Use of Temperature Sensitive Mutants to Study Yeast DNA Replication

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1984

(submitted May 23, 1984)

To my grandmother

and

my parents

Acknowledgements

I am deeply indebted to my research advisor, Judith Campbell, for valuable guidance and support during my graduate career. Her enthusiasm and encouragement will always be appreciated. I would also like to thank the other members of my committee, Norman Davidson, Michael Raftery, Jack Richards and Scott Emr for their contributions on my behalf.

I have benefitted from interactions with many people at Caltech. Among them, I would especially like to thank Dave Hershey and Puline Yen for their precious friendship and helpful advice; Ambrose Jong for his great work on the purification of thymidylate kinase presented in Chapter 4 of this thesis and, more recently, Scott Emr and Bernard Malisson for their interest and discussion. I would also like to thank Leanne Phares for typing this thesis.

Funding for my graduate study was provided by Caltech and by a Public Health Service Training Grant from the National Institutes of Health.

Finally, I would like to thank my family: my grandmother, parents, brother and sister for providing me with the unending love and encouragement which made it all possible, and my husband for his understanding and unquestioning support.

Abstract

An improved *in vitro* DNA replication system in Brij-treated *Saccharomyces cerevisiae* has been used to screen a random population of temperature-sensitive strains for mutants specifically defective in DNA synthesis. Twenty mutants defective in *in vitro* DNA synthesis have been isolated. 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 findings 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. Such an involvement was previously suggested by *in vivo* analysis for *CDC2* but was less clear for *CDC16*. The usefulness of our screening procedure is further 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.

The *in vitro* system has also been used to purify the CDC8 protein. *cdc8* mutant strains are temperature-sensitive for DNA chain elongation and the *CDC8* gene product is required for DNA synthesis *in vitro* in permeabilized yeast cells. Extracts of wild-type A364a yeast restore DNA synthesis in Brij-treated *cdc8* mutant. A small, heat-stable

protein responsible for this complementation has been partially purified from wild-type cells.

The CDC8 gene has been isolated on recombinant plasmids. The yeast-E.coli shuttle vector YCp50 was used to prepare a recombinant plasmid pool containing the entire yeast genome. Plasmids capable of complementing the temperature-sensitive cdc8-1 mutation were isolated by transformation of a cdc8-1 mutant and selection for clones able to grow at the nonpermissive temperature. The entire complementing activity is carried on a 0.75-kilobase fragment, as revealed by deletion mapping and DNA sequencing. This fragment lies 1 kilobase downstream from the well-characterized sup4 gene, a gene known to be genetically linked to CDC8, thus confirming the cloned gene corresponds to the chromosomal CDC8 gene. Two additional recombinant plasmids that complement the cdc8-1 mutation but that do not contain the 0.75-kilobase fragment or any flanking DNA were also identified in this study. These plasmids may contain genes that compensate for the cdc8-1 mutation.

By the following criteria, we have shown that thymidylate kinase, which catalyzes the phosphorylation of thymidine-5'-monophosphate to thymidine-5'-diphosphate in the pathway of synthesis of dTTP from dTMP, is the product of the CDC8 gene. First, transformed strains carrying the CDC8 gene on a stable high-copy-number plasmid express

higher levels of both the gene transcript and the kinase activity than does wild type. Secondly, extracts of strains bearing different alleles of cdc8 show no detectable thymidylate kinase activity. Third, the DNA sequence of CDC8 gene reveals an open reading frame that encodes a protein of 216 amino acids with the same amino terminal sequence as thymidylate kinase purified from yeast.

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Introduction

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 DNA replication mutants and then by complementation assays to purify and characterize the gene products required for DNA replication in yeast. We have used an improved in vitro DNA synthesis system to screen a randomly mutagenized population of temperature-sensitive strains originally isolated by L.H. Hartwell (1967) for defects in replication in vitro. The improved in vitro assay uses yeast cells made permeable to nucleoside triphosphates with the nonionic detergent Brij 58 (Hereford and Hartwell, 1971; Banks, 1973). Twenty mutants defective in in vitro DNA synthesis have been isolated. Seven of them fall into three complementation groups--cdc2, cdc8, and cdc16--involved in the control of the cell-division cycle. Because cdc7 mutants are defective in the initiation of DNA synthesis at 37°c in vivo (Hartwell, 1971; 1973; 1976) but can incorporate dTMP into DNA at 37°c to the same extent as wild type in this system, 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 suggests that these two mutations directly affect DNA synthesis at replication forks.

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* (Culotti *et al.*, 1971). Later *cdc2* mutants were shown to remain sensitive to hydroxyurea, an inhibitor of DNA synthesis, when shifted to the permissive temperature (Hartwell, 1976). The hydroxyurea experiment suggests that DNA replication at the nonpermissive temperature in these mutants is incomplete and is consistant with our *in vitro* results. Conrad and Newlon (1983) have shown that one third of the DNA remains unreplicated at the nonpermissive temperature in *cdc2* strains. This is also consistent with the result of the *in vitro* reduction in total incorporation we have observed if an initiation defect results in fewer active replicons. Thus our finding adds to the accumulating evidence that the product of the *CDC2* gene plays an important role in DNA synthesis in yeast. *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 (Hartwell, 1967). Our results suggest that this mutant probably does have a defect in DNA synthesis itself and is worthy of further study.

The usefulness of our screening procedure is further demonstrated by the isolation of replication mutants in previously undescribed complementation groups. One of the isolated *in vitro* replication mutants, for instance, strain 154 also has a defect in DNA synthesis *in vivo* and thus strain 154 is in fact a previously unidentified replication mutant. 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 described by Dumas *et al.* (1982).

The *cdc8* mutant was known to be temperature-sensitive for DNA chain elongation (Hartwell, 1971; 1973) and the *CDC8* gene product is required for DNA synthesis *in vitro* in Brij-treated yeast cells. Because extracts of wild-type A364a yeast restore DNA synthesis in Brij-treated *cdc8* and synthesis is inhibited by aphidicolin, an inhibitor of DNA replication *in vivo* (Plevani *et al.*, 1980; Sugino *et al.*, 1981), we have extended the use of the *in vitro* DNA synthesis system to purify the *CDC8* gene product by the complementation assay. Using conventional methods of

purification, we have purified the CDC8 protein 600-fold. The partially purified protein had a molecular weight of about 20,000 and is heat stable being resistant to incubation at 65°c for 5 min.

To carry out detailed biochemical and functional characterization of this enzyme, we decided to clone the CDC8 gene to overproduce the CDC8 protein. The yeast E.coli shuttle vector YCp50 bearing the yeast ARS1, CEN4, and URA3 sequences, to provide for replication, stability, and selection, respectively, was used to prepare a recombinant plasmid pool containing the entire yeast genome. Plasmids capable of complementing the temperature-sensitive cdc8-1 mutation were isolated by transformation of a cdc8-1 mutant and selection for clones able to grow at the nonpermissive temperature. The entire complementing activity is carried on a 0.75-kilobase fragment, as revealed by Bal31 deletion mapping and DNA sequencing. This fragment was shown to lie 1 kilobase downstream from the well-characterized sup4 gene, a gene known to be genetically linked to CDC8, thus confirming that the cloned gene corresponds to the chromosomal CDC8 gene. Two additional recombinant plasmids that complement the cdc8-1 mutation but that do not contain the CDC8 gene or any flanking DNA were also identified. These plasmids may contain genes that compensate for the lack of CDC8 gene product.

Northern blot analysis indicates that CDC8 gene cloned in YEp24, a stable high-copy-number plasmid vector, produces 10 times as much of a 0.9-kilobase RNA that hybridizes to the CDC8 gene as wild-type yeast cells. The DNA sequence of the CDC8 gene reveals an open reading frame that encodes a protein of 216 amino acids in length. Taken together with the size of the segment of DNA that gives complete complementation of the cdc8 mutation, it was clear that the molecular weight of the CDC8 protein is 24,792 instead of 40,000 which was suggested to be the molecular weight of the CDC8 protein (Arendes et al., 1983).

At the same time, it was shown by Sclafani and Fangman that the thymidine kinase gene of the Herpes Simplex Virus fully complemented the cdc8 defect when introduced into the cdc8-1 mutant (personal communication). Herpes thymidine kinase has previously been shown to have two additional catalytic activities associated with it, thymidylate kinase and deoxycytidine kinase (Chen and Prusoff, 1978). Because there is no thymidine kinase in yeast, we assumed that the CDC8 gene product might be thymidylate kinase and Ambrose Jong in this laboratory started to purify the thymidylate kinase. By studying the thymidylate kinase activity in extracts of cdc8 mutants, purifying the thymidylate kinase from transformants carrying the CDC8 gene on a plasmid DNA, and comparing the results of amino acid composition and amino terminal sequence analysis of the purified yeast

thymidylate kinase activity with the results obtained from the DNA sequencing analysis, we have shown that the CDC8 gene of yeast encodes thymidylate kinase.

We propose that either the CDC8 gene product may play a role in DNA replication in addition to precursor synthesis, as may be the case for bacteriophage T4 dCMP hydroxymethylase (Chao et al., 1977), or there may be degradative enzyme in vitro and precursor regenerating systems are necessary for efficient in vitro synthesis to explain the observation that DNA replication is temperature-sensitive in cdc8 strains in vitro (Hereford and Hartwell, 1971; Jazwinski and Edelman, 1976; 1979; Kuo and Campbell, 1982; Celniker and Campbell, 1982; Arendes et al., 1983).

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Chapter 1

Isolation of yeast DNA replication mutants in permeabilized cells

Reprint from Proc. Natl. Acad. Sci. USA
80, 6465-6469 (1983).

Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6465–6469, November 1983
Biochemistry

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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Communicated by Leroy Hood, July 15, 1983

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

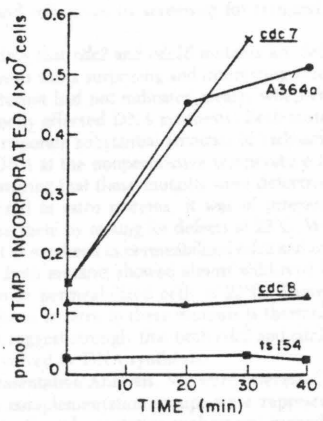

FIG. 1. Optimum time of Brij 58 treatment. Cells were treated with Brij 58 for the times indicated. Synthesis was then measured at 37°C. Values represent extent of synthesis and not initial rate (15). ts 154, temperature-sensitive mutant strain 154.

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

	dTMP incorporated per 10^6 cells, pmol	
Strain	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 Hartwell and colleagues by screening for terminal phenotype (20).

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.

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.

We thank Joan Kobori, Caren Chang, and Ambrose Jong for helpful comments on the manuscript. This work was supported by grants from the U.S. Public Health Service (GM 25508 and T 32 GM 07616), the American Cancer Society (MV-142), and the March of Dimes (1-794). J.L.C. is the recipient of Research Career Development Award CA00544.

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Chapter 2

Purification of the cdc8 protein of *Saccharomyces cerevisiae* by Complementation in an aphidicolin-sensitive *in vitro* DNA replication system

Reprint from Proc. Natl. Acad. Sci. USA
79, 4243-4247 (1982).

Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4243–4247, July 1982
Biochemistry

Purification of the cdc8 protein of *Saccharomyces cerevisiae* by complementation in an aphidicolin-sensitive *in vitro* DNA replication system

[permeabilized cells/yeast/cell division cycle (*cdc*) mutants/DNA elongation/eukaryotic DNA replication]

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Communicated by Norman Davidson, July 27, 1981

ABSTRACT DNA synthesis *in vitro* in Brij-treated *Saccharomyces cerevisiae* requires the product of the *CDC8* gene (Hereford, L. M. & Hartwell, L. H. (1971) *Nature (London) New Biol.* 234, 171–172). Extracts of wild-type A364a yeast restore DNA synthesis in Brij-treated *cdc8*, a mutant containing a thermolabile *cdc8* gene product. This constitutes a complementation assay by which the *cdc8* gene product can be monitored during purification. A heat-stable protein responsible for this complementation has been partially purified from both wild-type A364a cells and from a *cdc8* temperature-sensitive mutant. The complementation activity from the mutant is thermolabile when compared to the wild-type activity, indicating that *CDC8* is the structural gene for the protein.

The microbial eukaryote *Saccharomyces cerevisiae* is an ideal organism for probing the enzymatic mechanisms of DNA replication in higher cells. While displaying most of the features of higher eukaryotic chromosome structure observed during gene expression and DNA replication, yeast offers the advantage over higher cells of rapid growth and relatively simple organization. In addition, yeast cells can exist stably in both haploid and diploid states, thus facilitating the isolation of mutants and their genetic analysis. Tremendous progress has been made in elucidating the mechanism of DNA replication in bacteria, by analysis of replication mutants and by biochemical characterization of replication components. In these studies an important advance was the development of *in vitro* replication systems that allow purification of components of the complex replication machinery by complementation analysis. Such a combined genetic and biochemical approach for dissecting the replication machinery should also prove profitable in yeast.

Many temperature-sensitive mutants having defects in cell division have been isolated and characterized by Hartwell (1). Of these, several were found to be deficient in DNA synthesis, although only in *cdc8* mutants was it clear that the defect directly affected DNA replication (1, 2). We have examined the existing temperature-sensitive strains for mutants specifically defective in DNA replication, by assaying for replication *in vitro* in yeast cells made permeable to nucleoside triphosphates by the nonionic detergent Brij, as described by Hereford and Hartwell (3). Brij-treated cells retain the morphology of intact cells but are permeable to low molecular weight precursors of macromolecular synthesis. DNA synthesis in this system corresponds to *in vivo* replication by several criteria. In particular, DNA synthesis in the *S. cerevisiae* temperature-sensitive replication mutant *cdc8* is heat labile in this *in vitro* system (3). In addition, as shown in this communication, synthesis is inhibited by aphidicolin, an inhibitor of DNA replication *in vivo*. Using this screening procedure, we have identified nine additional replication mutants (unpublished).

It was not possible to predict *a priori* if detergent-treated cells would be permeable to macromolecules and thus amenable to *in vitro* complementation. During the course of these studies, we investigated whether Brij-treated cells of any of the DNA replication mutants could be complemented by extracts containing the wild-type gene product. *cdc8* alone showed complementation; DNA replication in Brij-treated *cdc8* cells could be restored by the addition of wild-type extract at the restrictive temperature. The ability to achieve complementation with the *cdc8* defect may, in fact, be due to the unique physical properties of the cdc8 protein described here.

In this communication we report the partial purification and characterization of the cdc8 protein, using a complementation assay to monitor activity. This is just the first step in ultimately generating a molecular description of eukaryotic replication by use of *in vitro* complementation to purify and to characterize the gene products involved.

MATERIALS AND METHODS

Strains. Parental strain A364a (*a ade1 ura1 gal1 tyr1 his7 lys2*) and strain 198 *cdc8*, derived from A364a, were provided by John Scott (University of California at Los Angeles).

Medium. YPD (yeast extract/peptone/dextrose) medium is described in ref. 4.

Preparation of Receptor. Strain *cdc8* was 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 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 at 30°C until the cells were permeable, as monitored by measuring alkaline phosphatase activity (3). The receptor preparations were prepared fresh for each assay and retained activity at 0°C for 2–3 hr.

Complementation Assay for cdc8 Activity. 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 the Tris/sucrose/Brij cell suspension; and the indicated amounts of donor fractions. After incubation for 30 min at 37°C the reaction was stopped by the addition of 3 ml of 1 M HCl/0.02 M sodium pyrophosphate, and the amount of trichloroacetic acid-insoluble radioactive material was determined (5). A unit of cdc8 activity is that amount of protein giving rise to incorporation of 1.0 pmol of total nucleotide in 30 min.

Other Methods. Protein was measured by the method of Lowry *et al.* (6).

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RESULTS

DNA Synthesis in Permeabilized Yeast Cells Mimics DNA Replication *in Vivo*. *DNA synthesis is thermosensitive in the thermolabile mutant* cdc8. Previous studies by Hereford and Hartwell (3) showed that yeast cells made permeable with Brij 58 could incorporate [α-^{32}P]dTTP into acid-insoluble material in a reaction requiring Mg^{2+}, ATP, and the other three dNTPs. The *in vitro* replication system apparently used the same synthesizing machinery as DNA replication *in vivo*, because DNA synthesis was defective in Brij-treated strain *cdc8* at the restrictive temperature. As shown in Fig. 1, we have reproduced this finding, using a modified procedure that involves more extensive treatment with detergent. The optimal time of Brij treatment is 40 min, a time at which maximal amounts of alkaline phosphatase are also measured. Whereas wild-type cells respond to the detergent treatment by supporting DNA synthesis when provided with dNTPs, no DNA synthesis over background is observed with Brij-treated *cdc8* cells. The level of synthesis in wild-type strain A364a is 5-fold higher than that observed in the mutant *cdc8* at 37°C. Residual synthesis in *cdc8* probably does not represent replication because ATP is not required.

Synthesis is inhibited by aphidicolin. Aphidicolin, a tetracyclic diterpenoid, has recently been shown to inhibit the growth of eukaryotic cells and virus-infected cells by inhibiting DNA replication (7, 8). Furthermore, Plevani (9) has shown that both yeast DNA polymerase I and DNA polymerase II are inhibited by aphidicolin, and Sugino *et al.* (10) have shown that in yeast mutants that are permeable to aphidicolin the drug inhibits growth.

Because inhibition of DNA synthesis by aphidicolin provides one additional criterion for DNA replication, we have examined its effect on DNA synthesis in Brij-treated yeast cells. As shown in Fig. 2, aphidicolin inhibits DNA synthesis in the permeabilized cell system; at a concentration of 20 μg/ml, DNA synthesis is reduced to 40% of normal. However, the addition of increasing amounts of aphidicolin up to 100 μg/ml does not eliminate this residual synthesis. This drug-resistant synthesis may reflect the fact that dNTPs are competitive inhibitors of aphidicolin (8) or may indicate the existence of a previously undetected aphidicolin-resistant DNA-synthesizing system in yeast.

Extracts of Wild-Type Yeast Restore DNA Synthesis in Permeabilized Cells of *cdc8*. Cell-free extracts prepared from a wild-type A364a (donor extract) can restore synthesis to *cdc8* cells made permeable by Brij treatment (receptor). As shown in Fig 3*a*, extracts of wild-type A364a stimulate DNA synthesis in permeabilized cells of *cdc8* at 37°C. In contrast, donor extracts from strain *cdc8* cannot restore the activity to the recipient *cdc8* preparation at 37°C (Fig. 3*a*). The difference in stimulatory activity in extracts from wild-type and *cdc8* indicates that the donor extract is specifically providing the *cdc8* gene product.

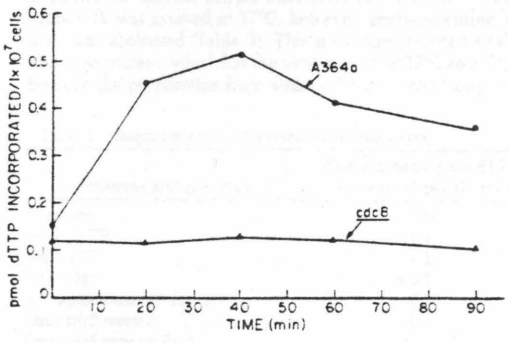

FIG. 1. Incorporation of [^{32}P]dTTP into DNA at 37°C plotted as a function of time of pretreatment for cultures of wild-type A364a and mutant *cdc8*. Strains were grown, harvested, and treated for the indicated time period with Tris/sucrose/Brij. DNA synthesis was then measured in the standard assay using [α-^{32}P]dTTP.

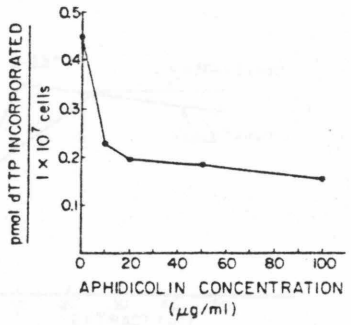

FIG. 2. Effect of aphidicolin on DNA synthesis in permeabilized A364a cells. DNA synthesis in Brij-treated A364a cells was measured with the addition of indicated amounts of aphidicolin dissolved in dimethyl sulfoxide.

The inability of an extract of *cdc8* cells to restore DNA synthesis to permeabilized *cdc8* cells at 37°C is not due to nonspecific loss of activity. When assayed at the permissive temperature, *cdc8* extracts can stimulate DNA synthesis in permeabilized *cdc8* cells (Fig. 3*b*). Thus temperature-sensitive mutant extracts contain a thermolabile gene product. The *cdc8* gene product in the permeabilized cells is apparently more labile than in the extract, perhaps because the Brij-treatment is carried out at 30°C, whereas the extracts are prepared at 4°C. This is an important observation, because the ability of *cdc8* extracts to serve as donors in the complementation assay at 23°C has allowed us to purify a thermolabile activity from the temperature-sensitive mutant.

These observations formed the basis for the complementation assay described in *Materials and Methods*. The requirements for the complementation reaction using partially purified cdc8 protein as donor are summarized in Table 1. In addition to a requirement for both receptor cells and a donor preparation, there is an absolute requirement for ATP. Complementation is inhibited by aphidicolin, just as synthesis is inhibited in wild-type permeabilized cells. A receptor extract prepared from a DNA replication mutant that falls in a different complementation group from *cdc8*, *ts12* (unpublished results), was not complemented in these permeabilized cells. This result not only shows the specificity for *cdc8* in this reaction, but suggests that this may not be a generally useful assay to isolate replication proteins. In permeabilized diploid cells, a 2-fold increase in overall DNA synthesis was routinely observed.

Partial Purification of the cdc8 Protein. Because the *cdc8* gene product is required for DNA synthesis in permeabilized yeast cells, we have been able to purify the cdc8 protein by using the complementation assay described above. The results of a typical purification are summarized in Table 2.

Analysis of glycerol gradient fractions by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that fraction V is greater than 25% pure by weight and

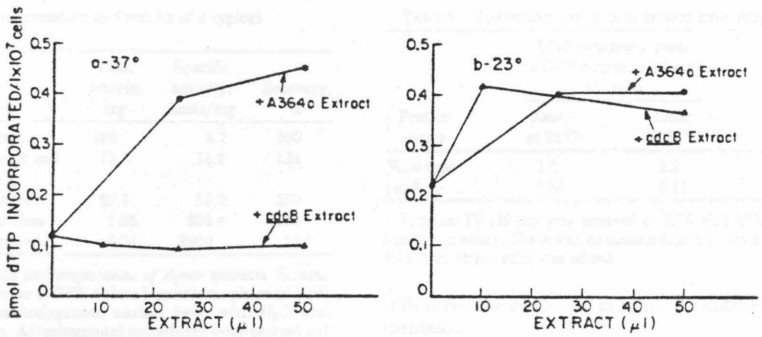

FIG. 3. Complementation of Brij-treated *S. cerevisiae cdc8* cells. Mutant *cdc8* was grown at 23°C to a density of 2×10^7 cells per ml and permeable cells were prepared (receptor). The standard complementation reaction mixture contained receptor cells and the indicated amounts of either wild-type (●) or *cdc8* (▲) extract. Incubation was for 30 min at either 37°C (*a*) or 23°C (*b*).

substantially purer on a molar basis. The glycerol gradient data allow a preliminary estimate of the sedimentation constant of the cdc8 protein that is consistent with a molecular weight (assuming it is a globular protein) of 10,000–20,000, which is also in reasonable agreement with the gel electrophoresis results.

Properties of the Purified cdc8 Protein. *Purified cdc8 protein can complement permeabilized* cdc8 *cells.* Although the cdc8 protein could be purified from extracts by virtue of its ability to restore DNA synthesis to permeabilized *cdc8* cells, the purified protein is much more efficient than crude preparations in stimulating synthesis. As shown in Fig. 4, 25 μg of *cdc8* protein leads to a 10-fold stimulation of activity in the complementation assay at 37°C. The stimulatory activity is linear for amounts of cdc8 protein ranging between 2.0 and 10 μg in the assay. Saturating amounts of cdc8 protein give a 30-fold stimulation of activity (data not shown). Results presented in Fig. 3, however, showed that only a 5-fold stimulation could be achieved with saturating amounts of extract. This is easily explicable if an inhibitor is removed during purification.

Complementation activity of the cdc8 protein purified from the temperature-sensitive mutant cdc8 *is thermolabile.* The cdc8 protein was also purified from extracts of *S. cerevisiae cdc8* through the phosphocellulose step as described above. The purification was monitored by complementation assay at 23°C, a temperature at which the cdc8 protein from this strain complements the mutant extract effectively (see Fig. 3*b*). When fraction IV was assayed at 37°C, however, complementing activity was abolished (Table 3). This is in sharp contrast to the wild-type protein, which has the same activity at 23°C and 37°C. Because the preparation from strain *cdc8* is thermolabile, the complementation activity is the product of the structural gene for *cdc8*.

Complementation by partially purified cdc8 protein is aphidicolin sensitive. After addition of aphidicolin, synthesis in the complementation assay using wild-type cdc8 protein was reduced to 40%, a value similar to that obtained with permeabilized A364a (compare Fig. 2). This argues for, though does not alone establish that, replicative synthesis occurs in this assay.

Heat stability of the cdc8 activity. As shown in Table 4, the activity restoring DNA replication in permeabilized *cdc8* at

Table 1. Requirements of the complementation assay

Omissions and additions	DNA synthesis, pmol dTTP incorporated/per 10^7 cells
Complete	0.61
Omit dNTP	<0.1
Omit ATP	0.12
Omit Mg^{2+}	<0.1
Add aphidicolin (20 μg/ml)	0.23
Omit *cdc8* receptor	<0.1
Omit *cdc8* gene product	<0.1
Omit *cdc8* receptor, add *ts12* receptor	<0.1

The assay conditions were as described in *Materials and Methods*, with the omissions and additions noted. Each reaction mixture (except the second-to-last) contained 10 μg of partially purified cdc8 protein.

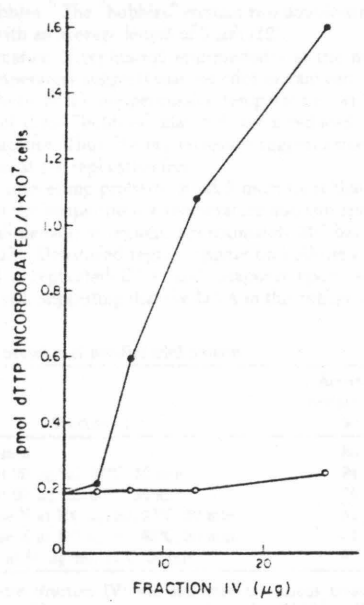

FIG. 4. Complementation by purified cdc8 protein and inhibition by proteinase K. Receptor *cdc8* cells were prepared, indicated amounts of cdc8 protein (fraction IV) were added, and incubation was carried out as in the legend to Fig. 3. The lower curve (○) represents reaction mixtures that contained cdc8 protein that had been treated with proteinase K (100 μg/ml) for 30 min at 65°C.

Table 2. Purification procedure and results of a typical purification

Fraction	Step	Total protein, mg	Specific activity, units/mg	Recovery, %
I	Crude extract	166	4.7	100
II	Streptomyocin and $(NH_4)_2SO_4$	73.7	14.2	134
III	DEAE-cellulose	22.7	52.0	150
IV	Phosphocellulose	1.36	924.4	160
V*	Glycerol gradient	0.04	2900	13.5

Growth, storage of cells and preparation of donor extracts. Strains A364a and *cdc8* were grown at 23°C to late logarithmic phase in YPD medium, collected by centrifugation, washed twice with H_2O, and stored in liquid nitrogen. All subsequent procedures were carried out at 0–4°C. For preparation of extracts, cell paste was thawed, diluted with a solution of Zymolyase 60,000 (0.5 mg/ml in H_2O), and adjusted to OD_{590} = 400. The cells were incubated at 30°C for 30 min, at which time spheroplast formation was complete. The spheroplasts were mixed on a Vortex mixer with glass beads and then disrupted in a chilled homogenizer. The supernatant was then collected by centrifugation at 10,000 × *g* for 40 min (fraction I). *Streptomyocin and ammonium sulfate fractionation.* A solution of 30% streptomyocin sulfate was added to extracts to a final concentration of 5%. After stirring for 30 min, the suspension was centrifuged at 20,000 × *g* for 20 min. Solid $(NH_4)_2SO_4$ (0.47 g/ml) was added to the supernatant fluid and the protein precipitate was collected by centrifugation. The precipitate was dissolved in buffer A [20 mM Tris·HCl (pH 7.6)/1 mM EDTA/1 mM 2-mercaptoethanol/20% (wt/vol) glycerol] and dialyzed against the same buffer (fraction II). *DEAE-cellulose column chromatography.* Fraction II (0.8 ml) was diluted with 0.8 ml of buffer A and applied to a 5-ml DE52 (Whatman) DEAE-cellulose column equilibrated with buffer A. The column was washed and then eluted with a 50-ml linear gradient of NaCl (0.1–0.6 M) in buffer A. Fractions containing the complementation activity were pooled and precipitated by the addition of ammonium sulfate (0.47 g/ml). The precipitate was collected by centrifugation, resuspended in 0.5 ml of buffer B [20 mM Tris·HCl (pH 7.0)/1 mM EDTA/1 mM 2-mercaptoethanol/20% (wt/vol) glycerol] and then dialyzed (fraction III). *Phosphocellulose P-11 column chromatography.* Fraction III was diluted with 0.5 ml of buffer B and applied to a 4-ml phosphocellulose P-11 (Whatman) column equilibrated with buffer B. The column was washed and protein was eluted with a 50-ml linear gradient of NaCl (0.1–0.6 M) in buffer B. Fractions containing complementation activity, which eluted at 0.13 M NaCl, were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, redissolved in buffer C (the same as buffer A except that 0.1 mM dithiothreitol is substituted for 2-mercaptoethanol), and dialyzed against the same buffer (fraction IV). *Glycerol gradient sedimentation.* Fraction IV was dialyzed against buffer C containing 5% (wt/vol) glycerol and layered on a 4.6-ml linear gradient of 10–30% (wt/vol) glycerol containing 0.02 M Tris·HCl (pH 7.5), 10 mM 2-mercaptoethanol, and 10 mM NaCl. Centrifugation was at 45,000 rpm at 2°C for 28 hr in a Spinco SW 50.1 rotor. The following proteins were used as standards: alcohol dehydrogenase, bovine serum albumin, hemoglobin, and cytochrome *c*.

* This step was performed on only a portion of fraction IV and the values in the table were obtained by multiplying by the appropriate factor.

37°C was highly stable, being resistant to incubation at 65°C. After 10 min at 65°C, 80% of the activity remained.

cdc8 activity resides in a protein. The lack of activity in fractions from *cdc8* extracts at the restrictive temperature, shown in Table 3, indicates that the activity resides in a protein or RNA. The activity is resistant to RNase A, however, ruling out that it is an RNA molecule. The activity is also resistant to papain, even at 65°C, a property possibly related to its resistance to heat denaturation (Table 4). However proteinase K, a protease generally considered to be more active toward proteins in native configurations than other proteases, does inactivate cdc8 activity (Table 4, Fig. 4). Controls showed that proteinase K had no effect on synthesis in permeabilized wild-type A364a cells under the conditions in which it inhibited *cdc8* complementation.

Table 3. Thermolability of cdc8 protein from *cdc8* cells

Protein source	DNA synthesis, pmol dTTP incorporated per 10^7 cells: Assay at 23°C	Assay at 37°C	Activity ratio, 37°C/23°C
Wild-type	1.2	1.2	1.0
cdc8	0.80	0.11	0.13

Fraction IV (10 μg) was assayed at 23°C and 37°C in the complementation assay. There was no measurable activity at 37°C when protein from strain *cdc8* was added.

DISCUSSION

The cdc8 protein is essential for DNA replication *in vivo* and is required for cell division. When strain *cdc8* grown at the permissive temperature, 23°C, is shifted to the restrictive temperature, 36°C, cells accumulate with a nucleus that remains undivided but is located at the isthmus between parent cell and bud. The first wave of DNA synthesis after shift up does not occur (1). The product of the *cdc8* gene is apparently required throughout the period of DNA synthesis, because when a synchronized culture of cells defective in the gene is shifted to 36°C within the S period, nuclear DNA replication ceases immediately (2). Mitochondrial DNA replication also ceases at the nonpermissive temperature in *cdc8* mutants (11). Electron microscopic examination of chromosomes in *cdc8* cells placed at the nonpermissive temperature for approximately two generation times shows a high proportion of molecules that contain replication "bubbles." The "bubbles" contain two double-stranded branches with an average length of 3 μm (12).

The formation of replication intermediates at the nonpermissive temperature suggests that the *cdc8* mutant can initiate DNA synthesis at the nonpermissive temperature, while the small size of these "bubbles" may indicate a reduced rate of chain propagation. Thus, the *in vivo* results suggest a role of the cdc8 protein at the replication fork.

Another interesting property of *cdc8* mutants is that DNA extracted at the nonpermissive temperature also contains clusters of small denatured regions, approximately 300 base pairs in length (13). Denatured regions appear on both newly replicated and unreplicated DNA, and disappear upon centrifugation in CsCl, suggesting that the DNA in this region may be

Table 4. Stability of purified cdc8 protein

Treatment	Activity remaining, %
65°C, 10 min	80
Papain at 20 μg/ml, 37°C, 30 min	94
Papain at 20 μg/ml, 65°C, 30 min	76
Proteinase K at 100 μg/ml, 37°C, 30 min	51
Proteinase K at 100 μg/ml, 65°C, 30 min	<1
RNase A at 50 μg/ml, 37°C, 30 min	97

Purified cdc8 (fraction IV) was subjected to various treatments. Treatment with RNase or papain was performed in 0.1 M Tris·HCl (pH 7.0). The partially purified protein activity was then determined by assay as described in the legend to Fig. 1, using 10 μg of fraction IV and with the indicated additions. All values cited are relative to controls carried through identical procedures except for the omission of the indicated agents. The control for heat treatment was maintained at 0°C.

noncovalently bound to protein (16). Such single-stranded regions could be envisioned as intermediates in recombination or replication. Biochemical studies to date indicate that *cdc8* strains are not deficient in precursor synthesis (3). Furthermore, *cdc8* does not code for one of the yeast DNA polymerases (14, 15).

Our findings agree with those of Hereford and Hartwell (3) that incorporation of dNTPs into permeabilized yeast cells corresponds to DNA replication *in vivo*. One of the strongest arguments for this is the finding that Brij-treated *S. cerevisiae cdc8* is incapable of carrying out DNA synthesis at the nonpermissive temperature. Also, we have shown that the synthesis is sensitive to aphidicolin, an inhibitor of yeast replication (9, 10). One point that is not resolved is whether nuclear or mitochondrial replication is being observed, because DNA synthesis occurs on the endogenous DNA template. Banks (16) suggests that all synthesis is mitochondrial, whereas our results indicate that some ρ^0 strains, that is, strains without mitochondrial DNA, are just as active *in vitro* as ρ^+ strains (unpublished). For the current work, resolution of this question is not important, because *CDC8* function is required for both nuclear and mitochondrial replication.

We have extended the earlier studies of Hereford and Hartwell (3) by showing that extracts of wild-type yeast can stimulate DNA synthesis in the permeabilized mutant *cdc8*. This was surprising because the Brij-treated cells retain the morphology of intact cells. The Brij-treated yeast cells are analogous to toluene-treated *Escherichia coli*, which also carry out DNA replication on endogenous templates in a reaction dependent on Mg^{2+} and ATP (17). When toluene-treated *E. coli* are treated with the detergent Triton X-100, DNA polymerase I and lactate dehydrogenase are free to diffuse from the cells, and repair synthesis in the cells is inhibited by antibody to DNA polymerase I (18), indicating that the cells are permeable to macromolecules. We therefore tested whether this yeast system was permeable to proteins by looking for complementation in our mutants and found that *cdc8* showed complementation. We were thus able to use permeabilized yeast cells as receptor extracts in an *in vitro* complementation assay to purify the cdc8 protein.

Using conventional methods of purification, we have purified the cdc8 protein 600-fold. We have shown that *CDC8* is the structural gene for the protein by purifying a thermolabile activity from the temperature-sensitive *cdc8* mutant. Perhaps the most striking property of this protein is its resistance to heating at 65°C and to high levels of papain even at 65°C. Even for proteinase K, a partially denatured protein is required for proteolytic inactivation. Such stability properties are usually attributed to proteins of small size.

What is the function of the cdc8 protein? Results presented here indicate that it participates in DNA replication. In addition, we have recently developed a fully soluble system that carries out replication *in vitro* of exogenous covalently closed circular yeast DNAs that, by many criteria, mimics *in vivo* replication (unpublished results). Synthesis *in vitro* is temperature sensitive in extracts prepared from *cdc8* mutants, and the defect can be complemented by the partially purified cdc8 protein.

Several small heat-stable proteins have been described that participate in replication in prokaryotes. One of them is the *E. coli* helix-destabilizing protein (M_r 18,500) (19, 20). Single-strand DNA-binding proteins are known to participate in events at the replication fork, and a mutation affecting such a protein could give rise to the *cdc8* phenotype. In fact, mutants of *E. coli* that contain a temperature-sensitive helix-destabilizing protein do not grow at high temperature (20). Other small heat-stable proteins have been found in various replication systems [e.g., the M_r 11,000 *E. coli* thioredoxin, a subunit of phage T7 DNA polymerase (21)]. There is also a class of small heat-stable proteins involved in eukaryotic DNA metabolism, namely the histones. Clearly the role of the cdc8 protein will be resolved only when homogeneous preparations of the protein are available.

We thank S. E. Celniker for helpful discussion. This investigation was supported by U.S. Public Health Service Grant GM 25508 and Research Career Development Award CA 00544.

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Chapter 3

Cloning of *Saccharomyces cerevisiae* DNA Replication Genes: Isolation of the *CDC8* Gene and Two Genes that Compensate for the *cdc8-1* mutation

Reprint from Mol. Cell. Biol. 3, 1730-1737 (1983).

MOLECULAR AND CELLULAR BIOLOGY, Oct. 1983, p. 1730–1737 Vol. 3, No. 10
0270-7306/83/101730-08$02.00/0

Cloning of *Saccharomyces cerevisiae* DNA Replication Genes: Isolation of the *CDC8* Gene and Two Genes That Compensate for the *cdc8-1* Mutation

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Received 11 April 1983/Accepted 13 July 1983

The *CDC8* gene, whose product is required for DNA replication in *Saccharomyces cerevisiae*, has been isolated on recombinant plasmids. The yeast vector YCp50 bearing the yeast *ARS1*, *CEN4*, and *URA3* sequences, to provide for replication, stability, and selection, respectively, was used to prepare a recombinant plasmid pool containing the entire yeast genome. Plasmids capable of complementing the temperature-sensitive *cdc8-1* mutation were isolated by transformation of a *cdc8-1* mutant and selection for clones able to grow at the nonpermissive temperature. The entire complementing activity is carried on a 0.75-kilobase fragment, as revealed by deletion mapping. This fragment lies 1 kilobase downstream from the well-characterized *sup4* gene, a gene known to be genetically linked to *CDC8*, thus confirming that the cloned gene corresponds to the chromosomal *CDC8* gene. Two additional recombinant plasmids that complement the *cdc8-1* mutation but that do not contain the 0.75-kilobase fragment or any flanking DNA were also identified in this study. These plasmids may contain genes that compensate for the lack of *CDC8* gene product.

The CDC8 protein of *Saccharomyces cerevisiae* is essential for chromosomal replication in vivo and is required for cell division. When the temperature-sensitive *cdc8* mutant is grown at the permissive temperature, 23°C, and then shifted to the restrictive temperature, 36°C, cells accumulate that contain a nucleus located at the isthmus between parent cell and bud, but which do not divide. The first wave of DNA synthesis after shift up does not occur (8). The product of the *CDC8* gene is apparently required throughout the period of DNA synthesis, since synchronized cultures of cells defective in the gene cease nuclear DNA replication when shifted to 36°C within the S period (9). Mitochondrial DNA replication also ceases at the nonpermissive temperature in *cdc8* mutants (22), and replication of the 2-μm circle plasmid is defective in *cdc8* mutants (17).

Recently the CDC8 protein has been purified to homogeneity, using in vitro replication systems (1, 15). The purified protein binds to single-stranded DNA and stimulates DNA polymerase I activity on single-stranded DNA templates. The CDC8 protein may be identical to protein C, discovered by Chang et al. (4).

To carry out detailed biochemical and functional characterization of an enzyme involved in DNA replication, it is necessary to obtain large quantities of purified protein. In *Escherichia coli*, one way to overcome the problem of low yield of replication proteins is to clone the gene coding for a given protein into temperature-inducible bacteriophage lambda vectors or into a high-copy-number plasmid. Overproduction of the replication proteins DNA polymerase I (12), DNA ligase (23), and dnaC protein (13) has been achieved. Overproduction of the *LEU2* gene product in yeast strains carrying the *LEU2* gene on an autonomously replicating, high-copy-number plasmid has also been achieved (10). Therefore, the same approach used in *E. coli* is applicable in yeast.

In this communication we report the molecular cloning of the *CDC8* gene as the first step toward overproducing the CDC8 protein and studying the regulation of expression of the gene. We were able to select stable hybrid plasmids containing the *CDC8* sequence from a yeast DNA library on the basis of their ability to complement a temperature-sensitive mutation in yeast transformation experiments. The identity of these genes with *CDC8* was confirmed by demonstration that the plasmids carrying them also carry the *sup4* gene, which is known to be linked to *CDC8* in the chromosome.

MATERIALS AND METHODS

Strains and plasmids. The recipient in the transformations used to isolate and study the *cdc8* gene was strain CLK6 (*ura3 trp1 cdc8-1*). This strain was constructed by mating strain 198 (*MATa cdc8-1*) with

strain SRGO5-1 (*MATα trp1-1 met8-1 ile-1 ilv-2*) from Steve Reed (University of California at Santa Barbara). After *MATα trp1 cdc8-1* spores were identified, they were crossed with strain SS111 (*MATa trp1-289 ura3-1 ura3-2 his3-532 ade2-10 gal2*) from Stewart Scherer, California Institute of Technology, Pasadena. Strain 198 was provided by L. H. Hartwell. S288C (wild type) was the source of DNA for the construction of the yeast DNA library. *E. coli* MC1061 F^- λ^- *araD139* Δ(*ara-leu*)*7697 lacX74 galU galK hsdR hsdM strA* was used to propagate the library. Plasmid YCp50, a gift of Stewart Scherer, is shown in Fig. 1 and described in Results.

Medium. Yeast extract-peptone-dextrose (YPD) or synthetic minimal medium used for the culture of yeast cells is described in reference 25. *E. coli* cells were grown in L broth or M9 medium (19).

Nucleic acids. Plasmid DNAs from *E. coli* were prepared as described previously (6). Total yeast DNA was purified by minor modifications of the procedures of Sherman et al. (25). Small-scale and rapid isolation of plasmid DNAs from yeast were carried out as described by Nasmyth and Reed (21). *Bam*HI linkers and *Xho*I linkers were obtained from New England Biolabs. Deoxyribonucleoside triphosphates were obtained from P-L Biochemicals.

Enzymes. All restriction enzymes were obtained from New England Biolabs or Bethesda Research Laboratories and were used according to the supplier's instructions. T4 DNA ligase and calf alkaline phosphatase were generously provided by C. C. Richardson and N. D. Hershey. *Bal* 31 nuclease, T4 polynucleotide kinase, and *E. coli* Klenow fragment were obtained from Bethesda Research Laboratories. Conditions for enzymatic reactions are as described by Maniatis et al. (18).

Construction of a pool of yeast DNA sequences in the centromere-containing vector YCp50. A pool of YCp50 plasmids bearing yeast DNA fragments was constructed as follows. Purified yeast DNA from S288C was cleaved with three different concentrations of *Sau*3A (0.02, 0.06, and 0.09 U/μg of DNA) so that its average size was approximately 10 kilobases (kb). It was then pooled and fractionated on 10 to 40% sucrose density gradients as described by Maniatis et al. (18). Fragments between 5 and 20 kb were purified and then ligated to YCp50 DNA that had been digested with *Bam*HI and treated with calf alkaline phosphatase. The DNA concentrations in the ligase reaction were 50 and 10 μg/ml. After incubation for 15 h at 14°C, the ligation reaction mixture was used to transform *E. coli* strain MC1061 to ampicillin resistance by the procedures described by Dagert and Ehrlich (5). The 0.1-ml ligation mixture produced 7.9×10^4 Amp^r colonies, of which 75% were Tet^s, indicating that 75% of the transformants contained inserts or deletions at the *Bam*HI site. The transformant colonies were scraped from the ampicillin plates, and the cells were pooled and collected by centrifugation and stored as described by Nasmyth and Reed (21).

Another recombinant plasmid pool containing yeast DNA sequences in plasmid YRp7 (21) was kindly supplied by Steven Reed and was also used in this study. YRp7 contains yeast *ARS1 TRP1* sequences but no centromere.

Yeast transformation. DNA transformation of lithium acetate-treated yeast cells, first described by Ito et al. (11), was used in this study. Strains were grown to an absorbance at 590 nm of 1 to 2 in YPD medium. Cells were harvested by centrifugation at 8,000 rpm (Sorvall RC5b centrifuge) for 6 min, washed once in water, and again collected by centrifugation. The pellet was suspended in 0.1 M lithium acetate (0.2 original volume) and incubated at 30°C for 30 min. The cells were collected by centrifugation and resuspended in 0.1 M lithium acetate (0.01 original volume). Cells were divided into 0.05-ml aliquots and DNA was added. After 30 min at 30°C, 0.6 ml of polyethylene glycol 4000 (Sigma Chemical Co.) in 0.01 M Tris-hydrochloride (pH 7.5) was added. Incubation was continued for 60 min at 30°C followed by 5 min at 42°C. Each 0.6-ml mixture was divided and spread on three petri plates containing the appropriate selective media. The hybrid molecules containing *ARS1* and *CEN4* transformed yeast at high frequency (4,000 to 5,000 transformants per μg of DNA), and the resulting transformants were highly stable.

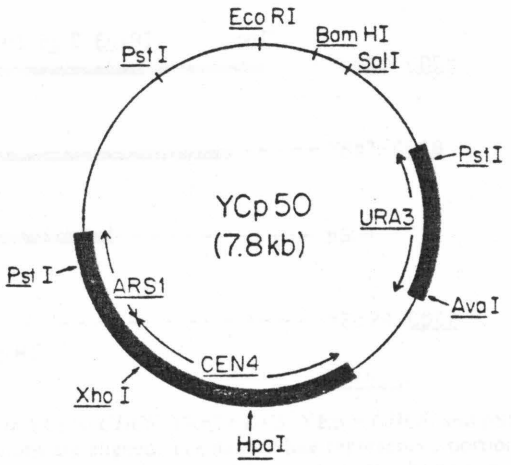

FIG. 1. Restriction map of the centromere-containing plasmid YCp50. The thin lines represent pBR322 DNA sequences; the thick lines represent yeast DNA sequences.

Localization of the minimal *cdc8*-complementing DNA fragment. The minimum sequence that complemented the *cdc8* mutation was identified by using *Bal* 31 deletion analysis of the originally isolated plasmids. Plasmid DNA (3 μg) containing the *cdc8* insert (Fig. 2) was cut with *Bam*HI restriction enzyme and then treated with 0.48 U of *Bal* 31 nuclease. After 2, 3, 4, 5, and 7 min, aliquots were removed, combined, and extracted with phenol. The DNA was incubated with the large fragment of *E. coli* DNA polymerase I and deoxyribonucleoside triphosphates to ensure that the ends were fully base paired. *Bam*HI linkers were then phosphorylated, using polynucleotide kinase, and phosphorylated linkers were incubated with the *Bal* 31-digested fragments in the presence of T4 DNA ligase. The respective DNA concentrations were 20 and 50 μg/ml. After ligation, a 10-fold excess of *Bam*HI restriction enzyme was added to cleave the linkers. The DNA fragments with *Bam*HI sticky ends were separated from the free linkers by gel filtration as described in reference 18. Ring closure was carried out

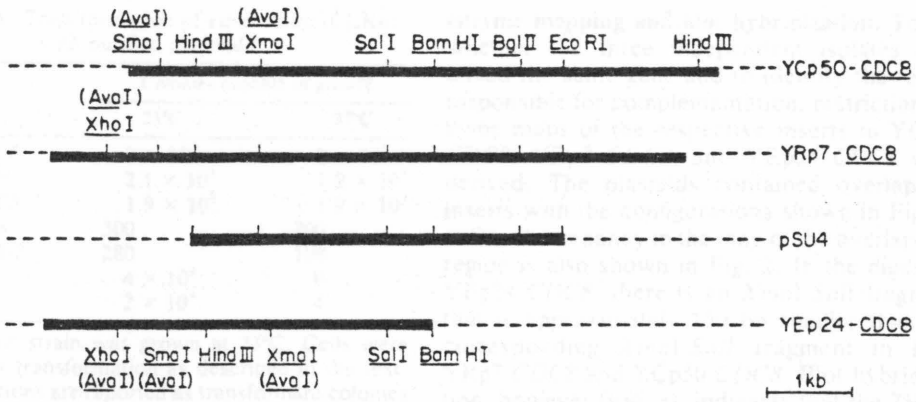

FIG. 2. Restriction map of *CDC8*-containing inserts in YCp50 *CDC8*, YRp7 *CDC8*, YEp24 *CDC8*, and pSU4. The four inserts are drawn so that their overlapping regions are aligned. The dashed line represents a portion of vector sequences. The sizes of the various restriction fragments were estimated from gel electrophoresis. pSU4 was originally constructed by ligating a yeast *Eco*RI-*Hin*dIII fragment containing the *SUP4* gene into plasmid pBR322 (7). The single *Xma*I site on pSU4 is located near the 3′ end of the cloned $tRNA^{Tyr}$ gene.

in the presence of T4 DNA ligase, and the ligation mixture was used for transformation of *E. coli* cells. Individual transformants were grown in 5-ml cultures, DNA was prepared, and the structure of the resultant plasmids was examined by gel electrophoresis after treatment with appropriate restriction enzymes. Plasmids containing deletions ranging from 50 to 500 base pairs (bp) were chosen for complementation testing by transformation of yeast.

DNA of the plasmid containing the 255-bp deletion (YpCLK255) was purified, and a second round of *Bal* 31 digestion was carried out from the opposite side of the *CDC8* fragment. YpCLK255 was digested with *Xho*I. *Bal* 31 digestions were carried out, and *Xho*I linkers were joined to the deleted DNAs. These deletions were religated and analyzed as above. The smallest insert that complemented *cdc8* was approximately 0.75 kb. The final plasmid is called YpCLK1.

Other procedures. Procedures for nick translation, gel electrophoresis, and blot hybridization were those described by Maniatis et al. (18).

RESULTS

Construction of a yeast DNA library in a centromere-containing vector. The vector chosen for the construction of the yeast library was the hybrid plasmid YCp50 (Fig. 1). YCp50 contains the replication origin of pBR322 and the genes for Amp^r and Tet^r. Insertion into the *Bam*HI site of YCp50 renders the plasmid Tet^s. YCp50 also contains a yeast replicator, *ARS1*, and a marker selectable in yeast, *URA3*. Finally, the plasmid contains the 1.8-kb yeast *CEN4* fragment that encompasses the centromere of chromosome IV. We chose to use the centromere-containing plasmid since the transformation frequencies and stability of transformants obtained are higher than with *ARS*- or 2-μm-containing vectors. This has the important consequence that each of the multiple transformations of yeast required for isolating and characterizing genes is accelerated by at least a day. Furthermore, at the outset, we did not know whether the *CDC8* gene would be lethal in high gene dosage. YCp50 has a copy number of 1.

The library was constructed by a minor modification of the procedure of Nasmyth and Reed (21), as described under Materials and Methods. Since the pool contained at least 1.7×10^4 recombinant clones, there are approximately six yeast genomes in this library.

Isolation of *CDC8*-containing plasmids. When used to transform CLK6 *ura3 cdc8*, DNA prepared from the yeast YCp50 hybrid pool yielded about 10^3 URA^+ transformants per μg, of which 0.04% were able to grow at the nonpermissive temperature (37°C). DNA was prepared from four of the yeast URA^+ CDC^+ transformants and introduced into *E. coli* by transformation to ampicillin resistance. Plasmid DNA was prepared from Amp^r transformants and analyzed by digestion with restriction endonuclease followed by agarose gel electrophoresis. Since the four plasmids all had identical restriction enzyme maps, only one, YCp50 *CDC8*, was chosen for further study.

In a second set of experiments, DNA prepared from the yeast YRp7 hybrid pool described previously by Nasmyth and Reed (21) was used to transform CLK6 *trp1 cdc8*. Five interesting TRP^+ CDC^+ transformants were isolated. Restriction enzyme mapping indicated that three of these were identical and homologous with the YCp50 clones (see Fig. 2), and they were therefore designated YRp7 *CDC8*. Two others, discussed separately below, contained two different inserts and were not homologous to the YCp50 insert.

Finally, L. H. Hartwell had independently

TABLE 1. Transformation of *cdc8* strain (CLK6) with purified plasmids[a]

Plasmid	Colonies capable of growth	
	23°C	37°C
YCp50 *CDC8*	2×10^3	2×10^3
YRp7 *CDC8*	2.1×10^3	1.9×10^3
YEp24 *CDC8*	1.9×10^3	1.9×10^3
YRp7 *SOC8-1*	300	200
YRp7 *SOC8-2*	280	198
YCp50	4×10^3	0
YRp7	2×10^3	4

[a] The *cdc8* strain was grown at 23°C. Cells were prepared for transformation as described in the text. Transformations are reported as transformant colonies per μg of transforming DNA.

isolated, from a yeast library of S288C DNA prepared in the vector YEp24 (2), two clones containing inserts that complement *cdc8* at 34°C (personal communication). These two plasmids, YEp24 *CDC8*, were identical to each other.

High-efficiency transformation of *cdc8* mutants with cloned plasmid DNAs; stability of transformants. Purified DNAs from plasmids YCp50 *CDC8*, YRp7 *CDC8*, and YEp24 *CDC8* were reintroduced into the CLK6 *cdc8* strain by transformation. In each case (Table 1), about 2×10^3 colonies per μg of transforming DNA were capable of growth at the restrictive temperature (selection for CDC$^-$ and URA$^-$ or TRP$^-$), consistent with complementation by autonomous replication rather than recombination. Similar results were obtained when selecting for URA$^-$ or TRP$^-$ at the permissive temperature (23°C).

Table 2 presents data obtained from experiments to determine the mitotic stability of transformants carrying the various plasmids isolated. As expected for a centromere vector (27), 98% of the transformants bearing *ARS1-CEN4* hybrid plasmids, YCp50 *CDC8*, maintained both URA$^-$ and CDC$^-$ phenotypes when grown under permissive conditions for 20 generations. However, only 15% of the cells transformed by YEp24 *CDC8*, a plasmid bearing the 2-μm origin of replication, retained both URA$^-$ and CDC$^-$ phenotypes after growing in rich media at room temperature for more than 10 generations. Unexpectedly, the *ARS1*-containing plasmid, YRp7 *CDC8*, gave rise to transformants that showed greater stability of both the TRP$^-$ and the CDC$^-$ properties than even the *CEN*-containing transformants. Since total yeast DNA made from TRP$^-$ CDC$^-$ yeast cells after growth under nonselective conditions yielded fewer than 10 Amp^r colonies when used to transform *E. coli*, the plasmid appears to have integrated into the chromosome in this particular transformant during growth under permissive conditions.

Characterization of cloned inserts by restriction enzyme mapping and blot hybridization. To see whether the three independent isolates contained the same gene and to identify the region responsible for complementation, restriction enzyme maps of the respective inserts in YCp50 *CDC8*, YRp7 *CDC8*, and YEp24 *CDC8* were derived. The plasmids contained overlapping inserts with the configurations shown in Fig. 2.

One discrepancy in the map of the overlapping region is also shown in Fig. 2. In the digest of YEp24 *CDC8*, there is an *Xma*I-*Sal*I fragment that is approximately 150 bp smaller than the corresponding *Xma*I-*Sal*I fragment in both YRp7 *CDC8* and YCp50 *CDC8*. Blot hybridization, however (Fig. 3), indicates that the 750-bp fragment shown below to contain the *CDC8* gene (Fig. 4) hybridized equally strongly to the *Xma*I-*Sal*I fragments from YEp24 *CDC8* and YRp7 *CDC8*, suggesting that the 150-bp difference in size in the YEp24 isolate was due to a deletion in an otherwise identical fragment. The region of the genome around *CDC8* shows a high frequency of sequence alterations when different strains are compared (3), and this may account for this polymorphism.

Linkage of the cloned genes to the *SUP4* locus. The apparent homology between the inserts isolated from YCp50, YRp7, and YEp24 libraries was not alone sufficient to prove that all three contained the *CDC8* gene. Since the *CDC8* gene has been shown to be closely linked to the *sup4* locus (16, 20), demonstration that the cloned gene came from the *sup4* region of chromosome X would constitute better proof that it was *CDC8*. To establish this linkage, use was made of the fact that the *SUP4* gene had been previously cloned and sequenced (7).

DNA from plasmid pSU4 (7), containing a functional *SUP4* gene, was digested with appro-

TABLE 2. Mitotic stability test of yeast transformants[a]

Plasmid	Transformed phenotype (%)	
	10 generations	20 generations
YCp50 *CDC8*	98	96
YRp7 *CDC8*	95	95
YEp24 *CDC8*	96	15
YRp7 *SOC8-1*	1	
YRp7 *SOC8-2*	2	

[a] Yeast transformants were grown in selective media and diluted approximately 1/1,000 (~10 generations) or $1/10^6$ (~20 generations) in either supplemented minimal media or rich media (YPD). After growth at room temperature, the mid-log-phase cultures were plated onto YPD plates, and the percentage of URA$^-$ CDC8$^-$ or TRP$^-$ CDC8$^-$ was determined by replica plating. The values given represent an average number of several independent determinations.

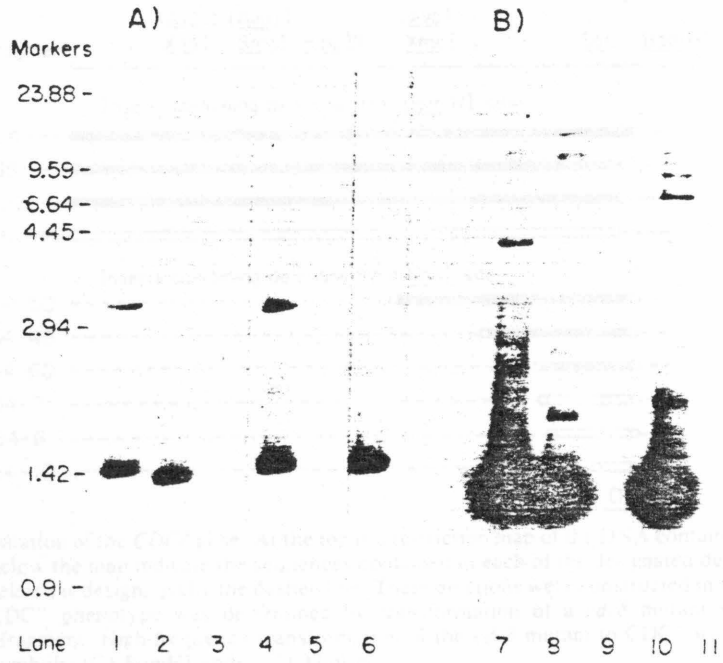

FIG. 3. Southern blot analysis of DNA sequence homology among the *CDC8*-related plasmids. The probe in (A) was the 750-bp *CDC8* fragment from YpCLK1 (Fig. 4), and the probe in (B) was the 1.17-kb *Aval* fragment shown in Fig. 2, to the left of the *Xmal-Sall* fragment. Each lane contains a different plasmid DNA that was transferred to a nitrocellulose filter after cleavage with restriction endonucleases *Sall* and *Aval* and electrophoresis on a 1% agarose gel. Lanes 1 and 7, YCp50 *CDC8*; lanes 2 and 8, YEp24 *CDC8*; lanes 3 and 9, YRp7 *SOC8*-1; lanes 4 and 10, YRp7 *CDC8*; lanes 5 and 11, YRp7 *SOC8*-2; lane 6, pSU4. Marker lengths are in kilobases.

priate restriction enzymes and analyzed by gel electrophoresis. The pSU4 plasmid contains an insert that overlaps the inserts in the *CDC8* plasmids by several kilobases (Fig. 2), raising the possibility that the putative *CDC8* clones also carried the *sup4* gene. The *SUP4* gene itself contains a unique *Xmal* recognition site (7), allowing us to infer the position of the *sup4* region on the *CDC8* clones. Comparison of restriction maps indicates that the *Xmal* site in the *sup4* gene forms one end of the common 1.5-kb *Xmal-Sall* fragment found in YCp50 and YRp7 derivatives and of the 1.35-kb fragment in YEp24 *CDC8*. Further support for the overlap of the *CDC8* and *SUP4* clones comes from blot hybridizations shown in Fig. 3. The 750-bp fragment from YpCLK1 (see Fig. 4) hybridized equally strongly to digests of the other *cdc8*-complementing plasmids and to the *SUP4* plasmid (Fig. 3). Previous mapping experiments show that there are no delta sequences on this probe (cf. Fig. 4 of reference 3). Similar results were obtained with the *Xmal-Sall* fragments from YEp24 *CDC8* or YCp50 *CDC8* as hybridization probes.

The mapping data were then confirmed by carrying out the converse experiment, namely, by showing that the originally isolated *SUP4* clone, pSU4, also contains the *CDC8* gene and is capable of complementing the *cdc8* mutation. Since the *SUP4* gene was on an integrating plasmid and since such vectors exhibit low transformation frequencies, it would be hard to distinguish complementation from reversion to temperature resistance with the original pSU4 plasmid. We therefore constructed a recombinant plasmid containing the *Bam*HI fragment of pSU4 (Fig. 5) inserted into the *Bam*HI site of YCp50. When the resulting plasmid was introduced into *cdc8* by transformation, an almost equal number of colonies was observed at 23 and 37°C. The efficiency of transformation with this plasmid was a little lower than that with the three plasmids described above and might be due to the presence of the mutant *SUP4* $tRNA^{Tyr}$ gene in the plasmid. We conclude that the cloned sequences shown in Fig. 2 are linked to the *SUP4* gene and probably carry the *CDC8* gene.

Minimal DNA fragment containing the *CDC8* gene. To identify the coding region for the *CDC8* gene, the DNA of the hybrid plasmids was subjected to deletion analysis in the regions considered likely, on the basis of the foregoing studies, to contain the gene. The sites chosen for *Bal* 31 deletion analysis and the final construc-

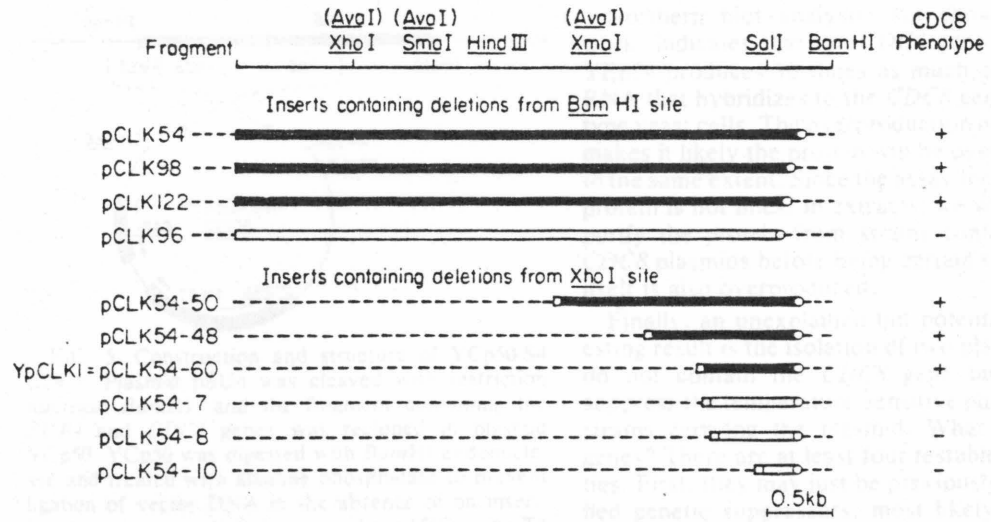

FIG. 4. Delimitation of the *CDC8* gene. At the top is a restriction map of the DNA containing the *CDC8* gene. The thick lines below the map indicate the sequences contained in each of the designated deletion mutants. The region of DNA deleted is designated by the dashed line. These deletions were constructed in vitro as described in the text. The CDC$^+$ phenotype was determined by transformation of a *cdc8* mutant with plasmid DNA containing each fragment. High-frequency transformation of the *cdc8* mutant to CDC$^+$ was indicative of *cdc8* gene function. Symbols: (○) *Bam*HI ends; (□) *Xho*I ends.

tion of a plasmid containing the minimal complementing sequence are described in Materials and Methods and summarized in Fig. 4.

The deletion analysis reveals that the smallest fragment capable of transforming the *cdc8* mutant to temperature resistance at high frequency is 750 bp long. This fragment contains no delta sequences and hybridizes to the *Xma*I-*Sal*I fragment from all of the libraries. This region lies <1 kb away from the *SUP4* gene. The close apposition of the two genes accounts for the suppression of meiotic recombination frequency reported between the two genes (16, 20). The transformation frequencies for a number of the deletion mutant plasmids was determined (Table 3). Since the high frequency of transformation observed is typical of efficiencies obtained by complementation rather than recombination, these data suggest that the small fragment is producing a functional *CDC8* protein or fragment thereof.

Detection of two additional genes that compensate for the *cdc8-1* mutation. Two of the plasmids detected in the YRp7 library contain genes that complement *cdc8-1*, but that do not contain any sequence homology with the cloned *CDC8* gene described in the preceding paragraphs. First, restriction enzyme mapping using both 4- and 6-bp recognition enzymes shows that the two plasmids isolated from the YRp7 library have a completely different restriction map from the plasmids discussed thus far and from each other (data not shown). Furthermore, no homology between these two plasmids and the 750-bp *CDC8*-containing fragment could be detected by blot hybridization (Fig. 3, lanes 3, 5, 9, and 11). Based on the restriction enzyme mapping, we conclude that these plasmids give high-frequency transformation of the *cdc8* mutants at the restrictive temperature by providing either a different protein that compensates for a deficiency in CDC8 protein or a suppressor of the missense mutation in *cdc8-1*. We have designated the genes responsible for this behavior *SOC8-1* and *SOC8-2*, suppressors of *cdc8*. These plasmids transform at a lower efficiency than the *CDC8*-containing plasmids (see Table 1), although still at a frequency 200 to 300 times greater than an integrating plasmid. Further investigations are essential to determine how they are compensating for the defect in *CDC8* (see Discussion).

DISCUSSION

We have cloned a gene that complements *cdc8* mutants and that is physically linked to the *SUP4* locus. The map position of the cloned gene confirms its identity with the *CDC8* gene. The library we have described here should also be useful in cloning genes that would be lethal in high dosage; high-frequency transformation is achieved with the centromere vector, but the intracellular copy number is 1.

An interesting finding is that the minimum fragment capable of complementing the *cdc8* mutation is 750 bp long. Since plasmids containing this small insert were able to transform with the same high frequency as plasmids containing

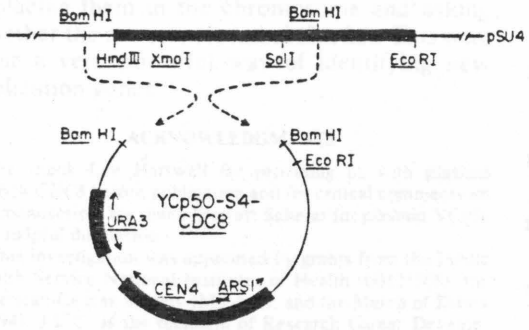

FIG. 5. Construction and structure of YCp50-S4 *CDC8*. Plasmid pSU4 was cleaved with restriction nuclease *Bam*HI, and the fragment containing the *SUP4* and *CDC8* genes was recloned in plasmid YCp50. YCp50 was digested with *Bam*HI endonuclease and treated with alkaline phosphatase to prevent ligation of vector DNA in the absence of an insert. Ligations were carried out overnight at 15°C, using T4 DNA ligase; the reaction mixture contained 1 μg of vector DNA and 0.5 μg of pSU4 in 50 μl of 66 mM Tris-hydrochloride (pH 7.6)–66 mM $MgCl_2$–10 mM dithiothreitol–1 mM ATP. The resulting plasmid, YCp50-S4 *CDC8*, was shown to contain two *Bam*HI sites, and the orientation of the fragment inserted was determined by restriction endonuclease digestion analysis.

large segments of flanking DNAs, it appears likely that this fragment contains the complete *CDC8* gene. However, such a coding region would normally only give a 27,000-dalton protein. Using complementation assays, others have purified the CDC8 protein to homogeneity and found a monomeric molecular weight of 34,000 to 40,000 (1). It is therefore possible that we have identified a fragment of the CDC8 protein that is active either in itself or in complementing the temperature-sensitive protein in the mutant. (The possibility that the small fragment of the gene is giving transformants at 37°C by virtue of recombination between the plasmid and the mutant gene rather than complementation is made unlikely by the high frequency of transformation and by the facts that cells that have segregated out the URA^- phenotype after growth on rich medium are once again temperature sensitive and that this segregation occurs with the frequency characteristic of autonomous plasmid loss rather than loss of integrated plasmids.) Nucleotide sequencing of the small fragment and purification of the overproduced *CDC8* gene product from cells containing the *CDC8* plasmid should resolve these questions. If, indeed, we have only identified an active fragment, this may be useful in determining whether the CDC8 protein can be described in terms of separate domains of activity, as is true of other single-stranded DNA binding proteins (14).

Northern blot analysis, not shown in this work, indicates that the *CDC8* gene cloned in YEp24 produces 10 times as much of a 0.9-kb RNA that hybridizes to the *CDC8* gene as wild-type yeast cells. The overproduction of the RNA makes it likely the protein will be overproduced to the same extent. Since the assay for the CDC8 protein is not linear in extracts, we will have to purify the protein from strains containing the *CDC8* plasmids before being certain the protein itself is also overproduced.

Finally, an unexplained but potentially interesting result is the isolation of two plasmids that do not contain the *CDC8* gene but that do suppress the temperature-sensitive phenotype in strains carrying the plasmid. What are these genes? There are at least four testable possibilities. First, they may just be previously unidentified genetic suppressors, most likely missense suppressors. Second, and more interesting, they may encode proteins that can bypass the need for the CDC8 protein. The CDC8 protein may be a single-stranded DNA binding protein (1), and perhaps there is a second such protein in yeast that can substitute for the CDC8 protein. We have identified an independent replication mutant that is deficient in a second single-stranded DNA binding protein that might serve such a function (C. L. Kuo, N. K. Huang, and J. L. Campbell, unpublished data). Third, they might encode proteins that are positively regulated by *CDC8* function. Finally, the most interesting possibility is that these sequences may represent genes for other replication proteins that interact with the CDC8 protein, and overproduction of the protein might compensate for an interaction weakened by the *cdc8* mutation. It is likely that the CDC8 protein does interact with other proteins since it has been shown in vitro to stimulate DNA polymerase I of yeasts (1). Such genes should be required for viability and this can be tested by creating deletion mutants in vitro.

TABLE 3. Frequency of transformation of *cdc8*(Ts) by hybrid plasmid DNA[a]

Plasmid	Insert size (kb)	Colonies/μg of DNA at 37°C
pCLK54	4.25	2.9×10^3
pCLK122	4.17	2.8×10^3
pCLK54-48	1.16	3.1×10^3
pCLK54-60	0.75	3×10^3
pCLK54-42	0.62	5
pCLK54-10	0.28	6

[a] The *cdc8*(Ts) strain, CLK6, was used for yeast transformation. Before selection for colonies capable of growth at the restrictive temperature, the transformed cells were allowed to grow for 3 h at 23°C on YPD plates before placing them at the restrictive temperature.

replacing them in the chromosome and asking whether the mutation is lethal (24, 26). This may offer a very powerful way of identifying new replication genes.

ACKNOWLEDGMENTS

We thank Lee Hartwell for providing us with plasmid YEp24-*CDC8* before publication and for critical comments on the manuscript. We thank Stewart Scherer for plasmid YCp50 and helpful discussion.

This investigation was supported by grants from the Public Health Service National Institutes of Health (GM25508), the American Cancer Society (MV-142), and the March of Dimes (1-794). J.L.C. is the recipient of Research Career Development Award CA00544.

ADDENDUM IN PROOF

The length of the RNA encoded by the *CDC8* gene is 0.9 kb. Furthermore, recent DNA sequencing in this laboratory indicates that the protein encoded by the *CDC8* gene is 216 amino acids in length. The molecular weight of the protein encoded by this sequence is 24,792. Taken together with the size of the segment of DNA that gives complete complementation of the *cdc8* mutation, these data make it unlikely that the CDC8 protein would have a molecular weight of 37,000, as previously estimated on the basis of mobility in SDS gels (1). The discrepancy between the molecular weight determined by DNA sequencing and that determined by analysis of the protein (1) could arise if the mobility of the protein is anomalous or if the molecular weight analysis of the protein is inaccurate for some other reason.

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Chapter 4

The *CDC8* Gene of Yeast Encodes Thymidylate Kinase

J. Biol. Chem. (1984), in press.

ABSTRACT

Thymidylate kinase catalyzes the phosphorylation of thymidine-5'-monophosphate to thymidine-5'-diphosphate in the pathway of synthesis of dTTP from dTMP. We have purified the enzyme approximately 5000-fold from a strain of the yeast Saccharomyces cerevisiae that overproduces the activity 6-fold. The protein appears homogeneous by SDS-polyacrylamide gel analysis and has a molecular weight of 25,000. The amino acid composition and the sequence of amino acids on the NH_2-terminus have been determined.

Our interest in thymidylate kinase stems from the fact that Sclafani and Fangman[1] recently presented genetic evidence that this enzyme is encoded by the CDC8 gene of yeast. In this paper we show by several biochemical criteria, thymidylate kinase is the product of the CDC8 gene. First, extracts of strains bearing six different alleles of cdc8 showed no thymidylate kinase activity. Secondly, strains carrying the CDC8 gene on a high copy number plasmid produce 6-fold higher levels of the kinase activity than does wild-type. Third, the DNA sequence of the CDC8 gene reveals an open reading frame that encodes a protein with the same amino terminal sequence as purified thymidylate kinase.

INTRODUCTION

cdc8 mutants of yeast have defects in cell division and in DNA replication. Temperature sensitive *cdc8* mutants shut off nuclear, mitochondrial and 2um DNA synthesis immediately when shifted to the restrictive temperature (Hereford and Hartwell, 1971, Newlon and Fangman, 1975, Livingston and Kupfer, 1977). DNA molecules isolated from *cdc8* cells at 36°C contain replication bubbles less than 3 um in size. The small size of the replication bubbles reflects a reduced rate of chain elongation (less than 1% that of the normal rate (Petes and Newlon, 1974)).

In order to gain an understanding of the role of the *CDC8* protein in replication, we have taken two approaches. First, we have partially purified the *CDC8* protein using *in vitro* complementation in Brij-treated cells (Kuo and Campbell, 1982). However, we were not able to assign a catalytic activity to this protein. We have also cloned the *CDC8* gene by complementation of the temperature sensitive defect *in vivo* (Kuo and Campbell, 1983) in order to overproduce the protein to aid in its purification and identification.

The first insight into the role of the *CDC8* came when Sclafani and Fangman showed that the thymidine kinase (TK) gene of the Herpes Simplex Virus (HSV), when introduced into *cdc8* mutants of yeast fully complemented the *cdc8* defect.[1] This was a surprising finding, since yeast has no thymidine kinase activity of its own and since the *CDC8* protein did not appear to be required for precursor synthesis. *cdc8* mutants exhibit temperature sensitive synthesis in permeabilized yeast cells,

where the precursors of synthesis are provided. It therefore seemed that some other activity associated with the HSV TK gene must be responsible for the complementation. Herpes thymidine kinase has previously been shown to have two additional catalytic activities associated with it, thymidylate kinase and deoxycytidine kinase (Chen and Prusoff, 1978). Sclafani and Fangman have taken a genetic approach and we present here a biochemical approach, both of which show that the product of the yeast CDC8 gene is thymidylate kinase. In this paper we report the purification of the thymidylate kinase activity to homogeneity and physical characterization of the protein. The DNA sequence of the CDC8 gene is also reported and comparison of DNA sequence and amino acid sequence of the purified protein conclusively demonstrates that the CDC8 protein is thymidylate kinase.

MATERIALS AND METHODS

Yeast Strains and Media-- Strains used in these studies are described in Table 1. Strain C6b was obtained by crossing SS111 to strain 198-1 cdc8-1. C6B was transformed by various plasmid vectors carrying the CDC8 gene: C6B/YEp24-CDC8, C6B/YRp7-CDC8, C6B/YCp50-CDC8, C6B/CLK-60, C6B/CLK-8 (Kuo and Campbell, 1983). YPD is complex medium for routine growth and contains 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose. SD is a synthetic minimal medium containing 0.67% Bacto-yeast nitrogen base without amino acids and 2% dextrose. Various constituents are added as required by the various strains for growth.

Chemicals -- ATP and dTMP were from P-L Biochemicals, [^{3}H-5-methyl]dTMP was from Amersham. PEI-cellulose plates were obtained from Brinkmann Instruments, Inc. Acrylamide and bisacrylamide were from Scientific Chemical Company, Inc. DE-52 was from Whatman. Hydroxylapatite was from Bio-Rad. Phenyl-Sepharose and Sephadex G-50 Superfine were purchased from Pharmacia Fine Chemicals. Zymolyase 60,000 was from Seikagaku Kogyo Co., LTD. (Japan).

Thymidylate Kinase Assay -- The reaction mixture (50 ul) contained: 75 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 7.5 mM ATP, and 0.5 mM [^{3}H]dTMP (40-60 cpm/pmole). The reaction was initiated by the addition of enzyme. After incubation at 37^{o}C for 30 min, a sample of the reaction mixture was spotted on a PEI-cellulose plate and the plate developed with 1 M formic acid, 0.8 M LiCl. Radioactivity was determined by placing fractions in vials and counting in the Beckman LS-230 scintillation counter. Under

standard conditions, the thymidylate kinase activity is proportional to the amount of protein added between 2.5 to 50 ug. The reaction rate is linear for at least 90 min at 37°C. The thymidylate kinase activity in crude extracts was stable for one month at -70°C. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of one nmole of dTDP at 37°C for 30 min.

Determination of Amino acid Composition and N-terminal Amino Acid Sequence -- Enzyme, purified through the HPLC step (Fraction VIa, Table IV), was hydrolyzed in 6N HCl for 22 hours at 110°C according to Moore and Stein (1954). The amino acid composition was determined using a Durrum Model D500 automatic amino acid analyzer. Acid-stable amino acids were determined with an HP1000 computer.

Purified thymidylate kinase (5 pmoles of Fraction VIa) was used for repetitive cycles of Edman degradation in the automatic gas-phase microsequenator (Hunkapiller, et. al., 1983; Hewick, et. al., 1981). The phenylthiohydantoin amino acids formed in the gas-phase sequenator were analyzed by HPLC as previously described (Hunkapiller and Hood, 1983).

Polyacrylamide Gel Electrophoresis -- The procedure for polyacrylamide gel electrophoresis was similar to the method of Weber and Osborn, (1969). Samples were run on 12.5% or 15% acrylamide, 0.6% methylene bisacrylamide slab gel (9 x 9cm) at 20 mA for 1 hr. Gels were stained by the method of Merril et al., 1981.

Other Methods -- Glycerol gradient sedimentation was performed as previously described (Kuo and Campbell, 1982),

except that 10-40% linear gradients were used. Protein concentration was determined by the Bio-Rad Protein Assay Kit based on the method of Bradford (1976).

RESULTS

dTMP Kinase Activity in Extracts of Yeast -- Sclafani and Fangman[1] recently showed that Herpes Simplex Virus thymidine kinase could complement *cdc8* mutants *in vivo*, suggesting that the *CDC8* gene product was either thymidine kinase or thymidylate kinase or deoxycytidine kinase, two other activities associated with the viral protein. They suggested that *CDC8* encoded thymidylate kinase, since yeast does not have thymidine kinase. In order to determine if the temperature sensitive, cell division cycle mutant, *cdc8*, was deficient in thymidylate kinase activity, an assay for the enzyme in crude extracts was developed (see Table II). (After we completed our assays, we learned that Sclafani and Fangman had obtained identical results independently).[1] Under standard assay conditions, the specific activity of thymidylate kinase in crude extracts of three different strains (PEP4, A364a, SS111) carrying the wild-type *CDC8* allele was determined. These values represent the normal amount of thymidylate kinase in yeast cells.

Six mutants, each containing a different mutant allele of *cdc8*, were then assayed for thymidylate kinase activity in crude extracts. Mutant cells were grown at non-restrictive temperature (23°C) in YPD medium. Thymidylate kinase assays were conducted at both the restrictive (37°C) and non-restrictive temperature. None of the mutants showed any enzyme activity at any temperature (Table II). The absence of thymidylate kinase in these mutants suggested that *CDC8* was either the structural gene for the enzyme, or that the *CDC8* gene regulated thymidylate kinase function or expression in some way. Mixing wild-type extracts

and cdc8 did not lead to inhibition of activity, making it unlikely that there was a soluble inhibitor in the cdc8 extract. Levels of deoxycytidine kinase, the other activity associated with Herpes TK, were normal in all strains.

The Level of Thymidylate Kinase in Strains Transformed with Plasmids Carrying the CDC8 Gene -- In order to investigate whether thymidylate kinase was encoded by CDC8, we made use of the fact that the CDC8 gene has been cloned in plasmids YEp24, YRp7 and YCp50 (Kuo and Campbell, 1983). The recombinant plasmids were capable of transforming the cdc8-1 mutant (C6b) to temperature resistance at high frequency (Kuo and Campbell, 1983). These transformants, designated C6B/YEp24-CDC8, C6B/YRp7-CDC8 and C6B/YCp50-CDC8, were grown in both rich medium (YPD) and defined media (SD-Ura or SD-Trp, depending on the marker on the plasmid). After growth at 30°C, samples of the culture were plated to examine the stability of plasmids. More than 98% of cells grown in either YPD or in selective media contained plasmid, indicating that the selection at 30 °C is sufficient to maintain the plasmids. (The YRp7-CDC8 plasmid was in the integrated form). Extracts were prepared from strains carrying each of the plasmids and assayed as described under Methods.

As shown in Table III, both C6B/YRp7-CDC8 and C6B/YCp50-CDC8 had the level of thymidylate kinase activity observed in wild-type cells. C6B/YEp24-CDC8 had a six-fold higher activity than any other cell (Tables II and III). These results are consistent with blot hybridization studies that indicate that C6B/YRp7-CDC8 and C6B/YCp50-CDC8 possessed an amount of CDC8 mRNA equivalent to

wild-type cells, suggesting an average of one copy of the gene per transformed cell. C6B/YEp24-CDC8, which has a copy number of 50-100 per cell, showed 10-20 times more CDC8 mRNA than wild type (data not shown) and a six-fold increase in kinase activity. Thus, the thymidylate kinase activity is proportional to the CDC8 mRNA levels in various transformed cells.

Previous deletion analysis of the cloned CDC8 gene revealed that the smallest fragment capable of transforming the cdc8 mutant to temperature resistance at high frequency is a 750 bp segment carried on plasmid pCLK-60, (Kuo and Campbell, 1983). Plasmid pCLK-8 contains an insert with 12bp deleted from the N-terminus of the functional CDC8 gene, and does not transform cdc8 to temperature resistance. Strain C6B/pCLK-60 showed normal levels of thymidylate kinase activity when grown at 37°C. Furthermore, the enzyme activity decreased to one-sixth of the normal level when cells were grown in YPD medium at room temperature, presumably due to the loss of plasmid under non-selective conditions. Interestingly, a low level of enzyme activity was detectable in C6B/pCLK-8, even though this strain did not contain the complementing form of the CDC8 gene. Since cdc8 mutants did not have any detectable thymidylate kinase activity, the enzyme activity found in the transformants could be due to increased amounts of mRNA or protein derived from the plasmid pCLK-8. In summary, the fact that the plasmid-containing cells contain thymidylate kinase in proportion to the gene dosage strongly suggests that the CDC8 product is thymidylate kinase. In order to establish this point we have taken the biochemical approach described in the following paragraphs.

PURIFICATION AND CHARACTERIZATION OF THYMIDYLATE KINASE

Purification Procedure for Thymidylate Kinase-- The strain used for the purification of thymidylate kinase was the overproducing, plasmid-carrying strain C6B/YEp24-CDC8 (Kuo and Campbell, 1983). This strain is a cdc8-1 mutant carrying plasmid YEp24 into which the wild-type CDC8 gene has been inserted. The level of thymidylate kinase activity in this strain was 6-10 fold as high as in the wild type (Tables II and III). This amplification of the level of thymidylate kinase enabled us to develop an efficient purification protocol.

The results of a typical purification are summarized in Table IV, and the details of the purification procedure are provided in the Miniprint Supplement. The protein is apparently homogeneous, as shown by polyacrylamide gel analysis (Fig. 1).

Molecular Weight Determination -- The molecular weight of the purified enzyme was estimated by glycerol gradient centrifugation and sodium dodecyl sulfate-PAGE methods. An estimate from glycerol gradient centrifugation suggested a native molecular weight for thymidylate kinase of 24,000 to 26,000 (Fig. 2a). For the purified and denatured enzyme, a molecular weight of 25,000 is estimated by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 2b). Taken together, these results suggest that thymidylate kinase is composed of one polypeptide chain with an apparent M_r of 25,000. This value is essentially the same as the one deduced from the CDC8 DNA sequence (see

below).

Amino Acid Composition -- The amino acid composition was determined by acid hydrolysis of the purified enzyme. The molar ratio of amino acids was very similar to that predicted from CDC8 DNA sequence except for two residues. The slight deviation of valine and isoleucine, for instance, could have arisen from incorrect accounting for the acid labile amino acids or from a contaminant in the enzyme fraction.

N-terminal Amino Acid Sequence -- The N-terminal amino acid residues of purified thymidylate kinase were determined by the Automated Microsequencing method (Hunkapiller and Hood, 1983). Eighteen residues were obtained as follows:

N-(R1)-(R2)-(R3)-Arg-Gly-Lys-Leu-Ile-Leu-Ile-Glu-
Gly-Leu-Asp-Arg-Thr-Gly-(R18)-Thr-Thr-Gln-Cys- - -.

The first three residues were obscured by the sequencing analysis due to high background in the early cycles of the automated process. The sequencable protein, i.e., protein with unblocked -amino group, starts at the fourth residue of the open reading frame and continues for eighteen amino acids. Only the eighteenth residue is ambiguous. The sequence from the fourth to the twenty-second residues of thymidylate kinase is the same as that deduced from CDC8 DNA sequence (see below). Thus, the result strongly suggests that thymidylate kinase is the gene product of CDC8, and also confirms that the reading frame of CDC8 gene in Table VI is correct.

Determination of Nucleotide Sequence of the CDC8 gene. The nucleotide sequence of the gene was determined and is shown in Table VI. The open reading frame, 216 amino acids in length,

would be translated into a protein of molecular weight 24,792. The open reading frame is confirmed by the amino acid sequence data presented above.

COMPLEMENTATION OF BRIJ-TREATED CELLS WITH PURIFIED THYMIDYLATE KINASE

Previously, we and others have reported that cdc8 mutants show thermolabile DNA synthesis in Brij-treated in vitro replication systems (Hereford and Hartwell, 1971; Banks, 1973, Kuo and Campbell, 1982). Furthermore, we showed that a protein could be partially purified that complemented the defect in Brij/sucrose-treated cdc8 cells (Kuo and Campbell, 1982).

Availability of the purified CDC8 gene product, thymidylate kinase, allowed us to ask why Brij/sucrose-treated cdc8 cells showed thermolabile DNA synthesis, even though exogenous dTTP was provided and what the nature of the previously observed complementation was. DNA synthesis was carried out in Brij/sucrose-treated wild-type or cdc8 cells at 23 or 37°C (Table VII). In the first experiment, dTMP was substituted for dTTP and synthesis was measured in wild-type cells. dTMP is just as efficient a precursor as dTTP. This is different from the result obtained with cells treated only with Brij as described by Hereford and Hartwell (1971). Such cells do not use dTMP efficiently as a precursor. Secondly, we examined the ratio of activity at 37°C and 23°C in both wild type and the cdc8 mutant. The wild type shows a ratio of 1, while the mutant had a value of

0.35. However, if purified thymidylate kinase is added to the cdc8 cells at 37°C, synthesis is restored almost to wild-type levels.

Why do we see complementation? Added dTTP may be degraded to dTMP such that a dTTP regenerating system, analogous to an ATP-regenerating system, is necessary for optimal activity. In this case, the precursors may flow in and out of the Brij-treated cells, allowing complementation by an exogenously added protein. Another possibility is that thymidylate kinase may participate more directly in replication by interacting with the functional replication complex to funnel nucleotides directly into the replication fork (see Discussion).

DISCUSSION

We have purified the dTMP kinase activity of yeast to homogeneity. Our results indicate that wild-type cells contain 2500 to 3500 molecules of dTMP kinase per cell. It has been suggested that there are 400 replication origins on the yeast genome (Newlon and Burke, 1980, Chan and Tye, 1980). Therefore, there are about 6 to 9 molecules of thymidylate kinase per replication origin. The corresponding number reported for DNA polymerase I is 1500 molecules in cells grown under similar conditions (Badaracco et al., 1983).

We have also shown that thymidylate kinase is the product of the CDC8 gene of yeast. First, cdc8 mutants contain no detectable thymidylate kinase activity at either 23°C or 37°C. This observation is identical to that of two other elongation mutants, cdc9 (DNA ligase) and cdc21 (thymidylate synthetase) (Johnston and Nasmyth, 1978; Bisson and Thorner, 1977) that show a temperature-sensitive elongation phenotype *in vivo*, but contain no detectable activity in extracts. It may be that there is an efficient degradation pathway in yeast for proteins not seen as native. The fact that normal or even elevated thymidylate kinase levels can be restored to cdc8 strains when they are transformed with the wild-type gene and the fact that the level of enzyme is proportional to the gene dosage in the transformants provides strong evidence that the CDC8 product is thymidylate kinase. Final proof comes from the fact that the molecular weight, amino acid composition, and N-terminal amino acid sequence of

thymidylate kinase are identical to those deduced from the DNA sequence of the cloned CDC8 gene. Sclafani and Fangman[1] have provided strong genetic evidence to complement our findings.

The finding that the CDC8 protein is required for precursor synthesis is not consistent with many early observations regarding the behavior of cdc8 mutants in in vitro replication systems. Hereford and Hartwell (1971) and Oertel and Goulian (1979) showed that synthesis was defective at the non-permissive temperature in permeabilized cells in such mutants, even though the precursors of synthesis, including dTTP, were provided. Kuo and Campbell (1982) reported similar findings and even purified a protein that could restore activity in vitro. Furthermore, synthesis was reported to be thermolabile in three soluble in vitro replication systems prepared from cdc8 cells (Jazwinski and Edelman, 1979; Kojo et al., 1980; Celniker and Campbell, 1981).

Similar observations have been made in animal (Reddy and Pardee, 1980) and in prokaryotic systems. For T4-infected E.coli cells, it has been reported that the T4 DNA replication complex contains at least some of the enzymes needed for synthesis of deoxyribonucleoside triphosphates and that mutations in genes coding for these enzymes could directly affect DNA synthesis (Collinsworth and Mathews, 1974; Dicou and Cozzarelli, 1973; North, Stafford and Mathews, 1976; Wovcha, Tomich, Chiu and Greenberg, 1973). Cells infected with either gene 42 (dCMP hydroxymethylase) or gene 1 (deoxynucleoside monophosphate kinase) mutants are defective in DNA synthesis in plasmolysed cells even though nucleoside triphosphates are provided (Dicou

and Cozarelli, 1973; Collinsworth and Mathews, 1974; North, Stafford and Mathews, 1976; Wovcha, Tomich, Chiu and Greenberg, 1973). A possible explanation for these results, as for the results in the yeast system, is that the proteins involved not only catalyze the synthesis of precursors, but also interact with the replication fork and are required for proper propagation of the replisome. In support of this idea, precursor enzymes have been isolated as part of a large complex containing DNA polymerase, and dTMP supported greater rates of synthesis than dTTP. Another result similar to the cdc8 results is in the E.coli system. Ribonucleoside diphosphate reductase (ribonucleoside diphosphate reductase catalyzes the reduction of the four ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates and thus generates all precursors for DNA synthesis. In studying the ribonucleoside diphosphate reductase mutant (nrdA), Manwaring and Fuchs (1979) found that the cells exhibited a sharp decrease in the enzyme activity and the rate of DNA synthesis upon shifting to 42°C, while at the same time displaying little or no decrease in the deoxyribonucleoside triphosphate pools. The ribonucleoside diphosphate reductase, they proposed, may have a more direct effect on DNA synthesis as a part of DNA replication complex than just production of deoxyribonucleotides. It is conceivable that the enzymes involved in the provision of essential precursors for DNA synthesis in prokaryotes interact with the functional DNA "replisome." If it is also true for yeast cells, the CDC8 protein (thymidylate kinase) may also play a direct role in DNA synthesis.

In order to test the possibility that CDC8 protein, in addition to its thymidylate kinase activity, also plays a direct role in DNA synthesis, we studied DNA synthesis using the permeabilized cell system in the presence and absence of purified thymidylate kinase. First, it appears that both dTMP and dTTP can serve equally well as substrates. Secondly, the purified thymidylate kinase restores synthesis at the nonpermissive temperature in cdc8 mutants to normal levels. As stated in the text, this could be due to resynthesis of degraded dTTP or it could be due to an interaction of thymidylate kinase with the replication machinary, which would require that the Brij-treated cells be permeable to the protein. While the latter possibility is favored by Sclafani and Fangman, we have carried out no experiments to distinguish these two cases and merely point out the possibilities and the analogies to the phage system as interesting.

Acknowledgments

We wish to thank Dr. R. A. Sclafani, W. Fangman, and L.H. Hartwell, Department of Genetics, University of Washington, Seattle, Washington, for information prior to publication. We are very grateful to Dr. J. Newman for help with the HPLC, to Dr. S. Kent for amino acid sequence determination and Mr. V. Farnsworth for amino acid composition measurement at the Biology Department of Caltech, Pasadena. This work was supported by the American Cancer Society, grant no. MV-142A, March of Dimes #1-794, and Judith L. Campbell was a recipient of U.S. Public Health Service RCDA CA00544.

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TABLES

Table I *S. cerevisiae* strains used.

Strains	Genotype	Source
A364a	*a ade1 ura1 gal1 tyr1 his7 lys2*	L. H. Hartwell (1973)
SS111	*MATa trp1-289 ura3-2 his3-532 ade2-10 gal2*	S. Scherer
PEP4	*trp1 pep4-3 prb11-1122 prc11-126*	E. Jones
cdc8	derived from A364a	L. H. Hartwell (1973)
C6b	derived from cdc8-1 *ura3 trp1* cdc8-1	Kuo and Campbell (1983)

Table II. Thymidylate Kinase activity at 23°C and 37°C in normal and mutant cells.

Strain	Growth temp	Spec. act. (Units/mg) 23°C	37°C
PEP4	30°C	16.5	34.8
C6B/YEP24-CDC8	30°C	126.9	233.8
A364a	30°C	22.3	47.1
SS111	30°C	21.8	37.9
cdc2	23°C	9.7	40.1
cdc8-1	23°C	<0.01	<0.01
cdc8-2	23°C	<0.01	<0.01
cdc8-3	23°C	<0.01	<0.01
cdc8-4	23°C	<0.01	<0.01
cdc8-5	23°C	<0.01	<0.01
cdc8-6	23°C	<0.01	<0.01

Cells were grown in 20 ml of YPD media or selective media to $O.D._{590}$ = 5, and harvested by centrifugation at 5,000 rpm, for 5 min in a Sorvall SE34 rotor at 4°C. The cell pellets were washed once with chilled glass distilled water and resuspended in 2 ml of 0.1 M Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM PMSF. Cells were broken by the addition of 1.5 ml of chilled glass beads followed by vortexing for 30 sec, 6 times vigorously. The supernatants were collected after centrifugation at 5,000 rpm for 5 min in a Sorvall SE34 rotor. Residual debris was removed by another 10 min centrifugation by Eppendorf microfuge. Samples of the supernatant were used for the determination of thymidylate kinase

activity. The complete system for the dTMP kinase reaction contains 50 mM Tris, pH 8.0, 10 mM Mg^{++}, 7.5 mM ATP and 0.5 mM [^{3}H-methyl] dTMP (50 cpm/pmole). The reactions were performed as described in Methods. A unit of dTMP Kinase is defined as the amount of enzyme which phosphorylates one nmole of dTMP per 30 min at 37°C.

Table III. Recovery of Thymidylate Kinase Activity in the *cdc8*-1 mutant carrying the cloned *CDC8* gene on plasmids.

Strain	Growth medium	Growth temp	Spec. act. (Units/mg)
C6B	YPD	23°C	<.01
C6B/YEp24-*CDC8*	YPD	30°C	242.1
	SD-Ura	30°C	275.0
C6B/YRp7-*CDC8*	YPD	30°C	31.5
	SD-Trp	30°C	28.8
C6B/YCp50-*CDC8*	YPD	30°C	28.7
	SD-Ura	30°C	31.5
C6B/pCLK-60	YPD	37°C	27.2
C6B/pCLK-60	YPD	23°C	4.8
C6B/pCLK-8	YPD	23°C	2.4

Table IV. Purification of Thymidylate Kinase

Step	Fraction	Total protein (mg)	Units ($x10^5$)	Units/mg ($x10^3$)	Purity -fold	Yield %
I	Crude Extract	1911.3	2.92	0.15	1	100
II	Ammonium sulfate	403.7	2.55	0.63	4	87
III	Phenyl-Sepharose	67.7	2.15	3.17	21	74
IV	DEAE-Cellulose	6.6	1.76	26.79	176	60
V	Hydroxylapatite	0.7	0.70	108.01	708	24
VI	Sephadex G-50	0.08	0.60	734.23	4894	21

Table V. Amino Acid Composition of Thymidylate Kinase

Residues	Mole Percent	Purified* Thymidylate Kinase	Predicted from CDC8 DNA Sequence
Ala	4.7	9	7
Arg	4.7	9	8
Asp + Asn	14.0	28	29
Cys	0	N.D.**	3
Glu + Gln	12.4	25	24
Gly	8.0	16	14
His	2.0	4	3
Ile	6.2	12	16
Leu	12.5	25	25
Lys	12.0	24	21
Met	0	N.D.	5
Phe	4.7	9	10
Pro	1.3	3	5
Ser	6.7	13	11
Thr	7.0	14	13
Trp	0	N.D.	3
Tyr	0	N.D.	6
Val	3.8	8	13

*Based on 199 amino acid residues.

**No data.

Table VI

DNA sequencing was carried out by the method of Maxam and Gilbert (1980) on genes that had been subjected to deletion analysis in the regions considered, on the basis of the previous studies (Kuo and Campbell, 1983), to contain the complementing sequence. Five of the deletion derivatives with sizes ranging from 300bp to 1150bp were used in sequencing. All of them contained a single Xho I site and a single Bam HI site introduced after Bal31 deletion reaction at the ends of the cloned fragments. After Xho I or BamHI cleavage, the linear DNA was labeled at its 5' ends or at its 3' ends. The labeled DNA was then digested with two labeled fragments that could be separated on polyacrylamide gel. The separated, end-labeled fragment was then eluted from the sliced gel and sequenced by the published procedure (Maxam and Gilbert, 1980).

CDC 8 SEQUENCE

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1       MetMetGlyArgGlyLysLeuIleLeuIleGluGlyLeuAspArgThrGlyLysThrThr   20
415     ATGATGGGTCGTGGCAAATTAATACTGATAGAAGGATTGGACAGGACTGGTAAAACCACG   474

21      GlnCysAsnIleLeuTyrLysLysLeuGlnProAsnCysLysLeuLeuLysPheProGlu   40
475     CAATGTAATATTCTTTACAAAAAATTGCAACCAAACTGTAAACTATTGAAGTTCCCCGAA   534

41      ArgSerThrArgIleGlyGlyLeuIleAsnGluTyrLeuThrAspAspSerPheGlnLeu   60
535     AGGTCTACCCGAATCGGAGGACTCATAAACGAATATTTGACGGATGATAGTTTCCAATTA   594

61      SerAspGlnAlaIleHisLeuLeuPheSerAlaAsnArgTrpGluIleValAspLysIle   80
595     TCAGATCAGGCAATTCACCTCTTGTTTTCGGCAAATAGATGGGAAATAGTTGACAAGATA   654

81      LysLysAspLeuLeuGluGlyLysAsnIleValMetAspArgTyrValTyrSerGlyVal   100
655     AAGAAAGATTTACTAGAAGGGAAGAACATTGTCATGGACAGATATGTTTATTCTGGAGTG   714

101     AlaTyrSerAlaAlaLysGlyThrAsnGlyMetAspLeuAspTrpCysLeuGlnProAsp   120
715     GCATATTCTGCCGCTAAGGGGACAAATGGAATGGATTTGGATTGGTGCTTGCAACCCGAT   774

121     ValGlyLeuLeuLysProAspLeuThrLeuPheLeuSerThrGlnAspValAspAsnAsn   140
775     GTAGGGTTGCTGAAACCGGATTTGACATTATTTTTAAGCACTCAAGATGTCGACAATAAC   834

141     AlaGluLysSerGlyPheGlyAspGluArgTyrGluThrValLysPheGlnGluLysVal   160
835     GCTGAAAAAATCTGGATTTGGTGACGAAAGATACGAAACTGTCAAGTTTCAAGAAAAAGTG   894

161     LysGlnThrPheMetLysLeuLeuAspLysGluIleArgLysGlyAspGluSerIleThr   180
895     AAGCAAACTTTTATGAAGCTATTGGATAAAGAGATAAGGAAAGGCGATGAGTCAATCACG   954

181     IleValAspValThrAsnLysAspIleGlnGluValGluAlaLeuIleTrpGlnIleVal   200
955     ATTGTAGACGTTACTAATAAGGACATTCAGGAAGTTGAAGCGCTTATTTGGCAAATCGTT   1014

201     GluProValLeuSerThrHisIleAspHisAspLysPheSerPhePheTrm           217
1015    GAGCCTGTTTTGAGTACGCATATTGATCATGATAAATTTTCGTTCTTCTAGGA          1067
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TABLE VII

IN VITRO COMPLEMENTATION OF *cdc8-1* MUTANT
BY PURIFIED dTMP KINASE

STRAIN	dTMP INCORPORATED PER 10^8 CELLS					
	- dTMP KINASE			+ dTMP KINASE		
	23°C	37°C	$Q_{\frac{37}{23}}$	23°C	37°C	$Q_{\frac{37}{23}}$
WILDTYPE	PMOL	PMOL		PMOL	PMOL	
dTMP	3.42	3.98	1.16	3.60	4.02	1.11
dTTP	3.24	3.78	1.16	3.58	4.05	1.14
cdc8 MUTANT						
dTMP	3.55	1.25	0.35	3.52	3.60	1.02
dTTP	3.50	1.16	0.33	3.48	3.40	0.98

FIGURE LEGENDS

Figure 1. SDS-PAGE electrophoresis of different purification steps of thymidylate kinase.

Electrophoresis was performed as described in Materials and Methods. Each lane contains 5-7 g of protein. Lane I: Postmitochondrial fraction, Lane II: Ammonium sulfate fraction (dialyzed), Lane III: Phenyl-Sepharose column fraction, Lane IV: DE-52 Cellulose fraction, Lane V: Hydroxylapatite column fraction, Lane VIb: Sephadex G-50 Superfine column fraction. Lane STD: Standard proteins from Sigma Chemical Co. contains 3 g of mixture of Bovine albumin (66,000), Egg albumin (45,000), Glyceraldehyde-3-phosphate dehydrogenase (36,000), Carbonic anhydrase (29,000), Trypsinogen (24,000) and -Lactalbumin (14,200).

Figure 2a. Glycerol gradient sedimentation of thymidylate kinase.

Thymidylate kinase was applied to a 10 to 40% glycerol gradient containing 25 mM Tris, pH 7.6, 5 mM DTT, 50 mM NaCl. Sedimentation was performed in a Beckman SW50.1 rotor at 47,000 rpm at 2°C for 24 hr. The gradient is shown from bottom to top (40 to 10%) of the tube. 38 fractions were collected after centrifugation. Fraction number 22 (arrow in the plot) shows the peak of thymidylate kinase activity. Standards sedimented in parallel gradients are catalase (25,000), alcohol dehydrogenase,

(80,000), BSA (Bovine serum albumin, 66,200) and Cyto.C (Cytochrome C, 12,300).

Figure 2b. Molecular weight determination of thymidylate kinase by SDS-PAGE electrophoresis.

Electrophoresis was performed as described in Materials and Methods. Pure thymidylate kinase was run parallel with standard proteins (1.5 ug per sample) in a 15% PAGE gel. The molecular weight of standard proteins was shown in the legend of Fig. 1. The arrow indicates the position of thymidylate kinase.

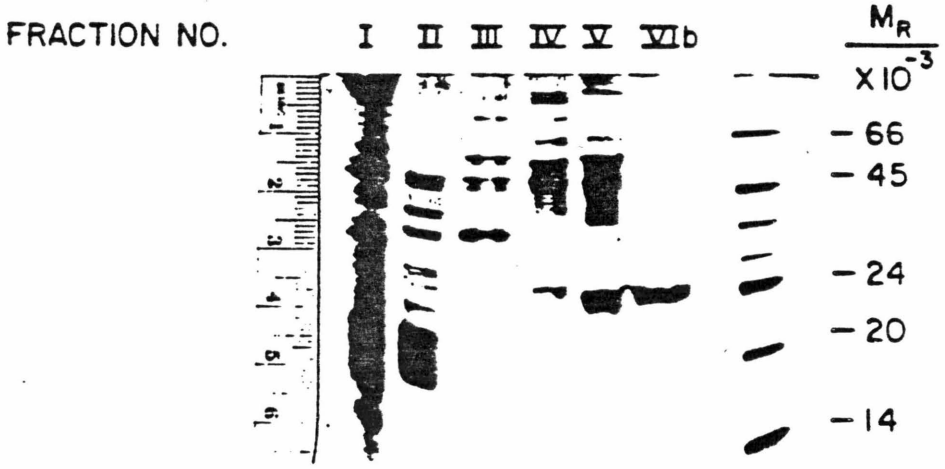

Figure 1

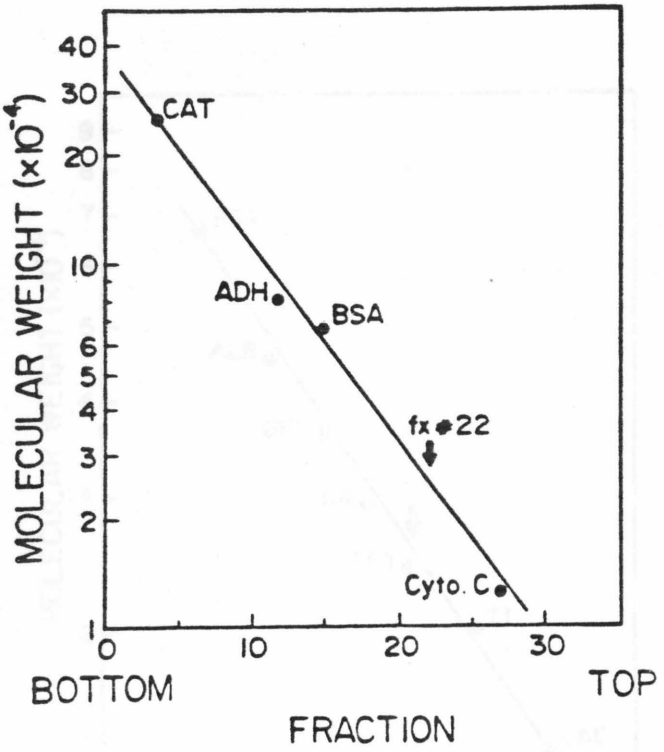
MOLECULAR WEIGHT (x10⁻⁴)
40
30
20
10
6
5
4
3
2
1
CAT
ADH
BSA
fx #22
Cyto. C
0
10
20
30
BOTTOM
TOP
FRACTION

Figure 2a

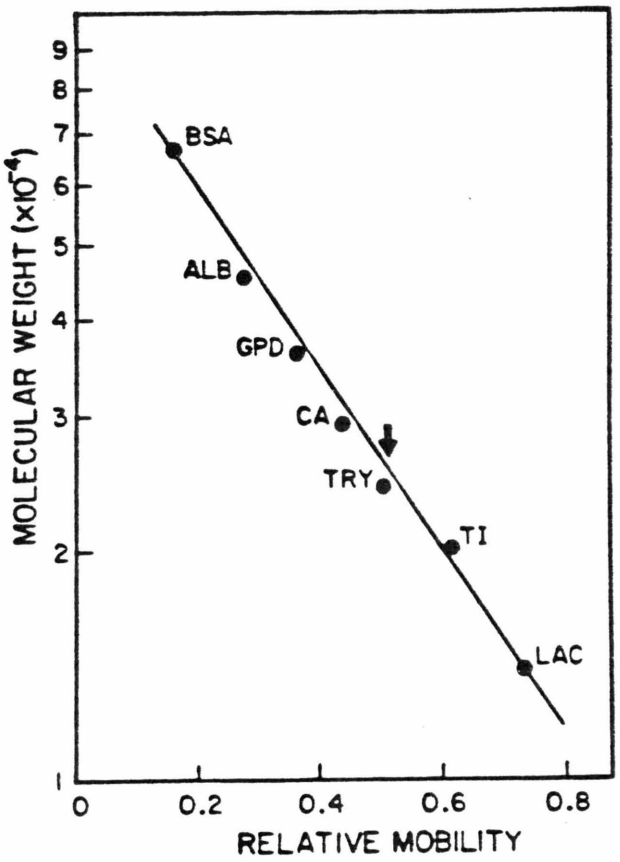
MOLECULAR WEIGHT (x10⁻⁴)
9
8
7
6
5
4
3
2
1
BSA
ALB
GPD
CA
TRY
TI
LAC
0
0.2
0.4
0.6
0.8
RELATIVE MOBILITY

Figure 2b

Miniprint Supplement

Preparation of Extracts -- The postmitochondrial fraction as described by (Daum, Bishi and Schatz, 1983) was used as starting material. The removal of the mitochondria is an essential step if homogeneous enzyme is desired, due to the presence of an abundant mitochondrial protein that co-purifies with thymidylate kinase in all subsequent steps. Yeast cells (12 l) were grown in YPD medium at 30°C and harvested in log phase, 2-4 x 10^7 cells/ml. Cells were washed with cold distilled water, collected by centrifugation and stored at -70°C. Cells (125 gm wet weight) were resuspended in 250 ml of 0.1 M Tris-H_2SO_4, pH 9.4, 10 mM dithiothreitol, and incubated for 10 min at 30°C. They were then washed with 1.2 M sorbitol and suspended in 850 ml of 1.2 M sorbitol, 20 mM KP, pH 7.4. 45 mg Zymolyase 60,000 was added and the suspension was incubated at 30°C with gentle shaking for 30 min. Spheroplasts were harvested by centrifugation for 5 min at 3000 rpm in a Sorvall RC-3 centrifuge. The spheroplasts were resuspended in 500 ml of cold 0.6 M mannitol, 10 mM Tris-HCl, pH 7.4, 0.02 mM dTMP, 1 mM phenylmethylsulfonyl fluoride and homogenized by 10-15 strokes in a tight-fitting glass homogenizer. The homogenate was diluted with 250 ml of the homogenization buffer and centrifuged for 15 min at 9000 rpm in a Sorvall GSA rotor. The mitochondria, nuclei, unbroken cells and their debris are found in the pellet. The pellet was rehomogenized once and the supernatants (about 850 ml) combined and saved (Fraction I).

Ammonium Sulfate Precipitation -- Solid ammonium sulfate,

390 gm per liter, was added slowly with stirring to Fraction I. After stirring at 0°C for 30 min, the precipitate was collected by centrifugation at 12,000 rpm for 30 min in a Sorvall GSA rotor. The supernatant was saved and the pellet was resuspended in buffer A (25 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM PMSF) with 60% ammonium sulfate and then centrifuged again. The supernatants were combined to give Fraction II.

Phenyl-Sepharose CL-4B Column -- Fraction II was diluted with 1700 ml of buffer A and loaded onto a phenyl Sepharose column (12.5cm^2 x 16cm) equilibrated with buffer A containing 0.25M ammonium sulfate. The column was washed with 1500 ml buffer A containing 0.25 M ammonium sulfate the protein was eluted with an 1800 ml gradient of ammonium sulfate (0.25M - 0 M) in buffer A. The elution profile is shown in Fig. 1. The thymidylate kinase eluted at about 100 mM ammonium sulfate in buffer A. The active fractions were pooled to give Fraction III.

DE 52 Column -- Fraction III was dialyzed against buffer A containing 60 mM NaCl for 3 hr and applied to DE-52 column (5cm^2x6cm). Protein was eluted with a 180 ml linear gradient of NaCl (60 mM to 360 mM) in buffer A (Fig. 2).

Hydroxylapatite Chromatography -- Fraction IV was applied to a 2 ml hydroxylapatite column (0.8 cm^2x2.5cm). After washing with 20 ml of 25 mM Tris-HCl, pH 7.6 0.1 mM KPi, the protein was eluted with a 20 ml linear gradient of KPi (0.1 mM KPi to 80 mM KPi). At this purification step, thymidylate kinase (M_r 25,000) could be easily observed in the SDS-PAGE gel (Fig. 3 and see Fig. 1 in the text).

HPLC -- Thymidylate kinase was isolated for the determination of amino acid composition and N-terminal amino acid sequence by HPLC using a Beckman Instrument with variable ultraviolet detection at 214 nm. A C4 VYDAC column (0.12cm^{2}4 x 25 cm) operating in a reversed phase mode was eluted with a gradient of 0% to 100% acetonitrile in 0.05% (v/v) trifluoroacetic acid (pH 2.1). The gradient was carried out at a flow rate 1 ml/min with 1 ml volume collection per tube. Ththymidylate kinase was eluted at fraction 39 as a sharp peak observed by SDS-PAGE gel and designated Fraction VIa (Fig. 5).

Sephadex G-50 Superfine Column -- Fraction V was placed in a dialysis tube and concentrated with polyethylene glycol (Carbowax) for 3 hours. The concentrated solution was applied to a Sephadex G-50 Superfine column (3cm^2 x 50 cm) (Fig. 4). The column was eluted with buffer A at a flow rate of 3 ml/hr. The peak of activity was pooled and designated Fraction VIb. A summary of this procedure is shown in Table IV in the text.

Miniprint Supplement Figures

Fig. 1 Phenyl-Sepharose CL-4B chromatography of thymidylate kinase.

A linear gradient of ammonium sulfate in buffer A was carried out with flow rate 40 ml/hr. The bar area indicates fractions pooled containing thymidylate kinase activity. Plotted are activity ( ), protein concentration (-) and ammonium sulfate concentration (--) versus fraction number.

Fig. 2 DE-52 Chromatography of thymidylate kinase.

10 ul aliquots of individual fractions were incubated in the thymidylate kinase assay. The bar area indicates fractions pooled containing thymidylate kinase activity. Plotted are activity (o), and [NaCl] (o) versus fraction number.

Fig. 3 Hydroxylapatite chromatography of thymidylate kinase.

2.5 ul samples were incubated in the thymidylate kinase assay. The bar area indicates fractions pooled containing thymidylate kinase activity. Plotted are activity ( ), and potassium phosphate (o) versus fraction number.

Fig.4 High-performance Liquid Chromatography of Thymidylate Kinase using a C4 VYDAC column.

A portion of the hydroxylapatite column pool was applied to a HPLC (C4 reversed phase, 0.4 x 25 cm column; room temperature; pressure 2000 psi). A linear gradient of 0-100% acetonitrile in 0.05% trifluoroacetic acid (v/v, pH 2.1) was carried out with a flow rate of 1 ml/min. (1 min/fraction; chart speed: 20 cm/hr; Detection system: UV absorption at 214 nm; Sensitivity: 0.1 absorbance units full scale). The position at which thymidylate kinase eluted was determined by polyacrylamide gel electrophoresis of fractions.

Fig. 5 Sephadex G-50 Superfine gel filtration.

1 ul samples were incubated in the thymidylate kinase assay. The bar area indicates fractions pooled containing thymidylate kinase activity. Plotted are activity ( ) versus fraction number.

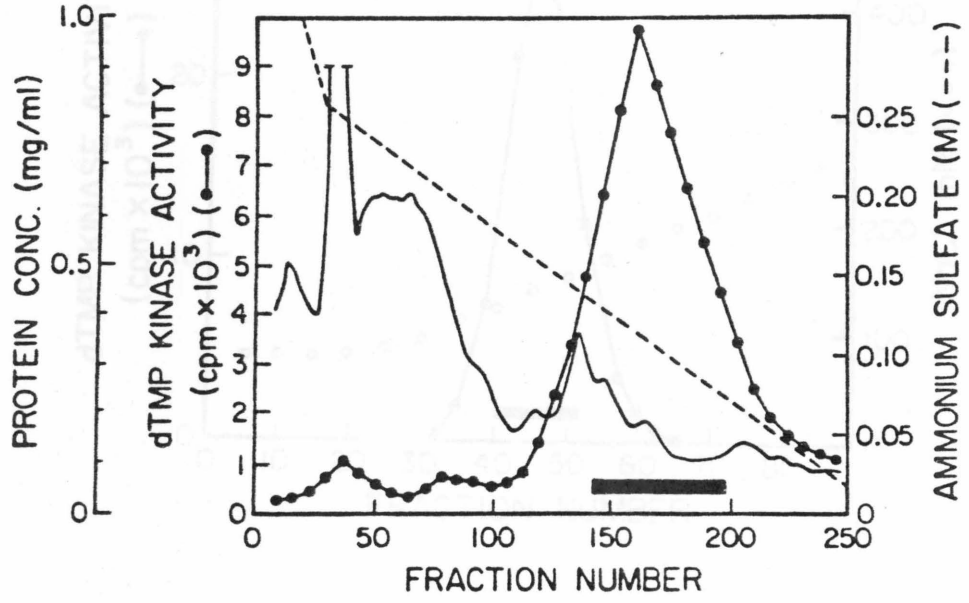
PROTEIN CONC. (mg/ml)
1.0
0.5
0
dTMP KINASE ACTIVITY
(cpm x 10³) (●—●)
9
8
7
6
5
4
3
2
1
0
AMMONIUM SULFATE (M) (---)
0.25
0.20
0.15
0.10
0.05
0
0
50
100
150
200
250
FRACTION NUMBER

Figure 1

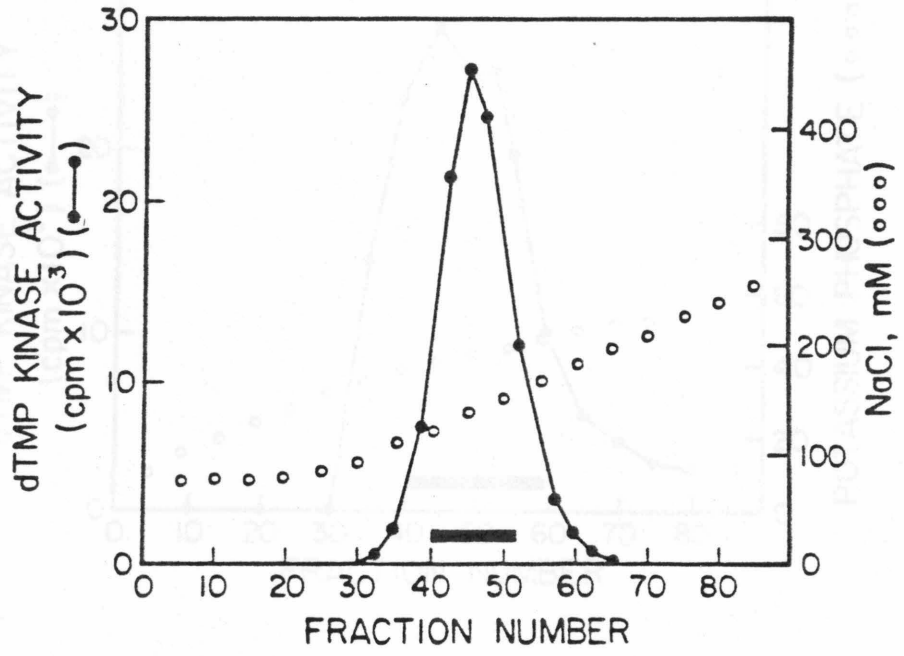
dTMP KINASE ACTIVITY
(cpm x 10³) (●—●)
NaCl, mM (o o o)
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
100
200
300
400

Figure 2

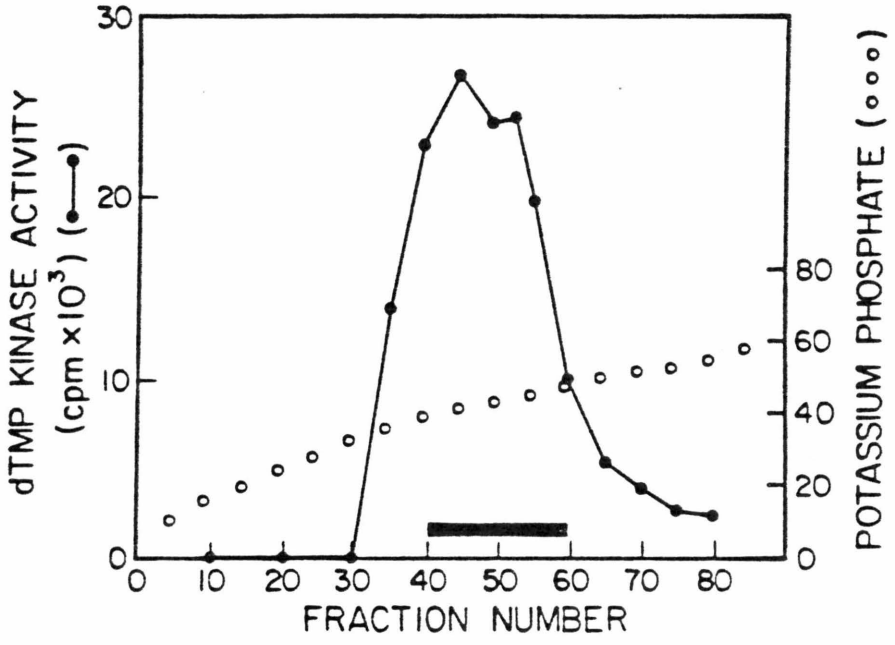
dTMP KINASE ACTIVITY
(cpm x10³) (●—●)
POTASSIUM PHOSPHATE (o o o)
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
0
20
40
60
80

Figure 3

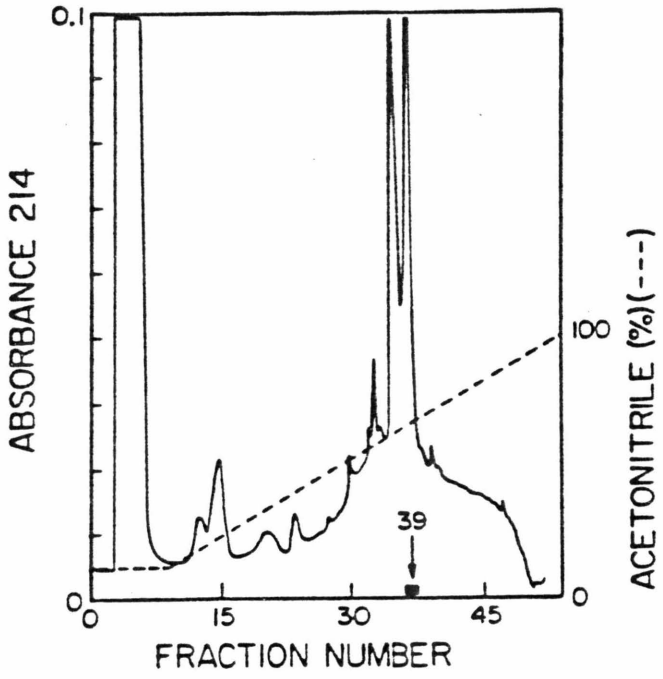
0.1
0
ABSORBANCE 214
100
0
ACETONITRILE (%)(---)
39
0
15
30
45
FRACTION NUMBER

Figure 4

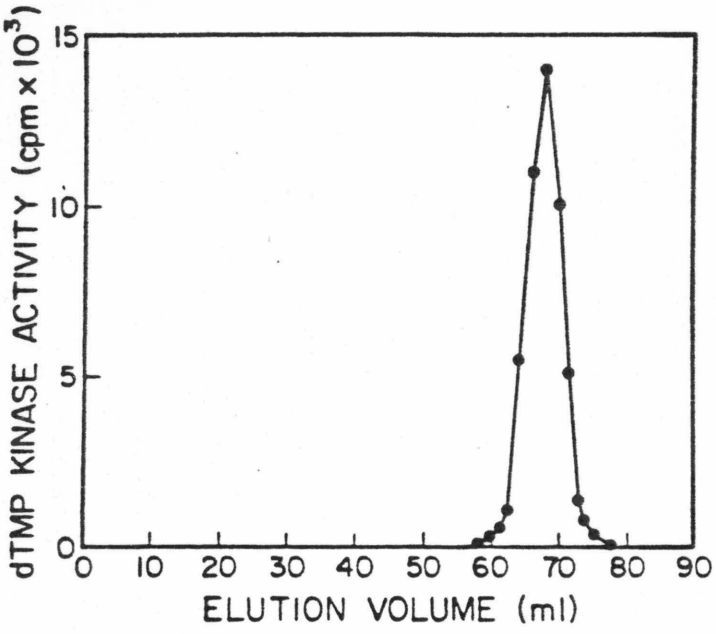
dTMP KINASE ACTIVITY (cpm x 10³)
15
10
5
0
0
10
20
30
40
50
60
70
80
90
ELUTION VOLUME (ml)

Figure 5