media. YPD medium, SD medium, sporulation agar, YPDG, and minimal agar are described in ref. 21.

Genetic Procedures. Complementation testing was performed by cross-streaking haploid strains of known genotype on YPD plates. After incubation overnight, the strains were replicated on appropriate synthetic medium (to select for auxotrophic markers) and then incubated at 37°C.

Standard procedures for genetic crosses in yeast were used, and all crosses were performed on YPD agar. Sporulated cells were removed from sporulation agar and suspended in H_2O. Asci were digested for 20 min at 32°C with a 1:20 dilution of glusulase. Tetrads were dissected and analyzed.

Preparation of Permeabilized Cells. Strains were grown at 23°C to a density of 2×10^7 cells per ml in YPD medium, collected by centrifugation, and washed twice with H_2O. The cells

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were suspended in 10 mM Tris·HCl, pH 7.2/2 M sucrose/1% Brij 58 to a final concentration of 2×10^8 cells per ml and incubated until the cells were permeable as monitored by alkaline phosphatase activity (18). The permeabilized cells can be frozen at −20°C for up to 2 wk and used in the assay for DNA synthesis when desired.

Screening Assay for DNA Synthesis. The standard DNA replication reaction mixture contained 50 mM Tris·HCl (pH 8.0); 10 mM $MgCl_2$; 1.5 mM 2-mercaptoethanol; 50 μM dATP, dGTP, and dCTP; 2 μM [α-^{32}P]dTTP (4,000–8,000 cpm/pmol); 1 mM ATP; 10 mM phospho*enol*pyruvate; 1% Brij 58; 0.05 ml of Tris·HCl/sucrose/Brij 58 cell suspension. After incubation for 30 min at 37°C the reaction was stopped and the amount of radioactive material was determined as described (15).

Labeling of DNA and RNA with [^{3}H]Adenine. Mutants showing a defect in DNA synthesis in the above procedure were assayed for DNA synthesis *in vivo*. Cells were grown in YPD medium at 23°C to 2×10^7 cells per ml. YPD medium was removed by centrifugation and washing and cells were suspended in SD medium containing the required amino acids (30 μg/ml) and [^{3}H]adenine at 7 μCi/ml (1 Ci = 3.7×10^{10} Bq) to a concentration of 5×10^7 cells per ml. After 30 min at 23°C, an aliquot of the cell suspension was removed. Cell number was determined and the amount of [^{3}H]adenine incorporated into DNA and RNA was measured as described in ref. 5. The remaining culture was then maintained at 37°C with shaking. After 3 hr, another sample was taken to determine cell number and radioactivity incorporated into DNA and RNA as described in ref. 5.

RESULTS

Characterization of DNA Synthesis in Permeabilized Cells. Hereford and Hartwell (18) showed that yeast cells made permeable by the addition of Brij 58 can incorporate [α-^{32}P]dTTP into DNA in a reaction requiring Mg^{2+}, ATP, and the three other dNTPs. Although cells of the mutant *cdc4* gave normal synthesis in the permeable cells when grown at the permissive temperature, synthesis did not occur in permeabilized *cdc4* mutants that had been kept at the nonpermissive temperature for more than one generation time before permeabilization. Because *cdc4* mutants are capable of completing an ongoing round of replication at the nonpermissive temperature but are incapable of initiating new synthesis (6), this result suggested that synthesis *in vitro* represented the propagation of replication forks active *in vivo* at the time of permeabilization. When permeabilized cells were prepared from *cdc8* mutants, which are deficient in elongation, no *in vitro* synthesis was observed whether the cells were grown at permissive or nonpermissive temperatures (18), suggesting that the same machinery used *in vivo* for carrying out DNA replication was also used *in vitro*.

A modification of the permeabilization procedure that was developed by Banks for studying *Ustilago* has been described (15, 19). Cells are made permeable to triphosphates by incubation in sucrose/Brij 58. By using this system, we were able to reproduce the results obtained by Hereford and Hartwell (18) and to extend them in several ways as described in ref. 15.

In Fig. 1, we present an additional aspect of this system—namely, that initiation mutants behave in the sucrose/Brij 58-treated cells in the same way that they do in the Brij 58-treated cells described by Hereford and Hartwell (18). Cells were grown at 23°C, incubated with Tris·HCl/sucrose/Brij 58 at 30°C for the indicated times, and synthesis was measured at 37°C. Fig. 1 reveals that wild-type cells respond to treatment with detergent and incorporate dTMP into DNA. In strain *cdc8*, however, even after several hours of treatment, there is no incorporation at 37°C. *cdc7* mutants are defective in the initiation of DNA

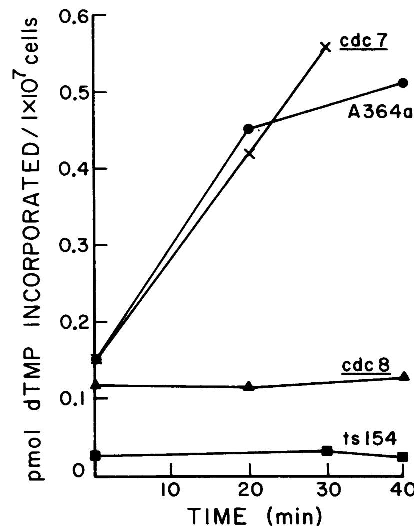

FIG. 1. Optimum time of Brij 58 treatment. Cells were treated with Brij 58 for the times indicated. Synthesis was then measured at 37°C. Values represent extent of synthesis and not initial rate (15). ts 154, temperature-sensitive mutant strain 154.

synthesis at 37°C *in vivo* (6–8) and behave quite differently from *cdc8* strains *in vitro*. The *cdc7* mutant incorporates dTMP into DNA at 37°C to the same extent as wild type. In a separate experiment, *cdc4* responded exactly as *cdc7*. Thus, mutants that inhibit entrance into S phase are not defective in synthesis in this type of *in vitro* system when the cells are grown at the permissive temperature. Only if they are incubated at the nonpermissive temperature *in vivo* for several hours do they show an *in vitro* defect (data not shown; see also ref. 19).

We also compared the amount of *in vitro* synthesis at 23°C and 37°C in strains A364a (wild type), *cdc7*, and *cdc8*. At 23°C synthesis is approximately the same in all three strains (Table 1). However, at 37°C synthesis is increased in wild type and in *cdc7* relative to 23°C, but it is decreased to background level in *cdc8*. Thus, *cdc8* replication is thermolabile *in vitro*, reflecting exactly the response of *cdc8* cells *in vivo*, but the *cdc7* mutant is not defective at any temperature *in vitro*.

Detection of DNA Replication Mutants. Because the preparation of permeabilized cells is easy and rapid, this *in vitro* replication system seemed to offer an efficient means of screening directly for mutants with defects specifically affecting DNA synthesis. The principle advantage of an *in vitro* system over a screening procedure using *in vivo* labeling techniques is that the latter cannot differentiate between mutants with a primary defect in replication and those mutants in which the defect in DNA synthesis is only secondary to a mutation affecting RNA

Table 1. Incorporation of [α-^{32}P]dTMP in permeabilized mutants, *cdc7* and *cdc8*, and in the parental yeast strain A364a

Strain	dTMP incorporated per 10^8 cells, pmol 37°C	23°C
A364a	4.83	4.25
cdc7	5.02	4.76
cdc8	1.07	3.98

Strains were grown, harvested, treated with Tris·HCl/sucrose/Brij 58, and assayed. DNA synthesis was measured at 37°C and 23°C. *cdc7* is temperature sensitive for the initiation of DNA synthesis, and *cdc8* is temperature sensitive for DNA replication at the restrictive temperature (37°C).

synthesis, protein synthesis, or a cell-cycle function such as a process required for the cells to enter S phase (for instance, *cdc28*, *cdc4*, or *cdc7*).

Approximately 400 temperature-sensitive mutants, strains *ts92–ts492*, were screened for a defect in DNA synthesis. The mutants were grown at 23°C and were harvested in logarithmic phase of growth. The cells were washed, permeabilized by treatment with Brij 58, and DNA synthesis was measured at 37°C. A strain was regarded as a potential DNA synthesis mutant if it had <25% of the activity of wild type, because that meant that it was as defective as (or more defective than) *cdc8*.

By this assay, 20 mutant strains showed a reproducible defect in DNA synthesis, and the results obtained for seven of these are shown in Table 2. The identification of such a large number of replication mutants confirms that such mutants appear frequently enough among heavily mutagenized populations (see below) of conditional mutants to make screening for them by *in vitro* replication assays a reasonable approach.

Identification of *cdc8*, *cdc2*, and *cdc16* Alleles Among Our Mutants. The mutant population we used was obtained from L. H. Hartwell, who had already shown that it was a representative collection (see ref. 20). This collection had previously been screened for cell-cycle mutants by assessing terminal phenotypes by time-lapse photomicroscopy (20). Therefore, it was possible to determine whether any of the temperature-sensitive mutants that we had identified corresponded to any *cdc* complementation groups, by comparing the numbers of our mutants with those identified by Hartwell and colleagues. First, we found that strain 198 was a *cdc8* allele. This turned out to be the same mutant we had used in the control experiments. This finding established the reliability of the screen. Second, two of our other mutants, strains 346 and 370, were found by this comparison to be *cdc2* mutants. Third, mutant strain 281 was found to be *cdc16*. There are a number of other mutants belonging to the *cdc2*, *cdc8*, and *cdc16* complementation groups in this collection. Mutants 256 and 336 (*cdc2*), 141 (*cdc8*), and 486 (*cdc16*) were either missing from the collection we received or were no longer temperature sensitive *in vivo* and were therefore not assayed. Mutant 172 (*cdc8*) could not be permeabilized with Brij 58. Mutant 284 (*cdc16*) showed less than 1/3rd the level of wild-type synthesis in the first screen and was indeed found to be even more defective than *cdc8* when reassayed. Mutants 246 and 249 (*cdc16*) were not as defective, but we have not checked that our isolates actually contain *cdc16* alleles. None of our remaining 16 mutants was among those identified by

Table 2. DNA synthesis in some representative temperature-sensitive mutants

Strain	DNA synthesis
A364a	1.00
cdc8	0.25
129	0.16
154	0.08
198	0.29
281	0.20
328	0.24
370	0.10
346	0.15
426	0.14

DNA synthesis was measured as the amount of [α-^{32}P]dTMP incorporated into DNA at 37°C. Values are the mean of at least three separate determinations. All reaction mixtures were adjusted to contain approximately the same number of cells at the same stage of permeability. All values are normalized to a value of 1.00 for A364a.

Hartwell and colleagues by screening for terminal phenotype (20).

The finding that *cdc2* and *cdc16* mutants are defective in *in vitro* synthesis was a surprising and interesting result, because previous studies had not indicated clearly whether these mutations directly affected DNA synthesis. Both mutants, for instance, incorporate substantial amounts of radioactive precursors into DNA at the nonpermissive temperature *in vivo* (22). After discovering that these mutants were defective at 37°C in permeabilized *in vitro* systems, it was of interest to further characterize them by testing for defects at 23°C. We compared the amount of synthesis in permeabilized *cdc2* and *cdc16* at 23°C and 37°C. Both mutants showed almost wild-type levels of incorporation in permeabilized cells at 23°C. Therefore, as for *cdc8*, synthesis *in vitro* in these mutants is thermolabile. Thus our results suggest strongly that both *cdc2* and *cdc16* genes are directly involved in DNA synthesis.

Complementation Analysis. We were interested in knowing how many complementation groups were represented among our mutants. Complementation analysis was carried out by preparing an α-mating type derivative of each of the 20 mutants. Pairwise matings were then conducted as described, and the resulting diploids were tested for temperature sensitivity. Mutants 129, 328, and 426 were found to fall into the same complementation group as 346 and 370—namely *cdc2*. Because of the high frequency of appearance of mutations in the *cdc2* group, these mutants were chosen for further study. Strain 154 was in a different complementation group from the others. It was chosen for further study because it had a severe defect in *in vitro* synthesis at both 23°C and 37°C, although more synthesis was observed at 23°C. The remaining mutants have not been further characterized and will be described elsewhere.

Cell-cycle theory predicts that a *cdc* gene product is necessary for only one of the discontinuous events in the cell cycle (20). Therefore one expects that at the nonpermissive temperature an asynchronous culture of a conditional mutant in a *cdc* gene will arrest growth with a homogeneous and characteristic morphology called a terminal phenotype. The three *cdc2* mutants identified, strains 129, 328, and 426, do not show the typical arrest morphology (two large buds) of the two previously identified *cdc2* alleles, strains 346 and 370 (10). It is possible, therefore, that these mutants contain secondary mutations and that when they are removed the typical *cdc2* terminal phenotype will be observed.

Segregation Analysis. It was also important in evaluating the usefulness of this method of detecting replication mutants to ensure that the temperature-sensitive phenotype and the defect in *in vitro* DNA synthesis were due to mutations in the same genes. Mutant strains 346 and 154 were each crossed with an appropriate nontemperature-sensitive strain and sporulated, and the segregation of the two traits, temperature sensitivity *in vivo* and *in vitro*, was assayed in six tetrads for each cross. In every tetrad, an example of which is shown in Table 3, the temperature-sensitive phenotype and the *in vitro* defect segregated together. This 2:2 pattern of ts^-/ts^+ (temperature sensitive/not temperature sensitive) indicates that the temperature-sensitive *in vitro* synthesis is due to a mutation in a single gene and that the same mutation is responsible for both the *in vivo* and *in vitro* thermolability.

DNA Synthesis *in Vivo* in the Recently Identified Replication Mutants. To verify that the new mutants actually had defects in DNA replication, the ability of mutant 154 to incorporate radioactive precursors into DNA and RNA *in vivo* was measured. At the permissive temperature all strains, wild-type A364a, *cdc8*, and mutant 154 showed similar levels of incorporation of [^{3}H]adenine into DNA. The incorporation into

Table 3. Tetrad data and *in vitro* DNA synthesis in the spores from crosses between temperature-sensitive mutants and wild-type strain

Spores	Temperature sensitivity	DNA synthesis
154		
1a	ts^-	0.26
1b	ts^-	0.35
1c	ts^+	0.82
1d	ts^+	0.94
2a	ts^+	0.86
2b	ts^+	0.82
2c	ts^-	0.34
2d	ts^-	0.22
346		
1a	ts^+	0.82
1b	ts^+	0.83
1c	ts^-	0.24
1d	ts^-	0.23

Strains were crossed with the wild-type strain D273-11a. The resulting diploid cells were removed and six tetrads from each cross were dissected. Each spore was analyzed for temperature sensitivity *in vivo* and *in vitro*. Assay for temperature sensitivity was carried out by incubating strains at 37°C. Procedures for DNA synthesis are as described in legend to Table 1. Data for only three representative tetrads are presented. All 12 tetrads were the same, that is 6 for strain 154 and 6 for strain 346. ts^+ indicates ability to grow at 37°C; ts^- indicates inability to grow at 37°C.

DNA and RNA at the nonpermissive temperature, however, was different in each of the mutants. These results are shown in Table 4. With respect to DNA synthesis, *cdc8* showed ≈25% of the level of wild type. Mutant 154 was even more defective than *cdc8*, showing only 15% of wild-type level. Both *cdc8* and mutant 154, however, showed a substantial amount of RNA synthesis, 44% and 33%, respectively, of the wild-type level and more than twice as much RNA synthesis as DNA synthesis. Thus, strain 154 is in fact a previously unidentified DNA replication mutant, confirming that the *in vitro* screen detects mutants with *in vivo* defects.

Further Characteristics of the Permeabilized Cell Screening System. One possible limitation of this screening method is that mutations affecting initiation may escape detection. However, one class of mutant we found, the *cdc2* mutants, has a phenotype consistent with a defect in initiation (23). Therefore, the system may be more versatile than we would have predicted. Furthermore, initiation mutants are defective if grown at the nonpermissive temperature, which could be incorporated into the screening procedure if desired (18).

A further consideration is whether chromosomal replication is affected in the mutants identified. Yeast contains, in addition to 16 (or 17) chromosomes, mitochondrial, ribosomal, and plasmid DNAs. The work of Banks (19) suggested that Tris·HCl/sucrose/Brij 58-treated cells might carry out only mitochondrial synthesis, because in a ρ^+ cell, all of the newly synthesized DNA was of mitochondrial rather than chromosomal density. To ensure that the *in vitro* system was capable of measuring chromosomal DNA synthesis and hoping to be able to screen for genes affecting only chromosomal DNA synthesis, we prepared ρ^0 derivatives of each of the strains in the collection of 400 temperature-sensitive mutants. ρ^0 strains contain no mitochondrial DNA. When we assayed them for DNA synthesis *in vitro*, we found about 50% as much synthesis on the average in the mitochondrial-lacking mutants in this *in vitro* system (Table 5). The important point to emphasize is that although some of the *in vitro* synthesis may be due to mitochondrial DNA, not all of it is, because many ρ^0 mutants are as efficient as wild type at *in vitro* synthesis (Table 5). Unfortunately, because of the inability to obtain reproducible results even on the same ρ^0 strains from experiment to experiment (Table 5 and data not shown), it was not possible to correlate lack of synthesis in any particular ρ^0 strain *in vitro* with specific mutations and we could not use ρ^0 strains to screen specifically for nuclear DNA synthesis defects. We attribute the variability in permeabilized ρ^0 to either (*i*) a lower permeability than ρ^+ cells; (*ii*) the fact that ρ^0 cells are much smaller (petites) than wild type and may, therefore, be unable to transport enough precursors to support the full level of synthesis; or (*iii*) increased variability in recovery of cells during the harvesting procedure. The strongest evidence, however, that chromosomal DNA synthesis is being observed *in vitro* and that mutants in chromosomal synthesis can be detected by the method described in this paper is the fact that *cdc2* mutants were among the mutants we identified. Conrad and Newlon (23) have shown that mitochondrial DNA synthesis continues for several hours after chromosomal DNA synthesis has ceased at the nonpermissive temperature *in vivo* in *cdc2* mutants. Because *cdc2* mutants are deficient *in vitro*, we must be observing defects due to chromosomal DNA synthesis.

Table 4. DNA and RNA synthesis

Mutant	Macromolecule synthesis	
	RNA	DNA
cdc8	0.44	0.24
Strain 154	0.33	0.15

RNA and DNA synthesis were measured as the amount of [^{3}H]adenine incorporated into RNA and DNA after 3 hr at 37°C as described (5). The absolute amount of ^{3}H incorporated into each of the mutant cultures was multiplied by the A_{590}/A_{590} ratio of A364a vs. mutant at the time of the shift to 37°C and then divided by the amount of incorporation in a culture of A364a. Thus, all values are normalized to a value of 1.0 for A364a.

Table 5. DNA synthesis in some representative permeabilized ρ^+ and ρ^0 temperature-sensitive mutants

Mutant strain	DNA synthesis	
	ρ^+	ρ^0
96	0.72	0.29
145	0.83	0.62
282	0.60	0.25
346	0.16	0.20

Strains were grown, harvested, treated with Brij 58, and assayed for DNA synthesis *in vitro* at 37°C. DNA synthesis values are normalized to a value of 1.0 for A364a. To isolate mutants containing no mitochondrial DNA, strains were grown for 4 days on YPD agar plates containing ethidium bromide. ρ^- phenotypes, cells that have no mitochondrial function, were identified and ρ^- were distinguished from ρ^0 by colony hybridization (24) using nick-translated yeast mitochondrial DNA prepared as described by Zeman and Lusena (25).

DISCUSSION

We have examined 400 temperature-sensitive yeast strains for mutants specifically defective in DNA replication by assaying for replication *in vitro* using cells made permeable to nucleoside triphosphates by the nonionic detergent Brij 58. We have identified 20 DNA replication mutants, 14 of which fall into new complementation groups. We can thus conclude that replication mutants appear quite frequently among random conditionally lethal mutant populations.

The reliability of the screening procedure was first demonstrated by the fact that strain 198, previously shown to carry

cdc8-1 and demonstrated to have a DNA elongation defect, was among the strains identified as having a replication defect in the *in vitro* screen. Second, complementation analysis showed that many different genes could be identified by this assay. The effectiveness of the screen was further demonstrated by confirming that strain 154, one of the recently identified mutants, had a defect in DNA synthesis *in vivo* and that the *in vitro* replication defect cosegregated with the *in vivo* temperature-sensitive growth phenotype in genetic crosses. It should be easy to adapt this assay into a mass screening protocol, as has been done in *E. coli* (26), now that the effectiveness of the permeabilized cells has been demonstrated.

One unexpected and interesting outcome of these studies was the demonstration that *cdc2* and *cdc16* mutants are defective in DNA synthesis in this type of *in vitro* replication system. *cdc16* mutants arrest synthesis with a terminal phenotype like that of other replication mutants, such as *cdc8*. *In vivo*, however, *cdc16* does not show a drastic decrease in the amount of incorporation of [^{3}H]uracil into alkali-resistant material at the nonpermissive temperature (5). Our results suggest that this mutant probably does have a defect in DNA synthesis itself and is worthy of further study.

cdc2 mutants have been shown to have an execution point early in S phase (10, 22) and arrest with a morphology like that of *cdc8* at the restrictive temperature (20). The original results of analysis of DNA synthesis in *cdc2* mutants showed that *cdc2* mutants incorporate substantial amounts of nucleic acid precursors at the nonpermissive temperature *in vivo*. Later it was found, quite unexpectedly, that *cdc2* cells remain sensitive to hydroxyurea, an inhibitor of DNA synthesis, after incubation at the restrictive temperature (8). To account for these two apparently contradictory findings, it was suggested that the gene product is required for the completion of DNA synthesis during S phase. Our *in vitro* results would not have been expected on the basis of the early *in vivo* incorporation studies but are entirely consistent with the hydroxyurea experiments, supporting the above explanation for the apparently contradictory results. Recently, Conrad and Newlon have shown that 1/3rd of the DNA remains unreplicated at the nonpermissive temperature in *cdc2* strains (23). It is not clear whether this is due to lack of initiation at some replicons or inhibition of elongation. The finding we report here, that *cdc2* mutants are defective in an *in vitro* replication system that clearly mimics *in vivo* replication, thus adds to the accumulating evidence that the product of the *CDC2* gene plays an important role in DNA synthesis in yeast.

Because the *in vitro* system can identify DNA synthesis mutants that do not show a substantial defect in precursor uptake into DNA *in vivo*, this method of isolating mutants is an essential complement to the *in vivo* screening procedure recently described by Dumas *et al.* (17). As might have been expected, *cdc2* and *cdc16* mutants were not among the alleles identified in that study.

We originally used a complementation assay based on the same permeabilized cell procedure used in this report to partially purify the CDC8 protein. However, none of the other mutants we have detected could be complemented in this system, probably because of the limited permeability of the cells to macromolecules. Recently, we have developed a fully soluble *in vitro* replication system (4). We have been able to use this system to purify the CDC8 protein to homogeneity by an *in vitro* complementation assay. *In vitro* replication extracts have been prepared from strain 154 and *cdc2* and both are markedly defective in *in vitro* replication. The CDC2 protein and the strain 154 protein have already been purified by complementation assay.

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1. Kornberg, A. (1980) *DNA Replication* (Freeman, San Francisco).
2. Jazwinski, S. M. & Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1223–1227.
3. Kojo, H., Greenberg, B. D. & Sugino, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7261–7265.
4. Celniker, S. E. & Campbell, J. L. (1982) *Cell* **31**, 201–213.
5. Hartwell, L. H. (1967) *J. Bacteriol.* **93**, 1662–1670.
6. Hartwell, L. H. (1971) *J. Mol. Biol.* **59**, 183–194.
7. Hartwell, L. H. (1973) *J. Bacteriol.* **115**, 966–974.
8. Hartwell, L. H. (1976) *J. Mol. Biol.* **104**, 803–817.
9. Hereford, L. M. & Hartwell, L. H. (1974) *J. Mol. Biol.* **84**, 445–461.
10. Hartwell, L. H., Culotti, J. & Reid, B. (1970) *Proc. Natl. Acad. Sci. USA* **66**, 352–359.
11. Johnston, L. H. & Nasmyth, K. (1978) *Nature (London)* **274**, 891–893.
12. Bisson, L. & Thorner, J. (1977) *J. Bacteriol.* **132**, 44–50.
13. Newlon, C. F. & Fangman, W. L. (1975) *Cell* **5**, 423–428.
14. Arendes, J., Kim, K. C. & Sugino, A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 673–677.
15. Kuo, C.-l. & Campbell, J. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4243–4248.
16. Johnston, L. H. & Thomas, A. P. (1982) *Mol. Gen. Genet.* **186**, 445–448.
17. Dumas, L. B., Lussky, J. P., McFarland, E. J. & Shampay, J. (1982) *Mol. Gen. Genet.* **187**, 42–46.
18. Hereford, L. M. & Hartwell, L. H. (1971) *Nature (London) New Biol.* **234**, 171–172.
19. Banks, G. R. (1973) *Nature (London) New Biol.* **245**, 196–199.
20. Hartwell, L. H., Mortimer, R. K., Culotti, J. & Culotti, M. (1973) *Genetics* **74**, 267–286.
21. Hicks, J., Sherman, F. & Fink, G. (1979) *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
22. Culotti, J. & Hartwell, L. H. (1971) *Exp. Cell Res.* **67**, 389–401.
23. Conrad, M. N. & Newlon, C. S. (1983) *Mol. Cell. Biol.* **3**, 1000–1012.
24. Grunstein, M. & Hogness, D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961–3964.
25. Zeman, L. J. & Lusena, C. V. (1975) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic, New York), Vol. 12, pp. 273–283.
26. Mark, D. F., Chase, J. W. & Richardson, C. C. (1977) *Mol. Gen. Genet.* **155**, 145–152.