The *Saccharomyces cerevisiae* CDC7 Protein Kinase, a Potential Link between START and the Initiation of DNA Replication

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To My Parents

Soo Nam and Yoo Deug Yoon

and

Soon Ah and Chul Mo Shin

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ABSTRACT

Two major controls of the eukaryotic cell cycle exist in the G1/S and G2/M transitions. In an attempt to understand the molecular basis of the G1/S regulatory pathway, biochemical characterization of the *Saccharomyces cerevisiae* CDC7 protein has been carried out. The temperature sensitive *cdc7* mutation arrests yeast cells at the G1/S phase boundary, after the completion of START and prior to the initiation of DNA synthesis. START is defined as the point of commitment to DNA replication in yeast. The open reading frame encoding CDC7 protein contains the 11 catalytic domains characteristic of protein kinases. That CDC7 protein indeed has protein kinase activity was demonstrated by incubating CDC7 immune complexes with histone H1. Kinase activity was elevated more than 10-fold in strains carrying the wild-type CDC7-overproducing plasmid. Overproduction of thermolabile cdc7 protein gave rise to overproduction of thermolabile kinase. In vivo ^{32}P-labeling of yeast cells revealed that CDC7 protein itself was a phosphoprotein. In addition, indirect immunofluorescence and biochemical fractionation studies showed CDC7 was located mainly in yeast nuclei.

The cell cycle-specific roles of CDC7 at the G1/S border were explored by following the phosphorylation state and kinase activity of CDC7 protein throughout the cell cycle. CDC7 was not active as a histone H1 kinase, nor was it phosphorylated in cells arrested by a *cdc28* mutation. Furthermore, phosphorylation of CDC7 protein was required for its activity. *CDC28* encodes the budding yeast homolog of the highly conserved p34^{cdc2}/MPF kinase subunit and its function is crucial to passage through START. The possibility that CDC7 is a substrate of the CDC28 kinase was tested using CDC28 immune complexes and bacterially produced, inactive CDC7 kinase. The CDC28 kinase specifically phosphorylated the CDC7 protein, which in turn led to activation of the CDC7 kinase. CDC28 and CDC7 could interact in vivo as well since *CDC7* hyperexpression suppressed the inviability of a *cdc28* mutation at 33°C but not at 36°C. Finally, possible mechanisms of action of CDC7 protein kinase in the initiation of DNA synthesis were examined by searching for

endogenous substrates among yeast replication proteins. The 34 kDa subunit of yeast replication protein-A (RP-A) is a substrate of both CDC7 and CDC28 kinases.

In conclusion, the biochemical evidence indicates that there is a phosphorylation cascade operating between START and the initiation of DNA replication involving yeast CDC28, CDC7, and RP-A.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

APPENDIX

CHAPTER I

INTRODUCTION

The eukaryotic cell cycle involves an ordered sequence of events that results in cell reproduction. Two major controls regulating the molecular events of a cell cycle exist in the G1 to S phase and G2 to M phase transitions. In the past decade, the study of cell cycle control has been focused on the ubiquitous $p34^{cdc2}$ protein and its associated regulatory components. $p34^{cdc2}$ was first identified in yeast as a product of the cell division cycle (*CDC*) gene *CDC28* (*Saccharomyces cerevisiae*)/*cdc2*$^+$ (*Schizosaccharomyces pombe*). *CDC28*/*cdc2*$^+$ encodes a pivotal protein-serine/threonine kinase that is conserved throughout phylogeny. In yeast, both genes are required al least twice in the cell cycle, once in G1 for commitment to DNA synthesis and in G2 for entry into M phase (Nurse and Bissett, 1981; Piggott et al., 1982). Thus, it appears that the same protein kinase is involved in regulating the two principal cell cycle transitions, G1/S and G2/M (Ghiara et al., 1991; Surana et al., 1991).

For the control of mitosis, a universal mechanism common to all eukaryotes has now emerged: entry into M phase is determined by activation of the M phase-promoting factor (MPF) (Dunphy and Newport, 1988; Nurse, 1990). MPF is a protein complex, minimally consisting of the $p34^{cdc2}$ protein kinase and one of the regulatory subunits known as mitotic cyclins. Cyclins are a family of proteins whose abundance oscillate during the cell cycle. Assembly of the $p34^{cdc2}$-cyclin complex as well as dephosphorylation of the $p34^{cdc2}$ protein leads to activation of the M phase-specific kinase, which in turn promotes the G2/M transition by phosphorylating key proteins involved in the major M phase events (Lewin, 1990; Nurse, 1990). As compared to the G2/M control, much less is known about the molecular events leading to the initiation of nuclear DNA replication at the G1/S border. The budding yeast *Saccharomyces cerevisiae* provides an ideal system to study the G1/S control, since it is an organism whose cell cycle is regulated mainly in G1. In fact , in *S. cerevisiae*, most mutant alleles of *CDC28* cause arrest at a point in G1 termed "START." START defines an important decision point at which yeast cells can undertake one of four possible developmental pathways (Pringle and Hartwell, *1981*) (*Figure 1*).

They may initiate DNA synthesis and begin the mitotic cell cycle. They may fuse with cells of opposite mating type to form zygotes, or they may enter stationary phase in the absence of nutrients. Diploid cells can undergo the meiotic cycle after N-starvation and completion of the *CDC4* step.

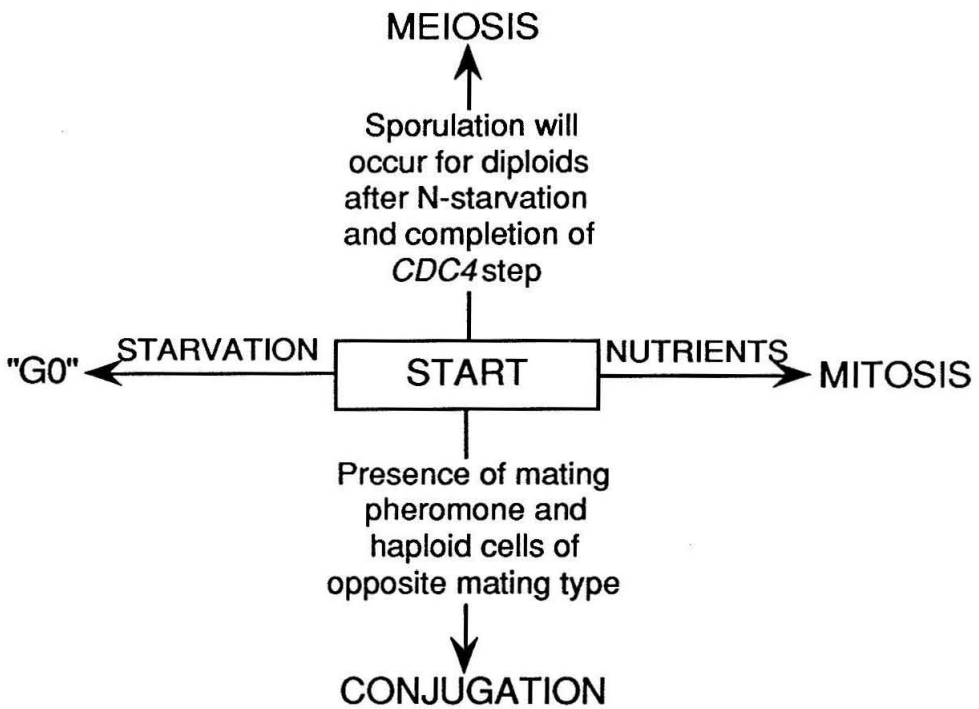

Figure 1. "START" as a decision point. Cells arrested in the G1 phase at START can, on release, develop in one of four different ways depending on the nature of the cell and its environment, as summarized in the diagram. "G0", non-proliferation. Reproduced from Wheals (1987).

Once past START, cells are committed to complete the ongoing round of DNA replication even in the absence of nutrients or in the presence of mating pheromones. Passage through START requires the activity of CDC28 protein, a budding yeast analog of the highly conserved $p34^{cdc2}$ protein kinase. START also requires three cyclin-like proteins encoded by *CLN1*, *CLN2*, and *CLN3*. The *CLN* genes were originally isolated as suppressors of *cdc28* mutations (*CLN1* and *CLN2*) or as dominant mutant alleles that ad-

vanced cells into START at a reduced size (*CLN3*) (Cross, 1988; Hadwiger et al., 1989; Sudbery et al., 1980). It has been proposed that the *CLN* genes encode components of a G1-specific form of the CDC28 kinase, whose activation is the signal for cells to pass START (Nash et al., 1988; Richardson et al., 1989; Wittenberg et al., 1990). A current model for cell cycle control in *S. cerevisiae* is shown in Figure 2. The *CLB* genes encode the G2 cyclins, i.e., mitotic cyclins, of the budding yeast. So far, the G1 cyclins have been fully characterized only in *S. cerevisiae*.

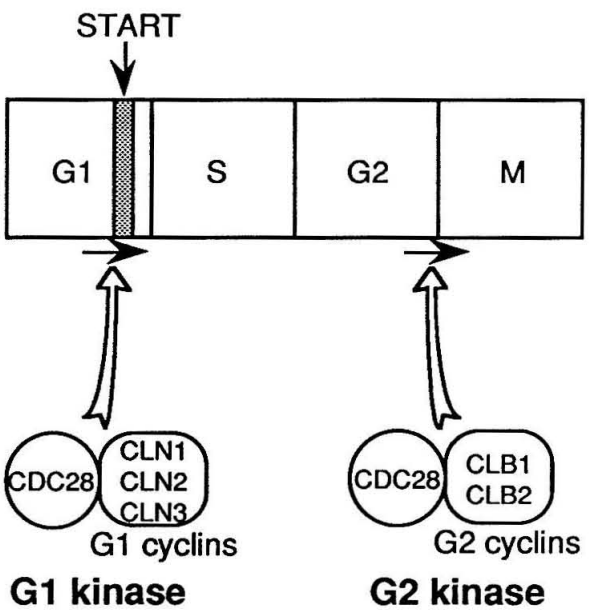

Figure 2. A model for cell cycle regulation in *S. cerevisiae*. It is proposed that the cell cycle in *S. cerevisiae* is controlled at two major transitions, namely, G1/S and G2/M. A successful execution of both transitions requires two distinct forms of a protein kinase. Although both forms contain a common catalytic subunit encoded by the *CDC28* gene, the proteins associated with it differ. While the fully active G1 kinase requires the interaction of CDC28 with G1 cyclins, the formation of G2 kinase involves association with G2 cyclins. The interaction with distinct cyclins may provide a way to modulate the substrate specificity or timing of action of the catalytic subunit CDC28. Adapted from Surana et al. (1991).

The discoveries of G1 cyclins have been a major breakthrough in elucidating the mechanism of START. What is lacking is any information on the molecular nature of the events set in motion by *CDC28* and the other START genes. While the commitment to DNA synthesis is made by passage through START, DNA synthesis does not begin immediately. A number of additional genetically defined steps must occur before the onset of DNA replication. It is likely that both induction of the genes encoding replication proteins and post-translational activation of replication proteins are preconditions for the initiation of DNA synthesis. The *CDC28*, *CDC4* and *CDC7* genes are thought to define one series of steps that must be completed before the initiation of DNA synthesis (Hartwell, 1976; Pringle and Hartwell, 1981). Morphological analysis of single and double mutants and reciprocal shift studies suggest that *CDC4* and *CDC7* act subsequent to *CDC28*/START (Hereford and Hartwell, 1974).

In addition to *CDC4* and *CDC7*, three other genes, *CDC34, DBF4*, and possibly *DBF2* also function in the post-START/pre-DNA synthesis period (Goebl et al., 1988; Johnston and Thomas, 1982; Chapman and Johnston, 1989; Johnston et al., 1990). *CDC34* and *CDC4* share the same terminal phenotype, a multibudded, uninucleate structure, in which the spindle pole body has duplicated but not separated. *CDC34* encodes a ubiquitin conjugating enzyme. One domain of *CDC4* is homologous to the β-subunit of transducin while another is similar to the *ets* oncogene (Fong et al., 1986; Peterson et al., 1984). *dbf4* mutants show a dumbbell shaped terminal phenotype typically associated with a DNA synthesis or nuclear division defect (Johnston and Thomas, 1982). Furthermore, the terminal phenotype of *dbf4cdc4* and *cdc7cdc4* double mutants is that of *cdc4*, implying, if they are on the same pathway, that *cdc4* precedes *cdc7* and *dbf4* (Hereford and Hartwell, 1974; Johnston and Thomas, 1982). *DBF4* encodes a 2.4 kb mRNA, but its function could not be deduced from its sequence (Chapman and Johnston, 1989). *DBF2* encodes a protein kinase with an execution point in G2/M (Johnston et al., 1990). There may also be an effect on G1, though the only evidence for this thus far is that recovery from α-factor is

retarded, as measured by a delay in the initiation of DNA synthesis after α-factor removal. The timing of *DBF2* function is obscured by the apparent redundancy of this protein kinase, since a gene encoding a second kinase with significant homology to *DBF2* and the ability to complement *dbf2* has been identified (Johnston et al., 1990).

CDC7 is believed to execute its function late in G1, since cells carrying a thermosensitive (ts) *cdc7* mutation block mitotic growth at the G1/S phase boundary at the restrictive temperature, just before the initiation of DNA synthesis (Hartwell, 1973; 1976). These cells resemble *dbf4* mutants, in that they arrest with large budded cells, i.e., dumbbells, with spindle pole body duplicated, and separated, but not completely elongated (Figure 3).

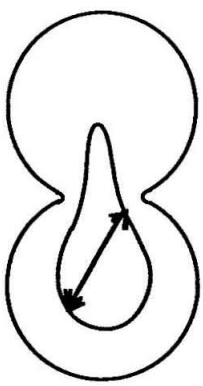

Figure 3. Terminal phenotype and nuclear-related behavior of cells carrying a *cdc7* mutation. Adapted from Wheals (1987).

Another reason for placing *CDC7* function very late in G1 is the fact that *cdc28* and *cdc4* mutants blocked at the nonpermissive temperature and then returned to the permissive temperature in the presence of the protein synthesis inhibitor cycloheximide do not progress further through the cycle and do not enter S phase, while *cdc7* mutants treated in the same way enter S phase and complete DNA synthesis in the presence of protein synthesis inhibitors (Hereford and Hartwell, 1974). Further protein synthesis is thus necessary for the initiation of DNA synthesis after *CDC28* and *CDC4*, whereas all of the proteins essential for DNA replication appear to be present at the *CDC7* stage. Further protein synthesis is also

required after return of *dbf4* mutants from the nonpermissive temperature for entry into S phase (Johnston and Thomas, 1982). *CDC7* thus mimics the final stage of the restriction point in mammalian cells, insofar as it was defined as the point in the cell cycle after which no further protein synthesis is necessary for initiation of DNA synthesis. Evidence suggesting the presence of CDC7 protein in a putative DNA replication complex also exists (Jazwinski, 1988). Since *CDC7* appears to be required only for the final stage of the G1/S transition, we speculated that *CDC7* might serve as a molecular link between START and the onset of DNA synthesis. In an effort to understand the regulatory apparatus operating at the G1/S border, we set out to determine the biochemical basis for the role of CDC7 protein in the G1/S transition.

The phenotype of *cdc7* mutants suggests that the CDC7 protein performs multiple functions in the life cycle of *S. cerevisiae*. As mentioned above, *cdc7* mutant cells show defects in the initiation of mitotic DNA synthesis. Though basal level expression requires an element in the promoter that is homologous to a c-fos activating sequence, *CDC7* mRNA remains at a constant level throughout the cell cycle when measured in cells released from α-factor arrest (Ham et al., 1989; Sclafani et al., 1988). In addition, a cell carrying a deletion of *CDC7* on the chromosome can divide up to eight times after loss of a plasmid harboring the *CDC7* gene, suggesting that the CDC7 protein exists in excess of the amount required for a single mitotic cell division (Sclafani et al., 1988).

In contrast to the behavior during mitosis, diploid homozygous *cdc7*-ts cells initiate premeiotic DNA synthesis normally at the restrictive temperature. However, these diploids do not form a synaptonemal complex, nor do they commit to recombination or form ascospores (Schild and Byers, 1978). Furthermore, the abundance of *CDC7* mRNA varies during the meiosis, with its peak occurring at recombination (Sclafani et al., 1988). The *cdc7* lesion seems to affect the mitotic and meiotic pathways in a different, distinct manner. The execution points of CDC7 are illustrated in Figure 4. The *CDC7* gene has also been implicated in an error-prone DNA repair pathway as a member of the *RAD6* epistasis group

(Njagi and Kilbey, 1982). Finally, *CDC7* may also be involved in transcriptional silencing of the HMR mating type cassette (Axelrod and Rine, 1991).

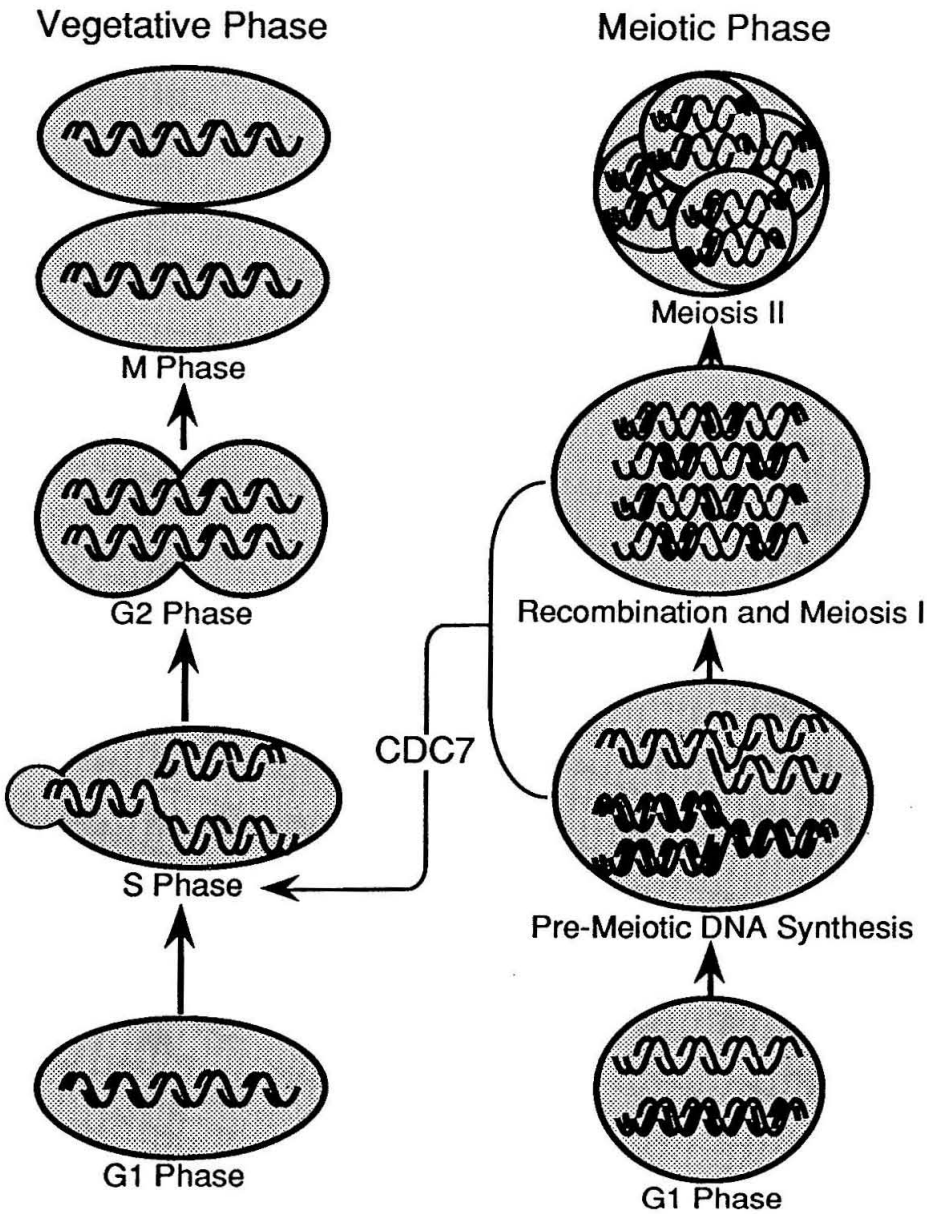

Figure 4. Execution points of CDC7 in the mitotic and meiotic cell cycles. Modified from Hoekstra et al. (1991).

How does *CDC7* affect these different DNA processes and what controls the timing of *CDC7* function in the cell cycle? A clue to investigate the molecular function of *CDC7*

was afforded by the demonstration that the open reading frame encoding CDC7 contains the 11 catalytic domains characteristic of protein kinases (Patterson et al., 1986). These catalytic domains range from 250 to 300 amino acid residues, corresponding to about 30 kDa. Subdomains VI and VIII are of particular interest in that they contain residues that are specifically conserved in either the protein-serine/threonine or the protein tyrosine kinases (Hanks et al., 1988). According to this indicator, *CDC7* encodes a protein-serine/threonine kinase. The CDC7 sequence, however, differs from that of other protein kinases in spacing between conserved subdomains. Very large inserts (in excess of 60 residues) occur in CDC7 between subdomains VII and VIII and between subdomains X and XI (Hanks et al., 1988). Thus, it was a matter of interest whether or not CDC7 carries a protein kinase activity. Since protein phosphorylation is an important regulatory mechanism of cell cycle progression, biochemical characterization of the putative kinase activity of CDC7 will further our knowledge of cell cycle control.

In order to demonstrate the kinase activity of CDC7, we isolated the *CDC7* gene by complementation of a *cdc7* mutation and subcloned it into *Escherichia coli*. Because of the reportedly low background of protein phosphorylation in *E. coli*, we thought that the putative kinase activity of CDC7 could be tested easily after overproducing the protein. The results of this work are described in Chapter II.

The initial strategy of characterizing CDC7 in *E. coli* turned out to be unfruitful. We could not detect CDC7-dependent kinase activity even after partially purifying the protein. Two plausible reasons for failure to detect active CDC7 in *E.coli* led us to refocus our studies on yeast. First, despite the fact that the amino acid sequence of CDC7 has homology to protein kinases, CDC7 may not function as a protein kinase. Second, CDC7 may not be active in *E. coli* because, like CDC28, activation of CDC7 kinase requires other proteins of yeast. We preferred the latter interpretation, and decided to carry out further analyses of CDC7 made in yeast. In Chapter III, I describe the generation of polyclonal antibodies against CDC7 and the subsequent use of these antibodies to characterize the

protein kinase activity, post-translational modifications, and subcellular locations of CDC7 in yeast. Demonstration that *CDC7* encodes a protein kinase led us to test the hypothesis that CDC7 phosphorylates a protein(s) in the replication apparatus. Furthermore, since CDC7 is a phosphoprotein with a protein kinase activity and functions only after completion of START, we tested the idea that CDC28 kinase activates CDC7 kinase via phosphorylation. These studies are described in Chapter IV.

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CHAPTER II

Biochemical Characterization of the Yeast CDC7 Protein in *Escherichia coli*

Abstract

The *cdc7* mutation of *Saccharomyces cerevisiae* arrests cells late in the G1 phase of the cell cycle, just prior to the initiation of mitotic DNA synthesis. The nucleotide sequence of the *CDC7* gene encodes a protein which has strong homology to protein kinases. To characterize the CDC7 protein, we introduced the gene into *Escherichia coli* by using a bacteriophage T7 expression system, and carried out enzymatic phosphorylation assays. An in vitro autophosphorylation assay and other protein kinase assays indicate that *E. coli* contains an endogenous autophosphorylatable protein of the same molecular weight as the CDC7 protein. In order to separate the *E. coli* kinase from the CDC7 protein, we partially purified the CDC7 protein using ^{35}S-labeled CDC7 protein as a probe. Unfortunately, we were not able to separate the *E. coli* kinase from the CDC7 protein. Since the CDC7 protein was not expressed at high levels in the T7 system, the gene was recloned into the *tac* expression system and was overproduced in *E. coli* after IPTG induction. Antibodies were raised against the overproduced protein, and antipeptide antibodies were made against the last 8 amino acids of CDC7.

Introduction

The mitotic cell cycle is made up of the events and processes that take place between the birth of the cell and its subsequent division into two daughters (vegetative cellular reproduction). Like the cell cycles of other eukaryotes, the yeast cell cycle is divided into a G1 phase (pre-DNA synthesis), an S phase (chromosomal DNA replication), a G2 phase (post-DNA synthesis), and an M phase (mitosis) (Pringle and Hartwell, 1981). Approximately 70 genes with a *CDC* (cell division cycle)-related phenotype have been detected in *Saccharomyces cerevisiae* and over 30 gene products that are required for a specific step in the cell division cycle have been identified by temperature sensitive (ts) mutations (reviewed in Wheals, 1987). Since a *cdc* mutation leads to a defect in a particular stage-specific function of the cell cycle, the *cdc* mutant cells are unable to complete the cell cycle, and should accumulate at the same stage (the terminal phenotype). Thus, an initially heterogeneous asynchronous culture should become synchronous and homogeneous (Pringle and Hartwell, 1981). A number of *cdc* mutants defective in some aspects of DNA synthesis have been detected, and some of them have been characterized. *CDC9*, for instance, is the structural gene for DNA ligase (Barker and Johnston, 1983). Mutations in two genes (*CDC4* and *CDC7*) appear to block a precondition for DNA synthesis because cells carrying these lesions can not start new rounds of DNA replication after a shift from permissive to nonpermissive temperature (Hartwell, 1973).

Since we have been interested in the regulation of yeast DNA replication and the *cdc7* mutation arrests cells late in G1, just prior to the initiation of mitotic DNA synthesis, we decided to identify the CDC7 protein and characterize its precise function. The terminal phenotype of the *cdc7* mutant appears as budded cells with a single nucleus but without an elongated spindle apparatus (Byers and Goetsch, 1974). It is not clear at this moment whether the CDC7 protein just promotes entry into S phase or if it is directly involved in the initiation of DNA synthesis. Furthermore, the initiation of meiotic replication is not affected in *cdc7* mutants, in contrast to the requirement for the *CDC7 gene product* to

initiate mitotic DNA synthesis (Schild and Byers, 1978). The protein synthesis requirement for mitotic DNA replication may be completed before the *CDC7*-mediated step because protein synthesis is not needed for DNA synthesis after arrest at the *cdc7* block (Hereford and Hartwell, 1974). Although it is not required for the initiation of meiotic replication, it is necessary for genetic recombination during meiosis and for the formation of ascospores (Schild and Byers, 1978). Thus, the *cdc7* mutants are defective in mitotic and meiotic cell cycles like nearly all of the *cdc* mutants that are defective in spindle pole body duplication or segregation, DNA replication, and chromosome segregation (Simchen, 1974). But the CDC7 protein may function in each pathway quite differently. *CDC7* is also implicated in error-prone DNA repair since the mutant *cdc7-1* shows a reduction in induced mutagenesis with DNA damaging agents (Njagi and Kilbey, 1982).

The *CDC7* gene has been cloned and its entire coding region has been sequenced (Patterson et al., 1986). The RNA transcript of *CDC7* is about 1,700 nucleotides (Patterson et al., 1986). Transcript levels do not vary during the mitotic cell cycle but they do during meiosis (Sclafani et al., 1988). The coding region has an open reading frame of 1,521 nucleotides, which encodes a Mr 56,000-58,200 protein with homology to protein kinases (Patterson et al., 1986). The CDC7 protein has significant homology with the *CDC28* gene product and other protein kinases (Patterson et al., 1986). The CDC28 protein kinase is presumably a component of a signal transduction pathway that conveys information from the exterior of the cell to the machinery that controls exit from G1 (Mendenhall et al., 1987). *CDC28* is required for movement past a point in G1 called "START" (Reed, 1980). The function of the CDC7 protein in the G1 phase to S phase transition and/or in the initiation of mitotic DNA replication may be accomplished by its protein kinase activity, as the CDC28 protein kinase may allow actively dividing cells to pass START by phosphorylating a Mr 40,000 protein during the G1 phase (Mendenhall et al., 1987; Reed et al., 1985).

Whatever the function of *CDC7* is, it must be very important in the control of the cell division cycle and/or in the mechanism of yeast DNA replication. In this chapter, we describe the cloning and overexpression of the *CDC7* gene, the characterization of its enzymatic activity, and the partial purification of the gene product in *Escherichia coli.*

Materials and Methods

Strains and Media

E. coli K38 (HfrC) and JM101 (supE, thi, Δ(lac-proAB), [F', traD36, proAB, lacI^{Q}ZΔM15]) were used for cloning and expression of the *CDC7* gene. *E. coli* cells were grown in LB, supplemented M9, or M9CA medium (Maniatis et al., 1982); ampicillin and/or kanamycin were added to media in a final concentration of 50 μg/ml or 40 μg/ml. *S. cerevisiae* strain used in this work was *cdc7* (**a** *ade1 ura3-52 tyr1 his7 lys2 gal1 cdc7-1*). Yeast extract-peptone-dextrose (YPD) or supplemented synthetic minimal medium was used for the culture of yeast cells (Sherman et al., 1986).

Chemicals

[^{35}S] methionine and [γ-^{32}P] ATP were obtained from ICN. Carrier-free [^{32}P] orthophosphate and [^{3}H] amino acid mixture were purchased from Amersham. Histones, casein, phosvitin, protamine, and Kemptide were products of Sigma.

Yeast Genomic Library

The recombinant plasmid pool containing yeast DNA sequences in a centromere-containing vector YCp50 (Kuo and Campbell, 1983) was used to complement the *cdc7-1* mutation.

Cloning

Restriction enzymes, calf intestinal alkaline phosphatase, *E. coli* Klenow fragment, T4 DNA ligase, and T4 DNA polymerase were purchased from New England Biolabs or Bethesda Research Laboratories and were used according to the supplier's instructions. T7 RNA polymerase/promoter expression system, SphI linker, and pKK223-3 were

generously provided by S. Tabor, M. Budd, and N. Gautum, respectively. Oligonucleotides were synthesized by Microchemical Facilities at Caltech and were purified by FPLC. Isolation of plasmid DNA, preparation of DNA fragments, and transformation of *E. coli* were carried out as described elsewhere (Maniatis et al., 1982).

Purification

One ml of cells (*E. coli* K38/pGP1-2/pT7-*CDC7*) was labeled with 1 mCi of [^{35}S] methionine in the presence of rifampicin (400 μg/ml) after heat induction and used as a radioactive probe to purify the CDC7 protein from a large culture as described below. Ten liters of cells (*E. coli* K38/pGP1-2/pT7-*CDC7*) were grown with aeration in a New Brunswick fermentor at 30°C in M9CA medium, pH 7.4. At OD_{600} = 0.5, the temperature was raised to 42°C. After 30 min, the temperature was lowered to 37°C for 120 min. Cells (35 g) were harvested, washed with 2 liters of 20% (w/v) sucrose, 50 mM Tris-HCl at pH 7.5, 10 mM EDTA at pH 8.0, and 150 mM NaCl (lysis buffer), at 0°C, resuspended in 150 ml of lysis buffer, dispensed into 30 ml-aliquots, and frozen in liquid N_2 until needed. Cells (30 ml) were thawed at 0°C and mixed with 1 ml of radioactive cells. Lysozyme was added to 0.2 mg/ml. After 45 min at 0°C, the cells were twice frozen in liquid N_2 and thawed at 0°C. The lysate was centrifuged and the supernatant was collected (fraction I, 25 ml). Ammonium sulfate (9.75 g) was added to fraction I at 0°C over 45 min. The precipitate was centrifuged and resuspended in 10% (v/v) glycerol, 50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 mM PMSF, and 1 mM benzamidine (buffer A) to a conductivity equal to that of buffer A containing 50 mM NaCl (fraction II, 40 ml). Fraction II was applied to a column of Whatman DE52 DEAE-cellulose (7 cm^2 x 9 cm) and the column was washed with 300 ml of buffer A containing 50 mM NaCl. Protein was eluted with a 600 ml-linear gradient from 50 to 400 mM NaCl in buffer A. The CDC7 protein was eluted from 90 to 230 mM NaCl (fraction III, 240 ml). Fraction III was concentrated by ammonium sulfate precipitation and loaded on a column of Whatman P11 phosphocellulose (3.14 cm^2 x 1.9 cm) equilibrated with 10% (v/v) glycerol, 50 mM

Tris-HCl at pH 7.2, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and 1 mM benzamidine (buffer B) containing 50 mM NaCl. The column was eluted with buffer B in a series containing NaCl at concentrations, 0.05, 0.1, 0.2, 0.4, 0.6, and 1.0 M. The CDC7 protein was eluted at 1.0 M NaCl.

Preparation of Antipeptide Antibodies

Peptide was synthesized by Microchemical Facilities at Caltech. Two New Zealand white rabbits (one for the free peptide, the other for the conjugated peptide) were immunized according to the following schedule: first, 250 μg peptide-coupled KLH (keyhole limpet hemocyanin) in complete Freund's adjuvant (1:1) at multiple subcutaneous sites on day 0; second, 250 μg in incomplete Freund's adjuvant (1:1) subcutaneously on day 21. The rabbit was bled 5 weeks after the first injection. After these initial injections, the rabbit was boosted with 250 μg peptide-coupled KLH emulsified with incomplete Freund's adjuvant (1:1) at 5 week intervals, and bled 10 days after the injection. The nonconjugated peptide was injected according to the same schedule except that 500 μg peptide was used per injection.

Other Methods

Protein concentrations were determined with the Bio-Rad protein assay kit. For Western blots, proteins were transferred from SDS-polyacrylamide gels to nitrocellulose as described in detail elsewhere (Towbin et al., 1979). The CDC7 protein was detected by the procedure explained in the Bio-Rad ImmunBlot assay kit.

Results

Isolation of a DNA Fragment Able to Complement the *cdc7* Mutation

The *CDC7* gene was isolated by transformation of a *ura3-52* derivative of strain *cdc7* with a YCp50 yeast genomic library constructed in this laboratory (Kuo and Campbell, 1983). *URA*$^+$ transformants were selected at 23°C and a transformant carrying the *CDC7* gene was identified by replica plating and testing for complementation of the

temperature sensitive phenotype (A. Jong). The gene was verified by standard procedures and the restriction map coincided with the published one (Patterson et al., 1986).

Expression of the *CDC7* Gene in the T7 RNA Polymerase/Promoter System

The bacteriophage T7 expression system was used to express the *CDC7* gene in *E. coli* as described previously (Tabor and Richardson, 1985). This system consists of two plasmids, pGP1-2 and pT7-7. pGP1-2 contains the T7 RNA polymerase gene under the control of the inducible λ P_L promoter and the gene for the heat-labile λ repressor, cI857. pT7-7 has a T7 RNA polymerase promoter, ϕ10, and a 16S ribosome binding site, followed by a polylinker containing ten different restriction enzyme sites. The *CDC7* gene was inserted into the polylinker region of the plasmid pT7-7 and expressed in *E. coli* after heat induction of T7 RNA polymerase and the addition of rifampicin to turn off *E. coli* RNA polymerase transcription. The plasmid which had the insert of the *CDC7* coding region was named pT7-*CDC7*. The addition of [^{3}H] amino acid mixture or [^{35}S] methionine resulted in specific labeling of the CDC7 protein. Figure 1 shows the profile of [^{35}S] methionine-labeled proteins in *E. coli*.

Protein Phosphorylation in *E. coli*

Protein phosphorylation has been recognized as a major regulatory process in a number of eukaryotic systems (Krebs and Beavo, 1979), and has recently been shown to occur in bacteria as well (Manai and Cozzone, 1979; Wang and Koshland, 1981). Despite the presence of some phosphoproteins in *E. coli*, Wang et al. have expressed the oncogene *v-abl* of Abelson murine leukemia virus in *E. coli* and demonstrated that this coding sequence gives rise to a Mr 62,000 protein which is phosphorylated on a tyrosine residue in bacterial cells carrying the *v-abl* gene (1982). The same approach was taken here to investigate whether the CDC7 protein was phosphorylated in *E. coli*. Cells containing pT7-7 or pT7-*CDC7* were labeled with [^{32}P] orthophosphate and cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 2A). The calculated size of the CDC7 protein is 58,200 or 56,000 daltons, depending on which of

two possible ATG codons initiates translation. As shown in Figure 2A, a Mr 58,000 protein was strongly phosphorylated in vivo. This protein may be, however, a bacterial gene product because it was present in the absence of heat induction and even in cells harboring the control plasmid only. Basically, the same result was obtained with cell lysates labeled in vitro with [γ-^{32}P] ATP (Figure 2B). It is not clear from these experiments whether the CDC7 protein was phosphorylated in *E. coli.*

In Vitro Assay for Autophosphorylating Activity

Homology between the *CDC7* gene product and other protein kinases is not distributed randomly throughout the coding sequences, but it is confined largely to two functional regions, the ATP-binding region and the phosphorylation site receptor region (Patterson et al., 1986). Consensus sequences are known to be present in both regions. The phosphorylation receptor residue in tyrosine kinases is either a tyrosine 7 residue after the arginine/lysine position in the consensus or an adjacent threonine (Hunter and Cooper, 1985). In cAMP-dependent protein kinases, a threonine 8 residue after the arginine/lysine position is autophosphorylated (Shoji et al., 1979). The CDC7 protein has a tyrosine 5 (Tyr 192) and a serine 7 (Ser 194) in this region. Autophosphorylation has been taken as good evidence that a protein can function as a protein kinase (e.g., Celenza and Carlson, 1986). To test whether CDC7 carried an autophosphorylating activity, an in situ autophosphorylation assay described by Celenza and Carlson (1986) was used. Mn^{2+}, Zn^{2+}, and Ca^{2+} as well as Mg^{2+} were used as cofactors. The results are shown in Figure 3. Again, in both the control and the CDC7-containing extracts, there was a labeled Mr 58,000 band regardless of the type of cations used. Thus, *E. coli* has an endogenous autophosphorylatable species with the same migration in SDS gels as the CDC7 protein. To see if the CDC7 protein comigrated with the endogenous activity, extracts were analyzed by two-dimensional gel electrophoresis according to O'Farrell (1975) and assayed for autophosphorylating activities. However, we could not detect any

autophosphorylatable proteins in this experiment due to the low sensitivity of this technique (data not shown).

Exogenous Protein Phosphorylation in *E. coli*

We also used exogenous proteins as substrates to detect CDC7-dependent kinase activity. *E. coli* extracts were prepared as usual, incubated in a buffer containing [γ-^{32}P] ATP, Mg^{2+}, and either different subgroups of histones, protamine, casein, or phosvitin. The reaction products were subjected to SDS-polyacrylamide gel eletrophoresis and visualized by autoradiography. To make sure that the phosphorylation of foreign substrates was achieved by proteins in extracts and not by proteins contaminating the substrates, the substrate proteins were incubated with [γ-^{32}P] ATP in the absence of extracts and used as control. The results are shown in Figure 4. Neither casein nor protamine were phosphorylated. But histones and phosvitin were phosphorylated even in extracts lacking the CDC7 protein. Since phosvitin was labeled in a control as well, its phosphorylation does not appear to be carried out by *E. coli* proteins. The reason that histones were phosphorylated by *E. coli* proteins remains to be clarified. Based on the requirement of cofactors, protein kinases may be classified into different types, for instance, cAMP-dependent protein kinase, etc. Since the protein phosphorylation in *E. coli* has been known to be cyclic nucleotide independent (Dadssi and Cozzone, 1985), we carried out another protein kinase assay, this time using a synthetic peptide Kemptide as substrate. To determine the effect of cyclic nucleotides on kinase activity, the extracts were fractionated on a DE52 column, and fractions were assayed in the presence of either cAMP or cGMP by using phosphate acceptor peptide Kemptide as substrate. No difference was seen whether cyclic nucleotides were added or not (data not shown).

Partial Purification of the CDC7 Protein

To determine the precise function of the CDC7 protein, we decided to purify the protein before assessing its activity. Since the CDC7 protein represented the major band on SDS-polyacrylamide gels when *E. coli* cells were labeled with [^{35}S] methionine in the

presence of rifampicin, we used this radioactive protein as a probe to purify the CDC7 protein from extracts of a large culture of unlabeled cells. The extracts were fractionated by ammonium sulfate, followed by chromatography on DEAE and phosphocellulose. After each chromatography, samples were taken from each fraction and counted to detect the fractions containing the CDC7 protein. The results were confirmed by SDS-polyacrylamide gel electrophoresis. On a DE52 DEAE-cellulose column, the CDC7 protein was eluted from 90 to 230 mM NaCl with a linear gradient from 50 to 400 mM NaCl, reaching a peak approximately 150 mM NaCl (Figure 5). The fractions containing the CDC7 protein were further fractionated on a column of P11 phosphocellulose with a NaCl step gradient. The CDC7 protein eluted at 1.0 M NaCl (Figure 6). Since CDC7 protein was not expressed at high levels in the T7 system, we decided to clone the gene in a different expression system and further overproduce the protein (see below).

Autophosphorylating Activities after DEAE-Cellulose Chromatography

We next assessed whether, after DEAE-cellulose chromatography, we had separated the CDC7 protein from the *E. coli* Mr 58,000 protein (p58). Cells containing pT7-*CDC7* or pT7-7 were fractionated on DEAE-cellulose columns, and then each fraction was assayed for autophosphorylating activities as described before (Celenza and Carlson, 1986). Extracts prepared from CDC7-containing cells showed an autophosphorylated protein of Mr 58,000 eluting from 100 to 170 mM NaCl (Figure 7A), and extracts made from the vector containing cells gave rise to an autophosphorylatable p58 from 130 to 190 mM NaCl (Figure 7B). Since both extracts showed autophosphorylatable proteins of Mr 58,000 within the region of 90 to 230 mM NaCl in which the CDC7 protein eluted on a DEAE-cellulose column, the results are still not conclusive to demonstrate that the CDC7 protein is autophosphorylatable.

Antisera to the *CDC7* Gene Product

The peptide homologous to the last 8 amino acids of the C-terminus of *CDC7* (as predicted from the DNA sequence) was synthesized and coupled to KLH by *succinimidyl*

4-(N-Maleimido methyl) cyclohexane 1-carboxylate. Cysteine was added to the C-terminus of the peptide to allow coupling to KLH (Liu et al., 1979). This conjugated peptide as well as the nonconjugated one, were used to immunize two rabbits. Both the conjugated peptide and the nonconjugated peptide provided antibodies that recognized the CDC7 protein expressed in *E. coli* on Western blots (data not shown).

Overexpression of the *CDC7* Gene in *E. coli*

It was very difficult to purify the CDC7 protein, mainly because it was not overproduced in the T7 expression system. We, therefore, recloned the gene by using the strong *trp-lac* (*tac*) promoter and succeeded in its overproduction. The expression vector pKK223-3 contains the *tac* promoter first described by DeBoer et al. (1983), a multiple cloning site, and the strong *rrnB* ribosomal RNA transcription terminators (Brosius et al., 1981). The *tac* promoter bears the -35 region of the *trp* promoter and the -10 region, operator, and ribosome binding site of the *lacUV-5* promoter (Amann et al., 1983). In a $lacI^Q$ host, the *tac* promoter is repressed but may be induced by the addition of isopropyl β-D-thiogalactoside (IPTG). The gene for *CDC7* was subcloned into this expression vector, and the resulting plasmid was named pKK-*CDC7*. Briefly, *CDC7* was brought under the control of the *tac* promoter by inserting the ClaI/EcoRI fragment from *CDC7* into the EcoRI site of the vector, and reconstituting the *CDC7* coding sequence with the oligonucleotide 5'-AA TTC ATG ACA AGC AAA ACG AAG AAT AT-3'. As shown in Figure 8, the CDC7 protein was overproduced in a $lacI^Q$ host, JM101, with its synthesis reaching a plateau 120 min after IPTG induction. The plasmid pKK223-3 was used as a control for this experiment.

Discussion

The T7 RNA polymerase/promoter expression system was originally chosen to produce the yeast *CDC7* gene in *E. coli* because this expression system had advantages over other systems. In order to overproduce the products of a cloned gene, the T7 system

makes use of a very strong promoter, ϕ10, coupled to the ribosome binding site and the initiation codon, ATG (Tabor and Richardson, 1985). When the mRNA is efficiently translated after heat induction and in the presence of rifampicin, a target protein can accumulate to about 50% of the total cell protein (Studier and Moffat, 1986). Although the CDC7 protein was the most abundant protein in *E. coli* upon labeling with [^{35}S] methionine (Figure 1), we could not detect it on Coomassie blue-stained gels. This might be because T7 RNA polymerase was not as active as it was supposed to be and/or certain cis- and trans-acting elements of the yeast *CDC7* gene were missing in *E. coli*. When we recloned the gene into the *tac* promoter/IPTG induction system, the protein was overproduced and represented a strong band on Coomassie blue-stained gels (Figure 8). The overexpression of the *CDC7* gene, therefore, seems to be dependent upon which expression system is used to introduce the gene into *E. coli*.

The nucleotide sequence of the *CDC7* gene predicted a protein that had significant homology to the CDC28 protein kinase, cAMP-dependent protein kinases, and oncogene kinases (Patterson et al., 1986). The phosphorylation site receptor domain of known protein kinases can be organized by itself into three regions including the middle spacer region in which tyrosine or threonine is phosphorylated or autophosphorylated (Hunter and Cooper, 1985). CDC7 contains 99 amino acid residues in this middle region, whereas other protein kinases have 16 to 21 amino acid residues. Since the organization of the phosphorylation site receptor domain in CDC7 is significantly different from all known protein kinases, this heterogeneity might modify the protein kinase activity of CDC7. To determine whether it is another *CDC* gene with protein kinase activity, we first looked for phosphoproteins and autophosphorylatable proteins in the *E. coli* cells containing the cloned *CDC7* gene. As shown in Figures 2 and 3, *E. coli* had an unknown autophosphorylatable protein of the same molecular weight as the CDC7 protein (Mr 58,000). Since bacterial protein kinases have been known to be cyclic nucleotide independent and to use mainly endogenous proteins as substrates, we carried out cAMP-

and cGMP-dependent protein phosphorylations and exogenous protein phosphorylation in vitro in order to find any differences between cells containing the cloned *CDC7* and cells carrying the vector alone. There was no cyclic nucleotide dependent phosphorylation in *E. coli*. Unexpectedly, however, histones were phosphorylated by *E. coli* extracts (Figure 4). Further experiments are needed to clarify this conflict.

We have tried to characterize the yeast CDC7 protein in *E. coli* because of the low background of protein phosphorylation in *E. coli* and the unique properties of prokaryotic protein kinases. This suggested that the putative protein kinase activity of CDC7 should be easily demonstrated even without purifying the protein. However, *E. coli* has turned out to contain an endogenous autophosphorylatable protein of Mr 58,000, the same molecular weight as CDC7. This *E. coli* protein even copurified with the CDC7 protein in DEAE-cellulose chromatography. In situ autophosphorylation assays after DEAE-cellulose fractionation showed that *E. coli* p58 eluted almost at the same salt concentrations as the CDC7 protein with a 50-400 mM NaCl linear gradient (Figure 7A and 7B). Furthermore, CDC7 may not be functional in *E. coli* because it requires other proteins or post-translational modifications of yeast to be activated and/or substrate proteins of CDC7 are absent in *E. coli*. Therefore, as described in the next two chapters, we turned to methods for studying CDC7 in yeast.

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Figure 1. Expression of the *CDC7* Gene by T7 RNA Polymerase/Promoter System.

E. coli K38/pGP1-2 containing pT7-7 (lane a) or pT7-*CDC7* (lane b) were grown in M9 medium supplemented with thiamine (20 μg/ml) and 18 amino acids (0.01%, minus cysteine and methionine). Cells (1.5 ml) were grown at 30°C until OD_{600} = 0.4. Temperature was shifted to 42°C for 20 min. Rifampicin (400 μg/ml) was added, and after 15 additional min at 42°C, cells were grown for 20 min at 30°C. After pulse labeling with 10 μCi of [^{35}S] methionine and chasing with cold methionine (4 μg/ml), cells were harvested, resuspended in 60 mM Tris-HCl at pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, boiled for 5 min and loaded onto a 12.5% SDS-polyacrylamide gel. To reduce the X-ray film exposure time necessary for visualization, the gel was treated with an autoradiography enhancer. The numbers on the left give the molecular weights of known proteins (in daltons).

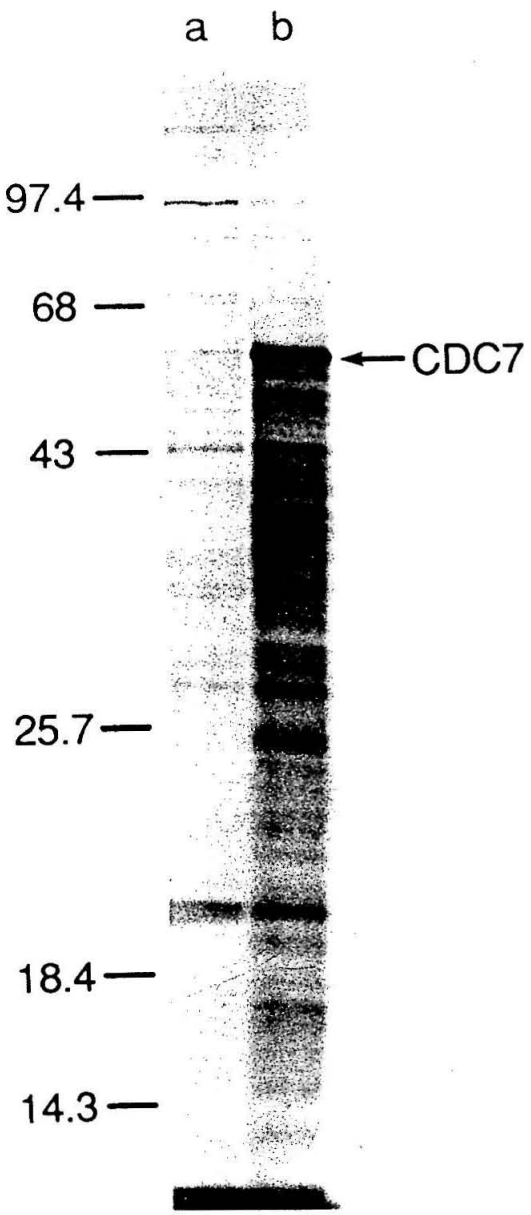

a
b
97.4
68
43
25.7
18.4
14.3
CDC7

Figure 2. In Vivo and In Vitro Protein Phosphorylation In *E. coli*.

(A) *E. coli* cells were grown on minimal medium containing 0.4% glucose, 50 μg/ml of all amino acids, 50 μg/ml ampicillin, 40 μg/ml kanamycin, and 1 mM phosphate at 30°C overnight. The next day, these cultures were diluted 1/30 into minimal medium without phosphate and grown at 30°C until OD_{600} = 0.5. A portion of culture was shifted to 42°C and incubated for 30 min. Carrier-free [^{32}P] orthophosphate was added at 500 μCi/ml for 5 min at 30°C. Cells were harvested by centrifugation and resuspended in sample buffer. Samples were boiled for 5 min and analyzed by electrophoresis on a 12.5% SDS-polyacrylamide gel. Lanes correspond to K38/pGP1-2/pT7-*CDC7* at 30°C (a) and at 42°C (b), and K38/pGP1-2/pT7-7 at 30°C (c) and at 42°C (d).

(B) Cells containing either pT7-*CDC7* or pT7-7 were grown in M9CA medium at 30°C until OD_{600} = 0.5. A portion of culture was shifted to 42°C and incubated for 30 min. Cells were harvested and cellular extracts were prepared. In vitro phosphorylation was carried out for 30 min at 30°C in a buffer containing 25 mM Tris-HCl at pH 7.5, 0.1 mM DTT, 10 mM $MgCl_2$, and 1 μM [γ-^{32}P] ATP. The reaction products were analyzed on a 15% SDS-polyacrylamide gel. Lanes a (30°C) and b (42°C) show K38/pGP1-2/pT7-*CDC7*, and c (30°C) and d (42°C) show K38/pGP1-2/pT7-7. The protein molecular weight markers are shown on the left of gels.

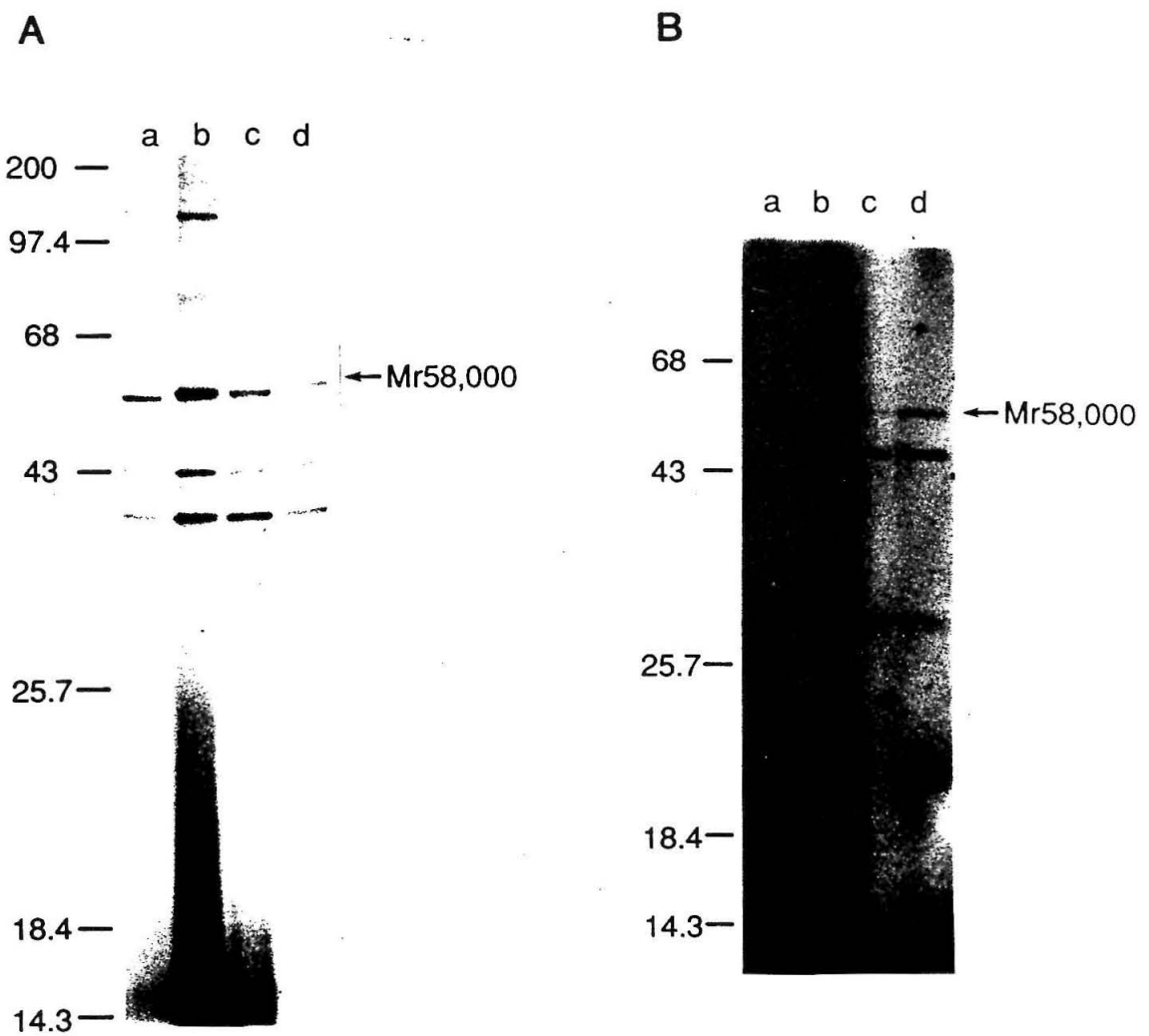

A
a b c d
200
97.4
68
43
25.7
18.4
14.3
Mr58,000
B
a b c d
68
43
25.7
18.4
14.3
Mr58,000

Figure 3. Autophosphorylation Assay on Nitrocellulose.

Cellular extracts were made after heat induction. Proteins (50 μg) were separated by 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The filter was blocked with 5% nonfat dry milk in 25 mM Tris-HCl at pH 7.5. Proteins were denatured with 7 M guanidine-HCl in 50 mM Tris-HCl at pH 8.0, 50 mM DTT, 2 mM EDTA, and 0.25% nonfat dry milk for 1 hour at 25°C, and then allowed to renature in 50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP-40, and 0.25% nonfat dry milk for 16 hours at 4°C. The filter was treated with 5% nonfat dry milk in 25 mM Tris-HCl at pH 7.5 for 30 min at 25°C and then was incubated in the same condition with 0.01 μM [γ-^{32}P] ATP, 25 mM Tris-HCl at pH 7.5, 0.25% nonfat dry milk, and either 10 mM $MgCl_2$, $ZnCl_2$, $MnCl_2$, or $CaCl_2$. After several washings with 25 mM Tris-HCl at pH 7.5 and 0.25% nonfat dry milk at 25°C, the filter was dried and visualized by autoradiography. The data with Ca^{2+} are not shown. Lanes a, c, and e contain extracts from K38/ pGP1-2/pT7-*CDC7* after heat induction. Lanes b, d, and f have extracts from K38/ pGP1-2/pT7-7 after heat induction.

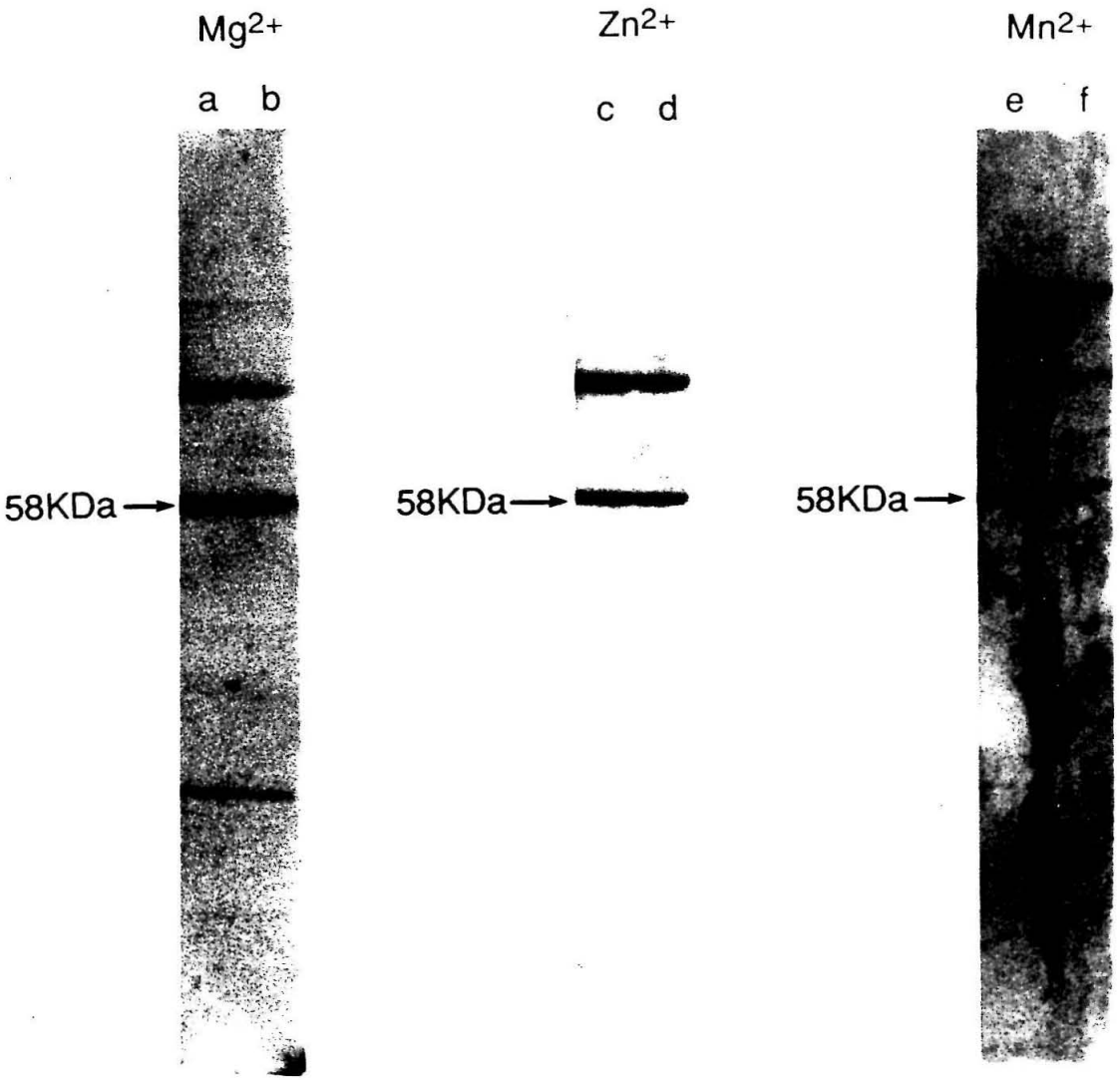

Mg2+
a b
58KDa
Zn2+
c d
58KDa
Mn2+
e f
58KDa

Figure 4. Exogenous Protein Phosphorylation with *E. coli* Extracts.

E. coli extracts from strains K38/pGP1-2/pT7-*CDC7* or K38/pGP1-2/pT7-7 were prepared and incubated for 30 min at 30°C with 1 μM [γ-^{32}P] ATP, 10 mM $MgCl_2$, 25 mM Tris-HCl at pH 7.5, and either 2 mg/ml casein (b,c), phosvitin (e,f), protamine (h,i), histone VI-S (k,l), or histone VIII-S (n,o). As a control, casein (a), phosvitin (d), protamine (g), histone VI-S (j), or histone VIII-S (m) was treated as above except that *E. coli* extracts were omitted. Lanes b, e, h, k, and n contain extracts made from K38/ pGP1-2/pT7-*CDC7*. Lanes c, f, i, l, and o have extracts from K38/pGP1-2/pT7-7. The molecular weight marker is shown on the left.

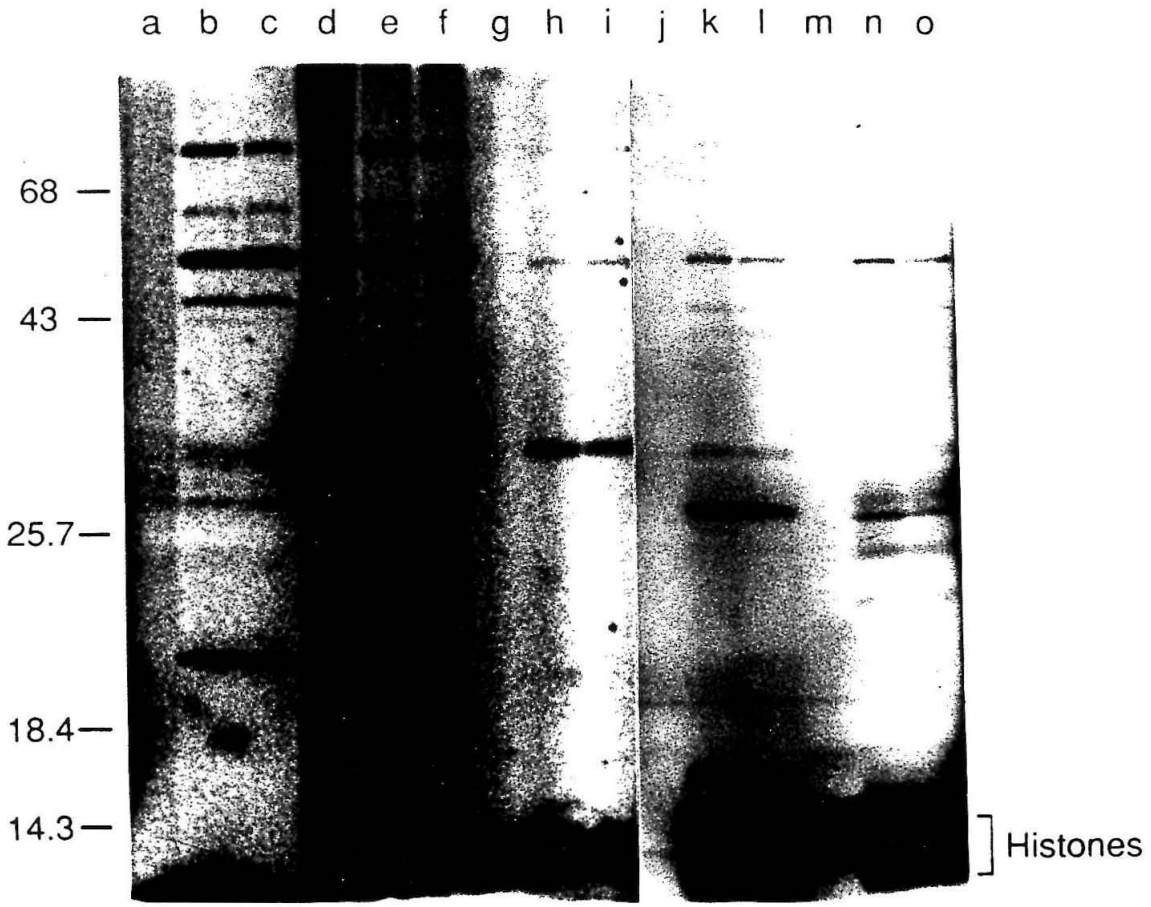

a b c d e f g h i j k l m n o
68
43
25.7
18.4
14.3
Histones

Figure 5. Purification of the CDC7 Protein in DEAE-Cellulose Chromatography.

The CDC7 protein was purified as described in Materials and Methods. After DEAE-cellulose chromatography, the putative fractions containing the CDC7 protein were determined by scintillation counting, concentrated by acetone precipitation, and analyzed on 10% SDS-polyacrylamide gels. Gels were treated with an autoradiography enhancer. Lane a contains fraction II and lanes b to t show the behavior of the CDC7 protein in DEAE-cellulose chromatography. Lanes b to t correspond to 80 to 250 mM NaCl in buffer A. The molecular weights of known proteins are shown on the left of the gel.

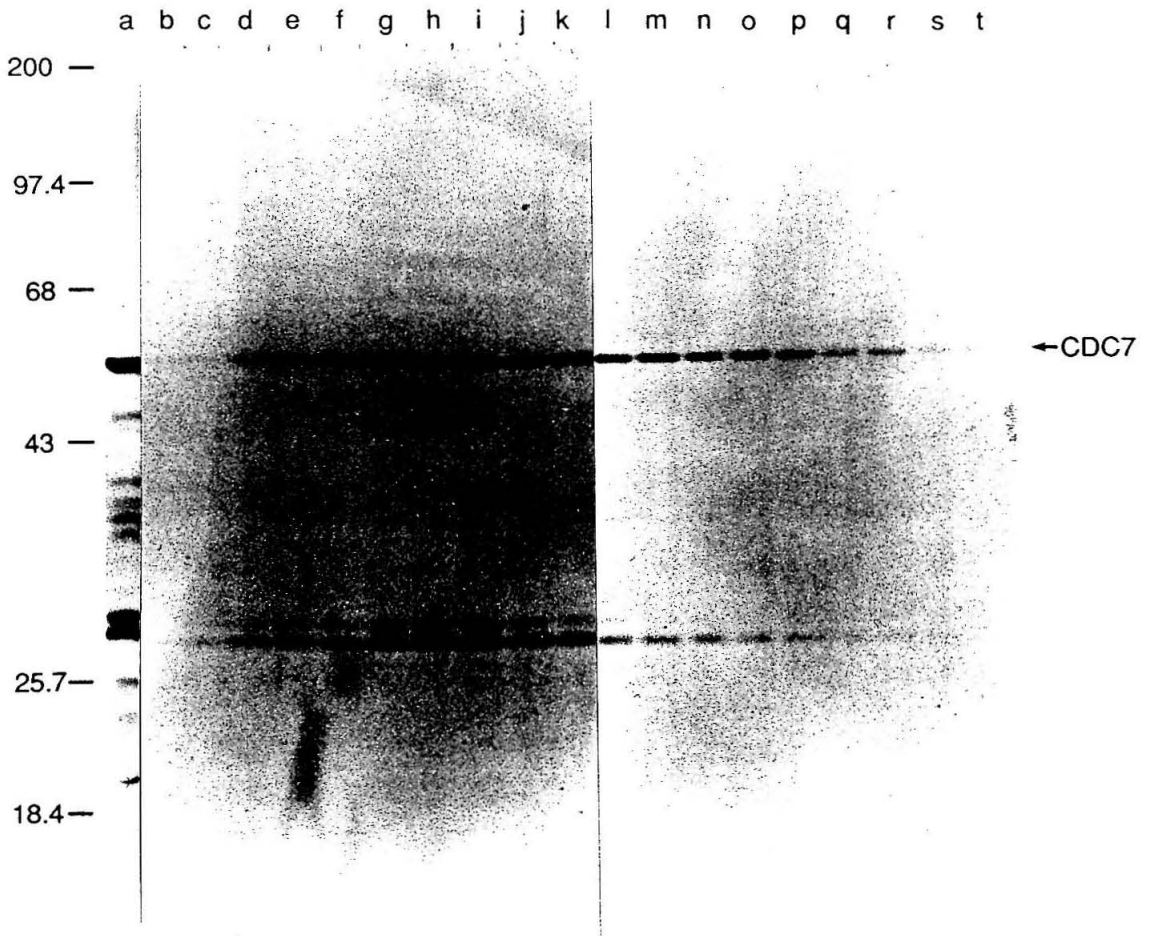

a b c d e f g h i j k l m n o p q r s t
200
97.4
68
43
25.7
18.4
←CDC7

Figure 6. Purification of the CDC7 Protein on a Phosphocellulose Column. After DEAE-cellulose chromatography, fraction III was concentrated by ammonium sulfate precipitation and applied to a column of P11 phosphocellulose. The column was eluted with a NaCl step gradient consisted of 0.05, 0.1, 0.2, 0.4, 0.6, and 1.0 M NaCl. Only fractions containing 0.05 (b), 0.6 (c), and 1.0 M NaCl (d) showed radioactivity high enough to be analyzed on SDS-polyacrylamide gels. Lane a contains fraction II. The gel was treated with an autoradiography enhancer. The molecular weight markers are shown in the left side of the gel.

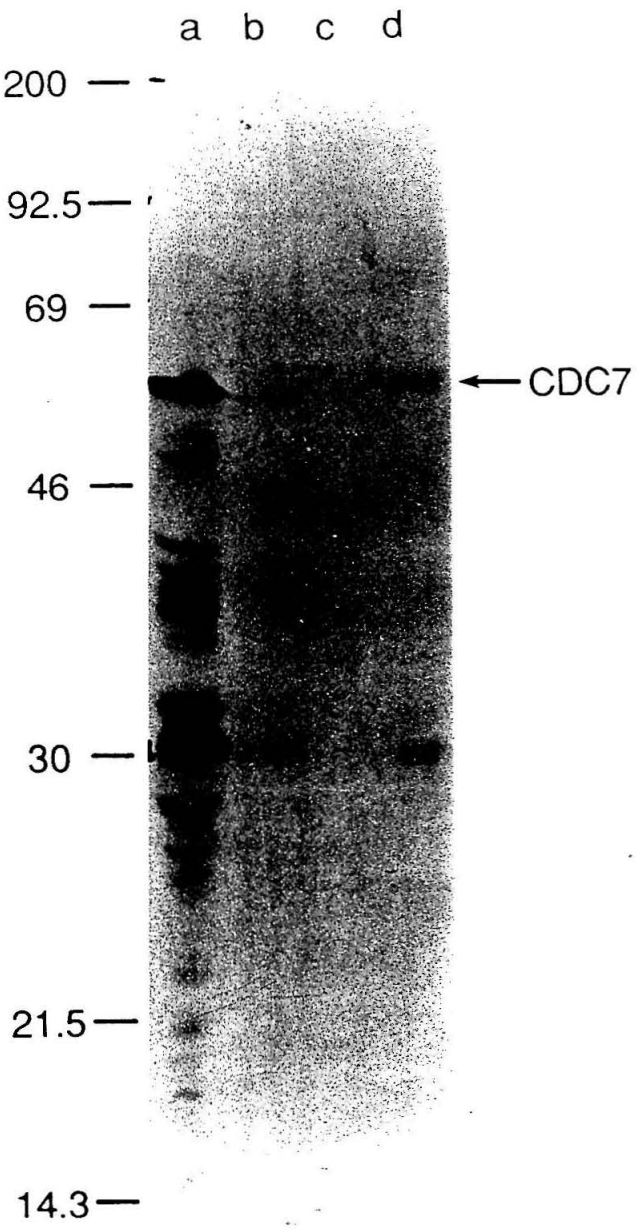
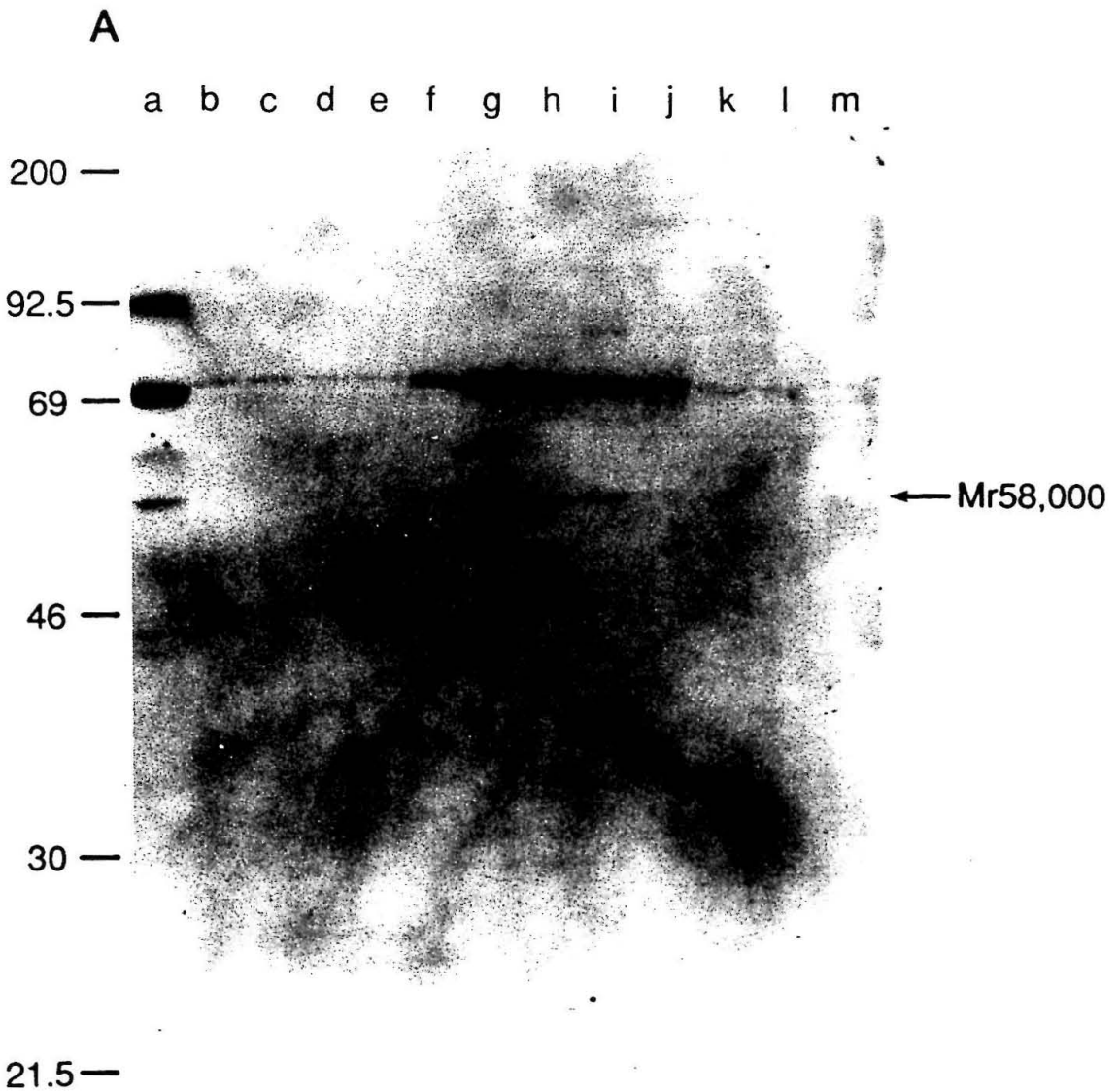

a
b
c
d
200
92.5
69
46
30
21.5
14.3
CDC7

Figure 7. Autophosphorylation Assay after DEAE-Cellulose Chromatography.

(A) Extracts were prepared from pT7-*CDC7* containing cells and fractionated on a column of DE52-cellulose as described in Materials and Methods. The volume of each fraction was 8 ml and 1.5 ml of each fraction were used for scintillation counting. The remaining 6.5 ml were concentrated by acetone precipitation and analyzed on a 10% SDS-polyacrylamide gel. An autophosphorylation assay on nitrocellulose was carried out as explained in legend to Figure 3. Lane a contains fraction II and lanes b to l contain fractions from 50 to 250 mM NaCl in buffer A after a 50-400 mM linear gradient. The numbers on the left indicate the molecular weights of known proteins.

(B) All the steps in this experiment are the same as those in Figure 7A except that extracts were made from pT7-7 containing cells. Lane a contains fraction II and lanes b to k correspond to fractions from 50 to 250 mM NaCl in buffer A. The molecular weights of known proteins are shown on the left of the gel.


A
a b c d e f g h i j k l m
200
92.5
69
46
30
21.5
Mr58,000

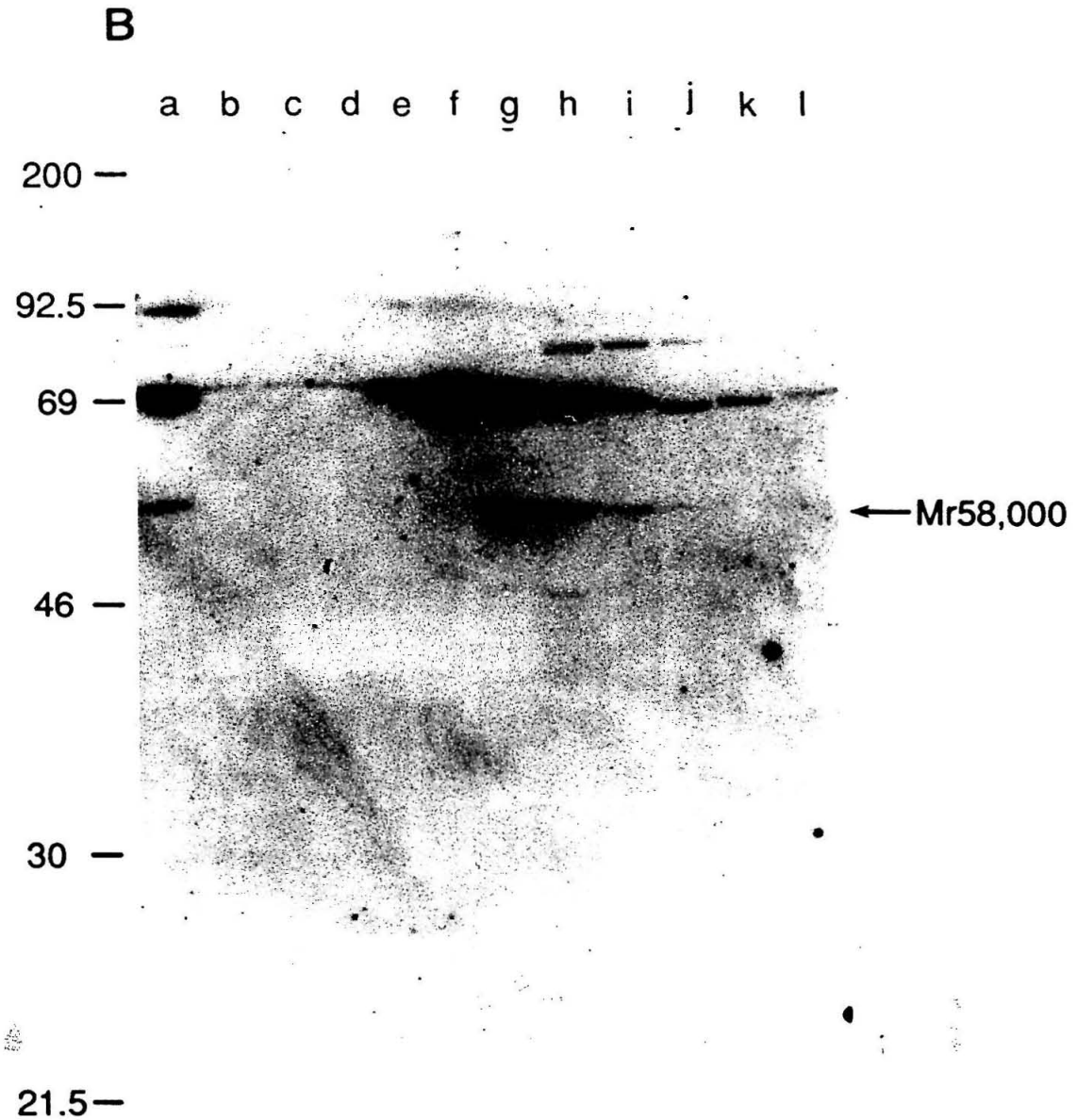
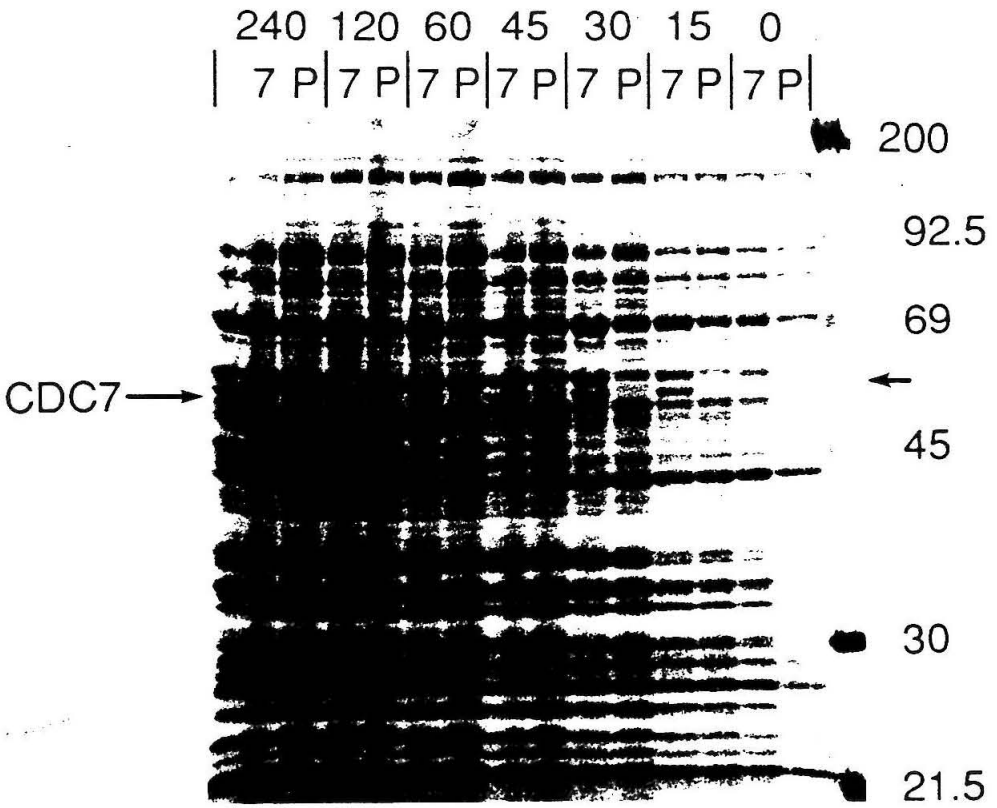

B
a b c d e f g h i j k l
200
92.5
69
46
30
21.5
Mr58,000

Figure 8. Overproduction of the CDC7 protein upon Induction with IPTG of the *tac* promoter.

Freshly transformed single colonies were used to inoculate cultures in LB medium in the presence of 50 μg/ml ampicillin. At OD_{600} = 0.4, IPTG was added to a final concentration of 1 mM. At the indicated times (in min) after IPTG induction, 0.7 ml of samples were withdrawn immediately, cells centrifuged, and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. The gel was stained with Coomasie blue; "p" indicates pKK223-3 and "7" indicates pKK-*CDC7*. The numbers on the right are the molecular weights of known proteins.


240 120 60 45 30 15 0
7 P 7 P 7 P 7 P 7 P 7 P 7 P
200
92.5
69
45
30
21.5
CDC7

CHAPTER III

The CDC7 Protein of *Saccharomyces cerevisiae* Is a Phosphoprotein that Contains Protein Kinase Activity

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Abstract

The CDC7 protein of *Saccharomyces cerevisiae* may be involved in the G1/S phase transition and/or in the initiation of mitotic DNA synthesis. The *CDC7* gene has two in-frame AUG codons as possible translation start sites, which would produce 58 and 56 kDa proteins, respectively. Both p58 and p56 derived from recombinant plasmids complement the temperature sensitive growth defect of the *cdc7-1* allele. To determine the biochemical function of the CDC7 protein, the *CDC7* gene was cloned and polyclonal antibodies were produced against the CDC7 protein. CDC7 immune complexes prepared from yeast using these antibodies phosphorylate histone H1. Kinase activity is thermolabile in strains carrying the *cdc7-1* temperature sensitive mutant allele and is elevated more than 10-fold in strains carrying plasmids overexpressing either p56 or p58, confirming that the kinase in the immunoprecipitates is the *CDC7* gene product. In addition we show that CDC7 is a phosphoprotein itself. Indirect immunofluorescence and biochemical fractionation show that the CDC7 protein is present at relatively high concentrations in the nucleus compared to the cytoplasm, suggesting that nuclear proteins may be substrates for the CDC7 protein.

Introduction

Two major events define the eukaryotic cell cycle: replication of the chromosomes during S phase and segregation of the chromosomes during mitosis. Replication and segregation are separated by two gap periods, G1 and G2, during which the cells prepare for these events. There are also two major control points for the cell cycle in G1 and G2, the point of commitment to DNA synthesis in G1 and the regulation of progression into mitosis in G2. Although we have made great strides in understanding mitotic control in the last three years, relatively little is known about the events in G1 that lead to S phase. In yeast, the point of commitment to DNA synthesis is defined as "START," early in G1. DNA synthesis does not ensue immediately after START, however, and it is not clear whether the lag is due to assembly of the replication apparatus or to a cascade of controls initiated at START. *CDC28*, *CDC4*, and *CDC7* define a dependent series of events set in motion at START (1-3). *CDC28* encodes a protein kinase subunit, the analog of the highly conserved cdc2/MPF kinase subunit involved in regulation of mitosis (3). *CDC4* is homologous to one subunit of the signal transducing protein, transducin (4). The DNA sequence of the *CDC7* gene predicts a protein which has homology to catalytic domains of the protein kinases CDC28/cdc2, NIM1, and a number of kinase-related transforming proteins (5). The CDC7 sequence differs from that of other protein kinases in the so-called phosphate acceptor domain, however (5,6).

We have been interested in the role of *CDC7* in the events linking START and the initiation of DNA replication. Mutations in *CDC7* appear to block a precondition for DNA synthesis because cells carrying these lesions cannot start new rounds of DNA replication after a shift from permissive to nonpermissive temperature (7,8). The *cdc7* mutation arrests cells at the late G1/S phase boundary, prior to the initiation of DNA synthesis and shows the dumbbell shaped terminal phenotype typically associated with a DNA synthesis or nuclear division defect (7,9). Cycloheximide does not inhibit initiation and completion of DNA synthesis when added after return of *cdc7* cells from the restrictive to the permis-

sive temperature (10), suggesting that all protein synthesis late in G1 essential for initiation of DNA replication can be completed during the *cdc7* block. The phenotype of *cdc7* mutants would be consistent with *CDC7* mediating an important signal for initiation of replication or with *CDC7* participating in initiation itself. The function of the CDC7 protein in the G1/S transition and/or in the initiation of mitotic DNA replication may be accomplished by its protein kinase activity, for instance, by periodically activating a crucial replication protein. This would imply that the function of *CDC7* itself must be periodically regulated. Since overall levels of CDC7 RNA and protein do not seem to be limiting for entry into S phase (11), fluctuation in CDC7 levels does not account for its periodic function. Thus, either the activity of CDC7 itself must change during the cell cycle, or its location in the cell may vary, or there is a periodic change in a protein with which it interacts, for instance, a substrate.

In order to investigate whether the activity of CDC7 changes during the cell cycle, we wished to define its biochemical activity and to devise an assay that could be used to follow CDC7 activity through the cell cycle. In this paper we show that the CDC7 protein contains protein kinase activity that can be measured specifically in nuclear extracts using histone H1 as substrate. We also show that the CDC7 protein is modified by phosphorylation, and that it is located in the nucleus.

Materials and Methods

Strains

Saccharomyces cerevisiae strains were SEY6210 (*MATα his3-Δ200 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9 leu2-3,112*) and YC7379 (*MATα ade1 his7 lys2 tyr1 ura3-52 cdc7-1*). Strain SEY6210 was obtained from S. D. Emr (California Institute of Technology, Pasadena, CA 91125) and strain YC7379 was constructed for this work.

Production of CDC7 Antisera

The *CDC7* gene was introduced into *Escherichia coli* using the p*tac* expression vector, pKK223-3 (12). Briefly, the gene was brought under the control of the *tac* promoter by inserting the ClaI/EcoRI fragment from *CDC7* into the EcoRI site of the vector, and reconstituting the *CDC7* coding sequence with the oligonucleotide 5'-AA TTC ATG ACA AGC AAA ACG AAG AAT AT-3'. The protein was partially purified by chromatography on DEAE-cellulose (Whatman) and heparin-agarose columns. Bands corresponding to the CDC7 protein were excised from SDS-polyacrylamide gels and used for immunization of two rabbits whose sera were free of anti-yeast IgG. Multiple injections were given until the CDC7 protein was detected on immunoblots of yeast extracts. A 1:600 dilution of antibody is sufficient to detect 50 ng of CDC7 protein by protein blotting.

Indirect Immunofluorescence of Yeast Cells

This procedure is based upon the techniques developed by Kilmartin et al. for whole mount yeast cells (13,14) and modified from the procedure as described previously (15).

Isolation of Yeast Nuclei

Yeast nuclei were prepared by the method of Hurt et al. (16). The nuclear pellet was resuspended in 50 ml of Percoll gradient buffer containing 50% Percoll (Pharmacia), 40 mM PIPES at pH 6.5, 5 mM $MgCl_2$, and 0.5 mM PMSF and centrifuged in a Beckman VTi50 rotor at 21,000 rpm for 40 min. Three bands were shown after centrifugation. The nuclear band was in the middle of the gradient and collected by inserting a needle into that position. The nuclear fraction was diluted with 3 vol of 40 mM PIPES at pH 6.5, 5 mM $MgCl_2$, and 0.5 mM PMSF and centrifuged at 12,000 rpm for 20 min to remove Percoll.

Immunoprecipitation and Protein Kinase Assays

Nuclear fractions were prepared as above except the Percoll gradient step was not performed. Crude nuclei were resuspended in 50 mM Tris-HCl at pH7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM PMSF, and 4M $(NH_4)_2SO_4$ was added to 0.4 M final

concentration. The lysate was spun at 100,000 *g* for 30 min. The supernatant was precipitated with ammonium sulfate (70% saturation) and centrifuged at 100,000 *g* for 20 min. The pellet was dissolved in 3 ml of 15% (w/v) sucrose, 50 mM Tris-HCl at pH 7.5, 50 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, and 0.5 mM PMSF, dialyzed against the same buffer, and frozen in aliquots at -70°C until needed. Immunoprecipitations and protein kinase assays were performed as described by Reed et al. (3) with some modifications. Ten µl of preimmune serum was added to 20 µl of a protein A-Sepharose (100 mg/ml) suspension and incubated for 1 hr at 4°C. The beads were collected by centrifugation and resuspended with 0.5 mg of cytosolic or nuclear fractions from various yeast cultures. After 1 hr at 4°C, the suspension was centrifuged and the supernatant was transferred to a new tube. The pellet contained preimmune complexes and was saved for further assays. The supernatant was used for a second immunoprecipitation with 10 µl of CDC7 antisera. Then, the preimmune and CDC7 immune complexes were washed twice in 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Tween 20, and 0.2 mM EDTA, followed by two washes in 0.5 M LiCl, and 0.1 M Tris-HCl at pH 7.5, and finally by two washes with 25 mM Tris-HCl at pH 7.5, 5 mM NaF, 10 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 mM PMSF (reaction buffer). The beads were resuspended in 30 µl of the reaction buffer. For kinase assays, 2 µg of histone H1 (Boehringer Mannheim Biochemicals) was added to 30 µl of redissolved immunoprecipitates along with 300 µM [γ-^{32}P]ATP (0.3 µCi/nmol). After 15 min at 37°C, reactions were terminated by the addition of SDS sample buffer, and phosphorylated products were analyzed by electrophoresis on SDS-polyacrylamide gels.

Results

Overexpression of *CDC7* in Yeast

The *CDC7* gene was isolated by transformation of a *ura3-52 cdc7-1* strain of *S. cerevisiae* with a YCp50 yeast genomic library constructed in this laboratory (17). To define the catalytic activity of the CDC7 protein, we used the cloned gene to facilitate isolation of the CDC7 protein from *E. coli* and yeast. The CDC7 protein expressed in *E. coli* using three different expression systems was not active as a protein kinase with any substrates tested (data not shown). Since we were unable to demonstrate CDC7 kinase activity in *E. coli*, we reasoned that there might be a specific modification of the protein or a specific cofactor only found in yeast. Therefore, we next undertook to isolate the protein from yeast. For this purpose, we cloned *CDC7* in a *GAL10* expression vector. The nucleotide sequence of CDC7 contains two in-frame ATG codons, one at nucleotide 1 and the other at nucleotide 55. The gene product arising from the first ATG in CDC7 is called p58, and the one from its second ATG is called p56. Since it is not known which ATG is used in vivo, we produced both p58 and p56. The entire *CDC7* gene was inserted into the unique EcoRI site of the *GAL* expression vector, pSEY18-Gal (S. D. Emr, California Institute of Technology, Pasadena, CA 91125), a high copy number vector that allows inducible expression of the CDC7 protein. The fragment carrying the *CDC7* gene was created by ligation of a synthetic linker (EcoRI-ATG ACA AGC AAA ACG AAG AAT-ClaI), representing the first seven amino acids of CDC7, to the 2.4 kb ClaI-EcoRI fragment of CDC7. In order to express p56, the first ATG was removed by cutting at the ClaI site, which lies between the two initiation codons, followed by further deletion with Bal31 of the DNA between that ClaI site and the second ATG. The plasmids encoding p58 and p56 were named pSYC758 and pSYC756, respectively. Both plasmids were able to complement the mitotic growth defect in *cdc7-1* cells at the nonpermissive temperature.

CDC7 protein expressed from these plasmids was analyzed by protein blotting and immunoprecipitation of ^{35}S-labeled yeast cells. As shown in Figure 1, yeast strains carry-

ing pSYC758 and pSYC756 produced *CDC7*-encoded proteins of 58 and 56 kDa, respectively. Strong signals were obtained only after galactose induction (lanes e, f and h). Thus, the CDC7 protein may be present at very low levels in wild-type cells.

Protein Kinase Activity of the *CDC7* Gene Product Isolated from Yeast

Because of the low levels of CDC7 protein present in whole cell extracts of wild-type cells and because the overproduced protein was difficult to solubilize, conventional purification was inefficient. CDC7 protein was therefore immunoprecipitated from both nuclear and cytosolic fractions of various yeast strains, and the redissolved immunoprecipitates were used in protein kinase assays employing calf thymus histone H1 as a substrate. As shown in Figure 2, histone H1 is clearly phosphorylated by immunoprecipitates prepared from nuclear extracts with CDC7 antisera, suggesting that the CDC7 protein contains protein kinase activity.

Since in order to preserve enzymatic activity, immunoprecipitates must be prepared under conditions where additional proteins may coprecipitate (although we removed proteins interacting nonspecifically with the rabbit IgG by first precipitating with preimmune sera), to insure that the kinase activity observed was due to the *CDC7*-encoded protein and not to a contaminating kinase, two controls were carried out. First, we demonstrated overproduction of kinase activity in extracts of *cdc7-1* mutant strains carrying plasmids pSYC758 and pSYC756 compared to extracts of the same strain carrying vector alone. Comparison of lanes c, d and e in Figure 2 indicates that the level of kinase activity was over 10-fold higher in the CDC7 overproducer than in a strain containing vector alone. The magnitude of overproduction was estimated from four independent experiments and densitometer tracings of the autoradiograms. Since these assays measure extents of phosphorylation rather than initial rates, this is a minimum estimate of the actual overproduction. Second, we demonstrated that the kinase activity was thermolabile in immunoprecipitates prepared from *cdc7-1* mutants. Comparison of lanes b and c in Figure 2 shows that the kinase activity is thermolabile in the strain carrying vector alone (Q_{23}^{37}=0.2). *Conversely, in*

the same strains carrying *CDC7*-containing plasmids, kinase activity at 37°C is at least 2-fold higher than at 23°C [lanes f and g, (Q_{23}^{37}=2)], indicating that the thermolability observed in lanes b and c is not due to nonspecific factors in the strain background affecting kinase activity at 37°C vs. 23°C and that the kinase is associated with the *CDC7* gene product. We could not detect CDC7-dependent phosphorylation of histones with preimmune complexes (data not shown).

CDC7 Is a Phosphoprotein

In immunoprecipitates from strains overproducing p58 and p56, in addition to phosphorylation of histone H1, a protein of 58 or 56 kDa, respectively, was also phosphorylated. As shown in Figure 3, phosphorylation of these 56 and 58 kDa proteins was detectable only in nuclear and not in cytoplasmic extracts. It is likely that these proteins are the *CDC7* gene product. The 48 kDa band that appears in this experiment was also seen in immunoprecipitates prepared with preserum and may represent a contaminant (such as IgG), or it may be an activator or a substrate of CDC7.

The observed phosphorylation of p56 and p58 by the CDC7 immunoprecipitates could be due either to autophosphorylation or to a coprecipitating kinase. The results suggested, however, that CDC7 might occur in vivo as a phosphoprotein. To investigate the latter possibility, cells carrying pSYC758 were grown on galactose, labeled with $^{32}P_i$, and immunoprecipitated with CDC7 antisera. As shown in Figure 3 (lane i), strains overproducing p58 do contain a 58 kDa phosphoprotein. This protein is not observed in the absence of overproduction (lane j) or in extracts precipitated with preimmune serum (lane k).

Localization of the *CDC7* Gene Product by Indirect Immunofluorescence and Subcellular Fractionation

Knowledge of the subcellular localization of the CDC7 protein may be useful in establishing its molecular function by narrowing the range of potential substrates and cellular proteins with which it interacts. Using indirect immunofluorescence microscopy with CDC7 antisera, we found intense immunofluorescence in the nuclei of CDC7-overproduc-

ing cells (Figure 4A). Fainter, but distinct immunofluorescence was also seen in the cytoplasm. In the experiment shown, cells were also stained with Hoechst 33258 to verify the position of the nuclei (Figure 4B). However, identical staining patterns were observed in other experiments where only anti-CDC7 staining and Nomarsky were used, and therefore the patterns are not due to leakage from the additional dye. Thus, the CDC7 protein appears to be present in both the cytoplasm and nucleus but to be relatively concentrated in the nucleus. Only faint CDC7-specific immunofluorescence was detected when cells carrying the vector pSEY18-Gal were stained with CDC7 antisera in an identical manner (data not shown). The difficulty in visualizing CDC7 antigen in the absence of overproduction suggests that the levels of CDC7 protein in yeast are low, in accord with the immunoblot analysis (Figure 1, lanes a and d).

The nuclear localization of the CDC7 protein was corroborated by biochemical fractionation. Figure 5 shows immunoblot analysis of CDC7 protein in cytosolic and nuclear fractions. Equal amounts of protein, rather than equal numbers of cell equivalents, were loaded in each lane, because of the difficulty of quantitating cell equivalents that are due to uncertainty in measurement of losses experienced during the purification of nuclei. These results demonstrate, however, that CDC7 is relatively concentrated in the Percoll gradient purified nuclei. When 5-fold excess of cytosolic protein is analyzed, CDC7 is detectable (not shown). We estimate that both p58 and p56 were present in at least 20-fold higher concentration in nuclear fractions than in cytosolic fractions (Figure 5). ABF1 and SSB1, nuclear proteins previously studied in this laboratory (18,19), were also observed in our nuclear fractions only (data not shown). Comparison of the CDC7 sequence with the nuclear targeting signal consensus revealed putative nuclear localization signals in at least six different positions.

Discussion

The phenotype of *S. cerevisiae cdc7* mutants suggests that the *CDC7* gene product has multiple functions. CDC7 may be involved in the initiation of mitotic DNA synthesis, premeiotic recombination, formation of ascospores, and error-prone DNA repair (20,21). To date, however, the molecular roles of CDC7 in those events have not been characterized. As a step toward investigating the functions of CDC7 in yeast, we have identified the CDC7 protein as a protein kinase in vegetatively growing yeast cells. [While our work was in review, a similar result was presented by others (22), although kinase activity could only be observed after overproduction.] We have also shown that the CDC7 protein is phosphorylated in vivo and is found in the nucleus as well as in the cytoplasm. The demonstration of protein kinase activity in the CDC7 protein is consistent with preliminary experiments showing that in vitro transcription and translation of the *CDC7* gene produces a protein kinase activity (23). CDC7 appears to be present at low abundance in yeast as predicted from the codon utilization analysis of the CDC7 open reading frame. We have, however, found a very faint band of p58 in immunoblots performed with nuclear extracts of wild-type yeast strains when alkaline phosphatase conjugates were used to visualize the CDC7 protein (unpublished data).

As suggested by the amino acid sequence, the CDC7 protein seems to carry a protein kinase activity. Using exogenous histone H1 as a substrate, we demonstrated protein kinase activity in CDC7 immunoprecipitates of nuclear extracts of wild-type cells without overproduction. The histone phosphorylation was thermolabile in *cdc7-1* extracts and phosphorylation was proportional to the amount of p58 present. Elevated kinase levels in the strain overproducing p56 suggest that p56, as well as p58, is active as a kinase. In addition to acting as protein kinases, both p56 and p58 also appear to be substrates of a phosphorylating activity in the immunoprecipitates. It is not yet clear if this represents autophosphorylation or coprecipitation of another kinase. Our finding that CDC7 protein is

a phosphoprotein in vivo, suggests that the in vitro phosphorylation may be physiologically significant.

According to indirect immunofluorescence and subcellular fractionation studies, both forms of CDC7 are concentrated in the nuclei of CDC7-overproducing cells. We are not sure whether the staining patterns reflect a role for CDC7 in both the cytoplasm and nucleus or if they simply result from the delocalization of the exclusively nuclear protein that is due to overproduction. The demonstration that CDC7 is associated with the nuclei of mitotic cells suggests that CDC7 may function in the mitotic cell cycle by phosphorylating proteins involved in the initiation of DNA synthesis and/or in the G1/S phase transition.

Although basal level expression requires an element in the promoter that is homologous to a c-fos activating sequence, CDC7 mRNA remains at a constant level throughout the cell cycle when measured in cells released from α-factor arrest (11,24). Furthermore, indirect evidence suggests that the CDC7 protein exists in excess of the amount required for a single mitotic cell division (11). Yet CDC7 is required at a discrete point in the cycle. It will be of interest to investigate whether phosphorylation of CDC7 protein, translocation to the nucleus, catalytic activity of the CDC7 protein kinase and/or availability of a crucial substrate is regulated through the cell cycle.

Acknowledgement

We appreciate the help of Michael W. Clark with the indirect immunofluorescence microscopy. We thank Ambrose Jong for preparing the *cdc7-1 ura3-52* strain used to isolate the *CDC7* gene. This work was supported in part by a Developmental Biology Grant from the Lucille P. Markey Charitable Trust and the National Science Foundation CHE-8509637.

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Figure 1. Characterization of CDC7 antibody by immunoblot and immunoprecipitation.

(Left) Stationary cultures of yeast strain SEY6210/pG12/pSEY18-Gal (lanes a and d), and the CDC7-overproducing strains SEY6210/pG12/pSYC756 (lanes b and e), and SEY6210/pG12/pSYC758 (lanes c and f) grown in synthetic complete (SC) medium (minus uracil and leucine) plus 2% glucose were diluted 1:10 in SC medium (minus uracil and leucine) containing either 2% glucose (lanes a, b, and c) or 2% galactose (lanes d, e, and f). Plasmid pG12 provides the GAL4 protein essential for expression from the *GAL10* promoter (25). After 8 hrs at 30°C, whole yeast lysates were prepared by disruption of the cells with glass beads and clarified by centrifugation. The pellets were resuspended in buffer containing 1% SDS and analyzed on a 10% SDS-polyacrylamide gel. Proteins were then electroblotted to a nitrocellulose membrane. This immunoblot was stained with anti-CDC7 antisera at a dilution of 1:250, and visualized by using the Bio-Rad ImmunBlot assay kit (goat anti-rabbit IgG horseradish peroxidase conjugate).

(Right) For labeling with [^{35}S]methionine (Trans ^{35}S-label, ICN), strain SEY6210/pG12/pSYC758 was grown in SC medium (minus uracil, leucine, and methionine) plus 2% glucose, to 2 x 10^7 cells/ml. Cells were harvested, washed twice and resuspended in the same medium containing 2% galactose. After 1 hr at 30°C, 250 μCi of [^{35}S] methionine was added and incubation continued for 40 min. Cells were lysed in 100 μl of 50 mM Tris-HCl at pH 7.5, 1 mM EDTA, and 1% SDS by vortexing in the presence of glass beads followed by boiling for 5 min. The lysate was diluted by the addition of 1 ml of buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.5% Tween 20), centrifuged, and the supernatant was used for immunoprecipitation with 5 μl of preimmune sera (lane g) or 5 μl of CDC7 antisera (lane h). Samples were analyzed by electrophoresis in 10% SDS-polyacrylamide.

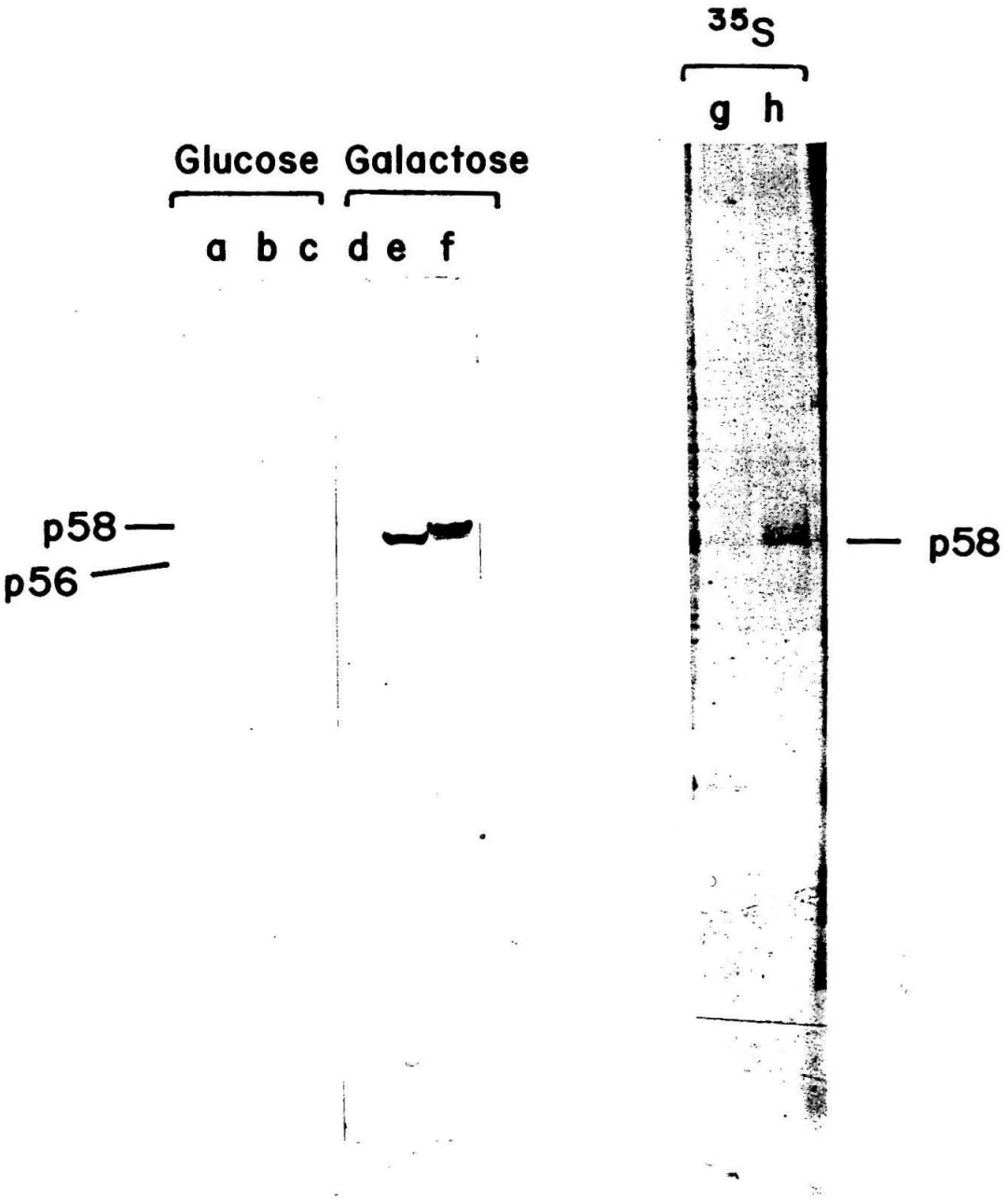

35S
g h
Glucose Galactose
a b c d e f
p58
p56
p58

Figure 2. CDC7-dependent phosphorylation of histone H1.

Nuclear extracts were prepared from strain YC7379 (*cdc7-1*), carrying pSEY18-Gal, pSYC756, or pSYC758. Cells were grown at 23°C and proteins (0.5 mg) were immunoprecipitated twice: proteins interacting nonspecifically with the rabbit IgG were removed by precipitating with preimmune sera and the supernatant was subjected to a second immunoprecipitation with CDC7 immune sera. The final pellet was preincubated at 23°C (lanes b and f) and 37°C (lanes a, c, d, e, and g). After 30 min, 2 μg of histone H1 and 300 μM [γ-^{32}P] ATP were added and incubation continued for 15 min. Reaction products were analyzed on a 10% SDS-polyacrylamide gel, stained with Coomassie blue, dried, and autoradiographed. Lane a, histone H1 alone; lane b, YC7379/pSEY18-Gal, 23°C; lane c, YC7379/pSEY18-Gal, 37°C; lane d, YC7379/pSYC756, 37°C; lane e, YC7379/pSYC758, 37°C; lane f, YC7379/pSYC758, 23°C; lane g, YC7379/pSYC758, 37°C.

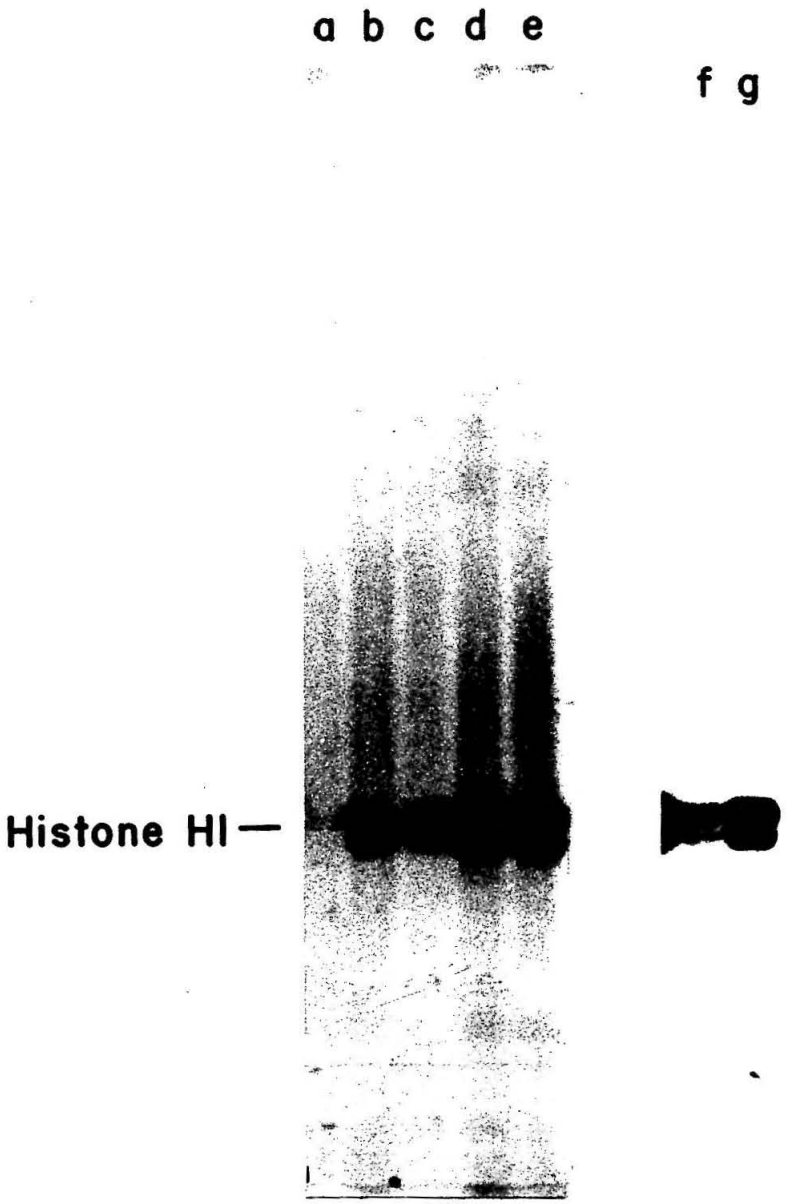

a b c d e
f g
Histone HI

Figure 3. Phosphorylation of the CDC7 protein in vivo and in vitro.

(Left) Proteins (0.5 mg) of nuclear (lanes a-d) and cytosolic (lanes e-h) fractions were immunoprecipitated twice as in Figure 2 and incubated for 30 min at 37°C in the presence of 10 μCi of [γ-^{32}P] ATP (3000 Ci/mmol; Amersham). Samples were analyzed on a 10% SDS-polyacrylamide gel. Lanes a and e, SEY6210/pG12/pSYC758; lanes b and f, SEY6210/pG12/pSYC756; lanes c and g, SEY6210/pG12/pSEY18-Gal; lanes d and h, YC7379/pSEY18-Gal.

(Right) ^{32}P-labeled CDC7 immunoprecipitates were prepared as follows. 5 x 10^7 cells of strain SEY6210/pG12/pSEY18-Gal (lane j) or SEY6210/pG12/pSYC758 (lanes i and k) were labeled for 1 hr with 0.5 mCi of [^{32}P] orthophosphate (New England Nuclear) in reduced phosphate minimal medium (26) containing 2% galactose. Cell lysis and immunoprecipitation were performed as described in the legend to Figure 1 except that either 10 μl of preimmune sera (lane k) or 10 μl of CDC7 immune sera (lanes i and j) were used.

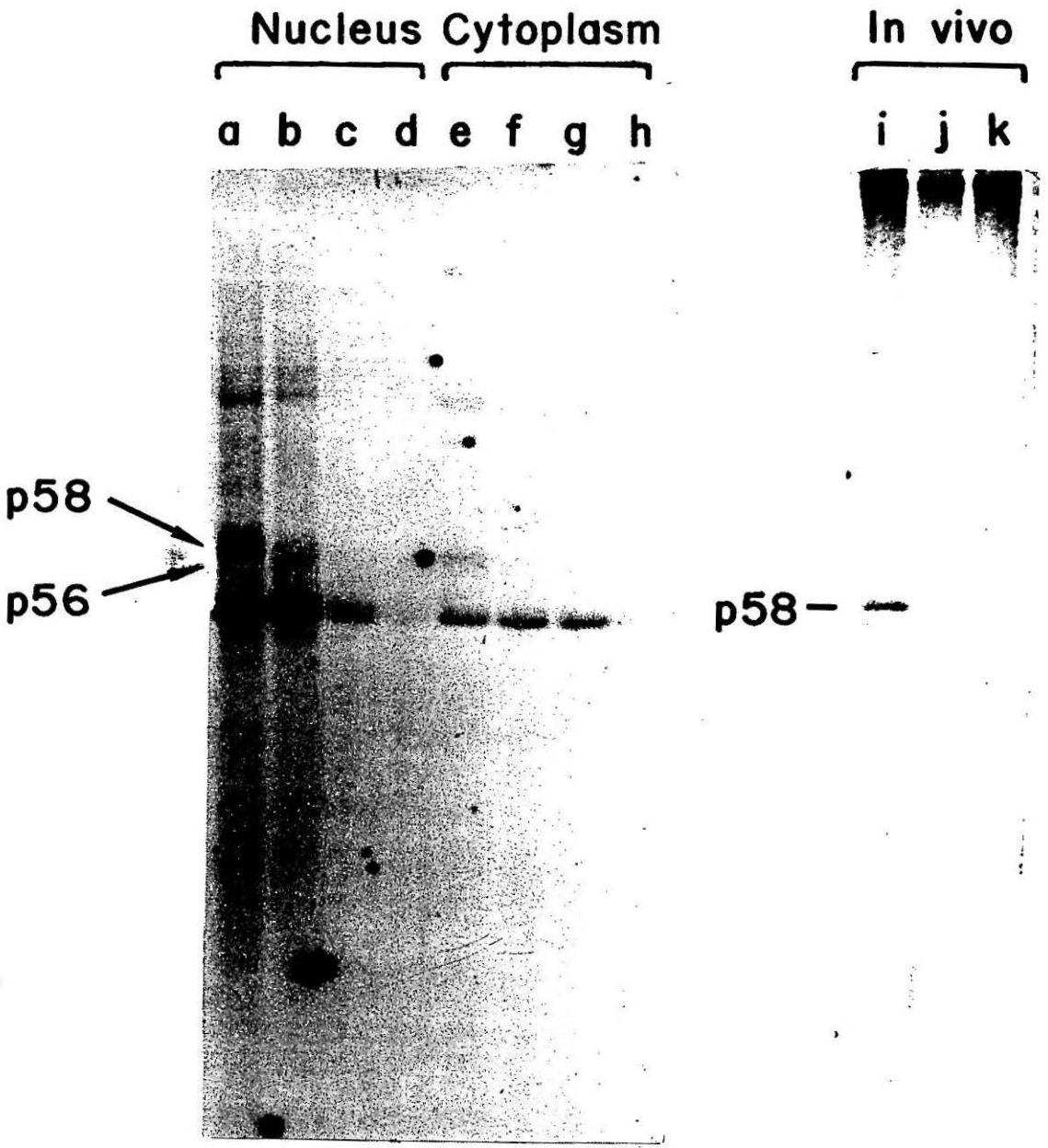

Nucleus
Cytoplasm
In vivo
a b c d e f g h
i j k
p58
p56
p58

Figure 4. Subcellular localization of the CDC7 protein.

Yeast strain SEY6210/pG12/pSYC758 was grown to stationary phase in SC medium (minus uracil and leucine) plus 2% glucose. Cells were harvested, washed twice with SC medium (minus uracil and leucine) containing 2% galactose, and resuspended in the same medium. At OD_{600}=0.4, cells were fixed with formaldehyde, digested with β-glucuronidase and zymolyase 100T, immobilized on polylysine coated slides, and stained with polyclonal anti-CDC7 antiserum (A) and Hoechst 33258 (B). (A) Viewed under rhodamine excitation wavelength. (B) Yeast nuclei were visualized by staining with the dye Hoechst 33258. (Bar = 10 μm)

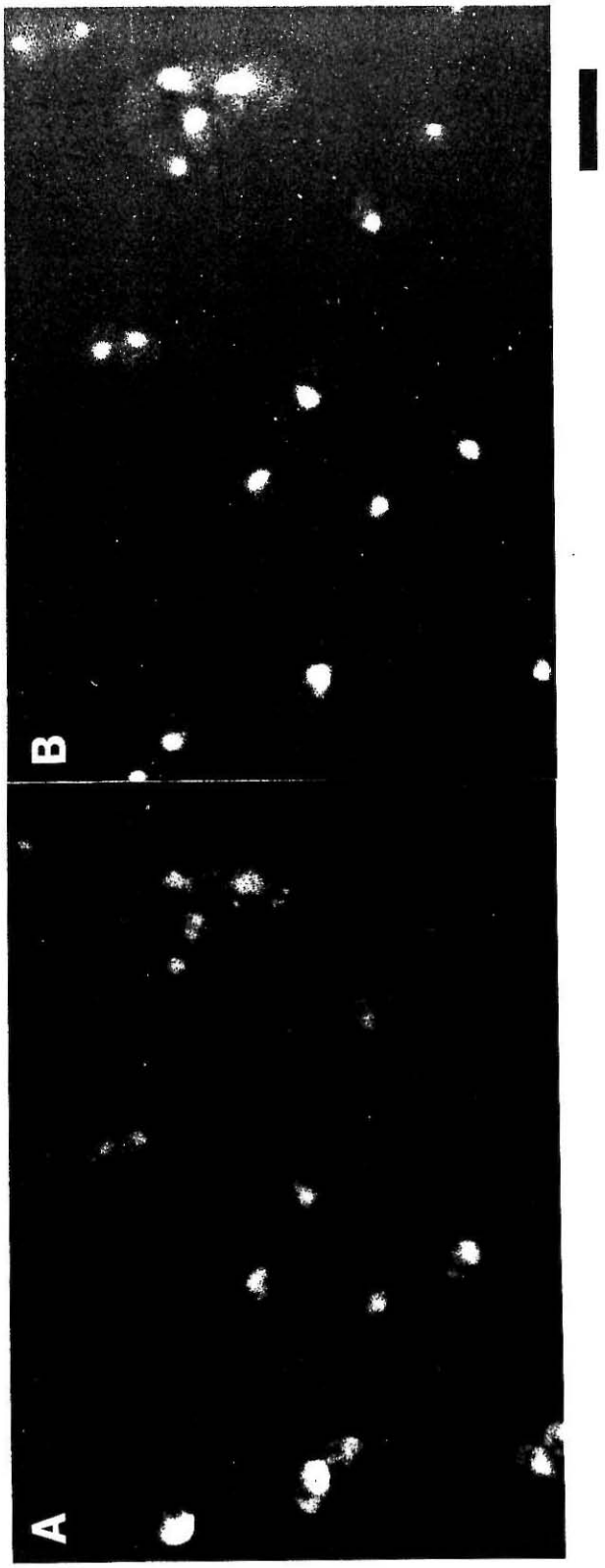
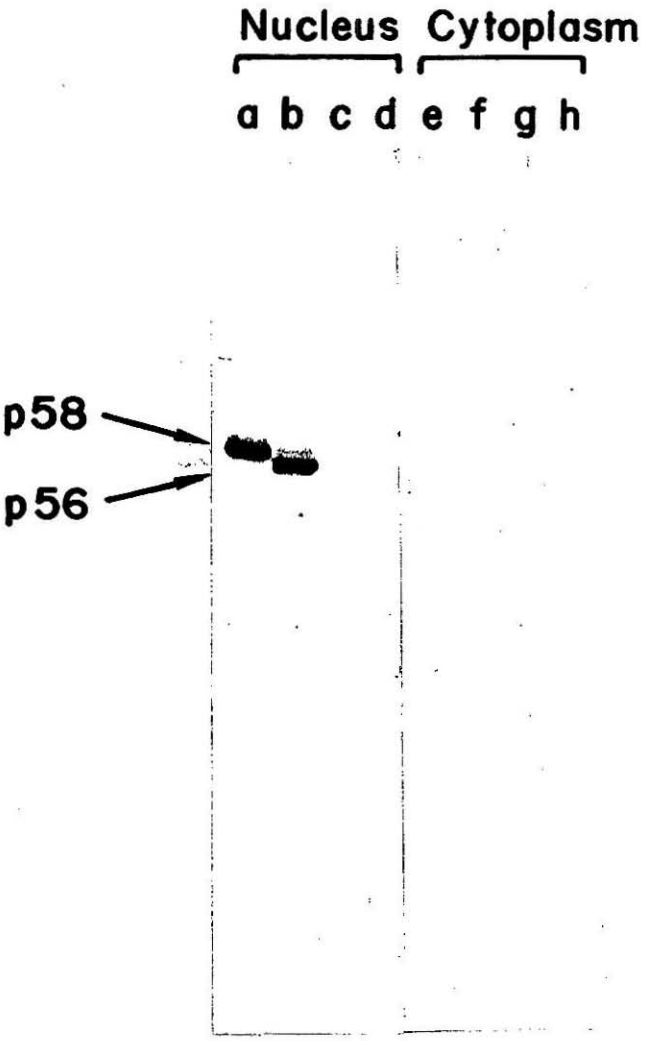

B
A

Figure 5. Localization of CDC7 by subcellular fractionation.

Proteins (100 μg) of nuclear (lanes a-d) and cytosolic (lanes e-h) fractions were separated by a 10% SDS-polyacrylamide gel, electroblotted to nitrocellulose, and stained with anti-CDC7 antiserum (diluted 1:250). Arrows on the left indicate the immunoblot bands for CDC7-p58 and CDC7-p56, respectively. Lanes a and e, SEY6210/pG12/pSYC758; lanes b and f, SEY6210/pG12/pSYC756; lanes c and g, SEY6210/pG12/pSEY18-Gal; lanes d and h, YC7379/pSEY18-Gal.


Nucleus
Cytoplasm
a b c d e f g h
p58
p56

CHAPTER IV

A Possible Phosphorylation Cascade Operating Between the Commitment to, and Initiation of DNA Replication Involving Yeast CDC28, CDC7, and the Replication Protein, RP-A

(Submitted in Cell for publication in 1991)

Abstract

The yeast Cdc7 function is required for the G1/S transition and is dependent on passage through START, a point controlled by the Cdc28 protein kinase. *CDC7* encodes a protein kinase and we show that Cdc7 kinase activity but not abundance varies in the cell cycle. We present several lines of evidence that activation of Cdc7 kinase is at least in part through a phosphorylation mechanism which involves Cdc28. We hypothesize that Cdc7 may lead to the initiation of DNA synthesis and S phase by phosphorylating one or more DNA replication proteins. A likely candidate is the single-stranded DNA binding protein, replication protein A, RP-A, because it is phosphorylated specifically late in G1. We present evidence that both Cdc28 and Cdc7 kinases participate in this phosphorylation.

Introduction

The eukaryotic cell cycle is composed of two major events, S phase, during which the chromosomes are replicated, and M phase, during which the chromosomes are segregated. These two portions of the cycle are separated by gaps called G1 and G2, which are devoted to regulating the transitions between replication and segregation. During G1, the events leading to chromosome duplication are monitored and executed. Checkpoints ensuring completion of DNA synthesis and readiness for nuclear division occur in G2 (Hartwell and Weinert, 1989). In the past few years, significant breakthroughs have been made in determining the molecular events that regulate and promote entry into M phase (Dunphy and Newport, 1988a; Lewin, 1990). A key regulatory molecule in this process is highly conserved in eukaryotes, namely, the p34/cdc2$^+$/MPF (mitosis promoting factor) protein kinase subunit (Dunphy and Newport, 1989b). Since much less is known about the events leading to the initiation of nuclear DNA replication at the G1/S transition, we have undertaken studies of this phase of the cell cycle.

In unicellular organisms such as yeast (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*), the major control point in G1 is called START, which may or may not correspond to the "restriction point" in mammalian cells (Pardee, 1989; Pringle and Hartwell, 1981). START defines the point in the cycle after which the cell is committed to a round of DNA synthesis and no longer can undergo the alternative developmental program of conjugation. Several yeast mutants, the best studied of which is *cdc28*, arrest at a point in G1 at which conjugation is still possible and thus are said to affect START (Pringle and Hartwell, 1981; Reed, 1980). *CDC28* encodes the homolog of the p34/cdc2$^+$/MPF protein kinase subunit. Thus, it appears that the same protein kinase is involved in regulating both the G1/S and G2/M transitions during the yeast cell cycle (Ghiara et al., 1991; Nurse and Bissett, 1981; Piggott et al., 1982; Surana et al., 1991).

We have very little information on the molecular nature of the events set in motion by *CDC28* and the other START genes. While the commitment to DNA synthesis is made by passage through START, DNA synthesis does not begin immediately. A number of additional genetically defined steps must occur before the onset of DNA replication. It is likely that both induction of the genes encoding replication proteins and post-translational activation of replication proteins are preconditions for the initiation of DNA synthesis. The *CDC28*, *CDC4* and *CDC7* genes are thought to define one series of steps that must be completed before the initiation of DNA synthesis (Hartwell, 1976; Pringle and Hartwell, 1981). *CDC28* encodes a protein kinase, *CDC4* encodes a protein with homology to the β-subunit of transducin and the *ets* oncogene (Fong et al., 1986; Peterson et al., 1984) and *CDC7* encodes a protein kinase (Patterson et al., 1986; Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991). Though these activities suggest a signal transduction cascade, little information is available about their functional interrelationships.

Our focus has been to determine the biochemical basis for the position of the Cdc7 protein in the putative G1 regulatory cascade. The *CDC7* controlled events have been shown genetically to depend on completion of START, since the *CDC7* step cannot be completed during block of cells with the mating pheromone α-factor, which like *cdc28* mutations, arrests cells at START (Hereford and Hartwell, 1974). The Cdc7 protein is thought to execute its function late in G1, since cells carrying a thermosensitive (ts) *cdc7* mutation arrest mitotic growth at the G1/S phase boundary at the restrictive temperature, just before the initiation of DNA synthesis (Hartwell, 1973; 1976). These cells show a dumbbell shaped terminal phenotype typically associated with a DNA synthesis or nuclear division defect (Hartwell, 1973). Another reason for placing *CDC7* function very late in G1 is the fact that *cdc28* and *cdc4* mutants blocked at the nonpermissive temperature and then returned to the permissive temperature in the presence of the protein synthesis inhibitor cycloheximide, they do not progress further through the cycle and do not enter S phase,

while *cdc7* mutants treated in the same way enter S phase and complete DNA synthesis in the presence of protein synthesis inhibitors (Hereford and Hartwell, 1974). Further protein synthesis is thus necessary for the initiation of DNA synthesis after *CDC28* and *CDC4*, whereas all of the proteins essential for DNA replication appear to be present at the *CDC7* stage. Which crucial proteins are needed is not known, but the cyclins are likely to be among them (Wittenberg et al., 1990). *CDC7* thus mimics the final stage of the "restriction point" in mammalian cells, insofar as it was defined as the point in the cell cycle after which no further protein synthesis is necessary for initiation of DNA synthesis. Evidence suggesting the presence of Cdc7 protein in a putative DNA replication complex has been presented (Jazwinski, 1988).

In contrast to this behavior during mitosis, diploid homozygous *cdc7*-ts cells initiate premeiotic DNA synthesis normally at the restrictive temperature. However, these diploids do not form a synaptonemal complex, nor do they commit to recombination or form ascospores, indicating that the *cdc7* lesion affects the mitotic and meiotic pathways in a different, distinct manner (Schild and Byers, 1978). Furthermore, the *CDC7* gene has also been implicated in an error-prone DNA repair pathway as a member of the *RAD6* epistasis group (Njagi and Kilbey, 1982). Finally, *CDC7* may also be involved in transcriptional silencing of the HMR mating type cassette (Axelrod and Rine, 1991).

At present, little is known about how *CDC7* affects these different DNA processes. A clue as to the molecular function of *CDC7* was afforded by the demonstration that the open reading frame encoding *CDC7* contains the 11 catalytic domains characteristic of protein kinases (Patterson et al., 1986). We and others have shown that the Cdc7 protein isolated from vegetatively growing yeast cells is active as a kinase that phosphorylates histone H1 in vitro (Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991). In addition, the Cdc7 protein is modified by phosphorylation and is located in the nucleus (Yoon and Campbell, 1991). The demonstration that Cdc7 is a protein kinase associated

with the nuclei of mitotic cells suggests that Cdc7 may function in the mitotic cell cycle by phosphorylating proteins involved in the initiation of DNA synthesis and/or in the G1/S transition. Recently, Din et al. (1990) have shown that both *CDC28* and *CDC7* are required in vivo for phosphorylation of the 34 kDa subunit of the replication protein RP-A, and that this phosphorylation occurs at the G1/S transition. RP-A was originally isolated from human cells in the process of fractionating cellular extracts into components capable of reconstituting SV40 DNA replication in vitro (Fairman and Stillman, 1988; Wobbe et al., 1987; Wold and Kelly, 1988). RP-A is an SSB essential for both the initiation and elongation reactions (Wold and Kelly, 1988; Kenny et al., 1989;1990). Because of its documented cell cycle specific phosphorylation pattern, RP-A is a good candidate for direct phosphorylation by either the Cdc28 or Cdc7 kinase, and in this paper we show that both kinases do indeed phosphorylate RP-A in vitro.

Although we had some clues to the role of *CDC7* from previous work, very few clues were available as to what limits *CDC7* function to a specific point in the cell cycle. *CDC7* is not periodically transcribed, and cells carrying a deletion of *CDC7* on the chromosome can divide up to eight times after loss of a plasmid carrying the *CDC7* gene (Sclafani et al., 1988). Thus periodic activation of Cdc7 kinase rather than abundance may govern its stage-specific function. Since Cdc7 appears to be required only for the final stage of the G1/S transition, we speculated that the catalytic activity of the Cdc7 kinase might be regulated by Cdc28 as part of a cascade linking START and the onset of DNA synthesis. In this paper we present evidence to support this proposal. First, we demonstrate that Cdc7 activity, not Cdc7 protein levels vary during the cell cycle. Second, we show that phosphorylation of Cdc7 protein is required for its activity. Cdc28 kinase specifically phosphorylates the Cdc7 protein and activates Cdc7 kinase. To complement the biochemical data, we show that *CDC7* hyperexpression suppresses the temperature sensitive phenotype of a *cdc28-13* mutation at 33°C. We propose that, taken together with numerous preceding

genetic results, these biochemical studies on Cdc7, Cdc28 and RP-A define a pathway through S phase linking START and initiation of DNA replication.

Results

The Activity of Cdc7 Kinase Varies in the Cell Cycle and Is Dependent on Cdc28 Function

We have shown previously that the Cdc7 protein prepared as an immune complex from nuclear extracts of vegetatively growing cells, contains a histone H1 kinase activity (Yoon and Campbell, 1991). Genetic studies, however, suggest that Cdc7 is not active throughout the entire cell cycle, but that it functions in vivo only after completion of START. Demonstration by Sclafani et al. (1988) that *CDC7* is not periodically transcribed and that there appears to be sufficient Cdc7 in the cell to support eight cell cycles, indirectly suggested that fluctuation in abundance of Cdc7 does not account for its periodic activity. In order to directly investigate the levels of Cdc7 protein and kinase at different stages of the cell cycle, the activity and abundance of Cdc7 were determined in extracts of synchronized cells. A standard α-factor block and release protocol was followed. As shown in Figure 1, Cdc7 activity, as measured by ability of Cdc7 immune complexes to phosphorylate histone H1, is low in α-factor arrested cells. After removal of α-factor, Cdc7 kinase activity increases during G1 and remains high throughout the rest of the cell cycle. Activity then drops sharply, at a position when cells with large buds are maximal, near the end of the first cell cycle. In the second cell cycle after release from α-factor, activity rises again late in G1. Thus, Cdc7 activity varies in the second cell cycle with a timing consistent with its function in late G1. The first cell cycle is also consistent, but the first cell cycle after α-factor release is known to be atypical, and the second cell cycle is a better indicator of cell cycle timing. The activity levels are clearly not determined by the abundance of Cdc7 pro-

tein, however, since the levels of Cdc7 protein, as measured by Western blotting, remain constant in all samples (Figure 1).

In order to precisely define the timing of Cdc7 kinase appearance, we measured Cdc7 kinase activity in immunoprecipitates of nuclear extracts of cells arrested at various stages in the cell cycle either by use of *cdc* mutants or by the use of cell cycle specific inhibitors. Yeast cells were blocked with the mating pheromone α-factor, hydroxyurea, or nocodazole, which inhibit START, DNA synthesis and mitosis, respectively. In addition, cells were arrested at the *cdc28*, *cdc4*, and *pol1* steps, which represent START, G1, and S phase, respectively. As shown in Figure 2, there was no active Cdc7 kinase in *cdc28* blocked cells and greatly reduced, though detectable, levels in α-factor arrested cells (Figure 2, lanes 2 and 6).

In contrast, cells blocked at the *cdc4* step and beyond in the cycle, even the nocodazole treated cells, contained active Cdc7 kinase (Figure 2, lanes 3, 4, 7 and 8). When *cdc28* cells were grown at the permissive temperature, 25°C, and assayed for kinase activity, normal levels of kinase were observed as shown in Figure 2, lane 5. These findings suggest that the Cdc7 kinase is inactive before START, but that passage through START provides a kinase active at least in vitro. Since substrate was provided in the in vitro assay, availability of substrate could not account for the inactivity of Cdc7 in the extracts, although such secondary factors may contribute to the overall regulation of G1 in vivo. Thus, we favored the interpretation that activation of the Cdc7 kinase through post-translational modification during the cycle was an important component of its cell cycle regulation, and sought more direct evidence that this might be the case.

The Activity of Cdc7 Protein Is Correlated with Changes in Phosphorylation

The fact that Cdc7 was active at all arrest points except G1/START, even in *cdc4* arrested cells, suggested to us that *CDC7* might be more intimately connected to START

than had been appreciated from interpretation of genetic experiments using reciprocal shifts and double mutants to order the function of the G1 *CDC* genes (Hereford and Hartwell, 1974). We became particularly interested in whether Cdc28 might directly activate the Cdc7 kinase, that is, whether Cdc7 kinase might be one of the crucial substrates of the Cdc28 kinase. We have previously reported that Cdc7 is a phosphoprotein and that this phosphorylation can be detected by in vivo $^{32}P_i$ labeling of cells expressing elevated levels of Cdc7 protein (Yoon and Campbell, 1991). To determine whether the absence of Cdc7 kinase activity correlates with absence of phosphorylation, we carried out blocking experiments similar to those in Figure 2 and characterized the phosphorylation state of Cdc7 during the block. *cdc28, cdc4, cdc6,* and *pol1* strains were transformed with a plasmid expressing *CDC7* under control of the *GAL1,10* promoter and were labeled with $^{32}P_i$ in vivo during a block at the nonpermissive temperature. [*cdc6* arrests late in G1 with a phenotype similar to *cdc7* (Hartwell, 1976).] As shown in Figure 3, Cdc7 was present as a phosphoprotein in all except *cdc28* blocked cells (lanes 2-5). *cdc28* mutant cells grown and labeled at 25°C did contain a phosphorylated band of Cdc7 (Figure 3, lane 1). Thus, there is a positive correlation between when the kinase is active and the phosphorylation state of Cdc7 protein (Figures 2 and 3). These results also show that the kinase that phosphorylates Cdc7 is not limiting and can phosphorylate the Cdc7 protein present in cells expressing elevated levels of Cdc7 protein. This is consistent with the fluctuation in Cdc7 activity during the cell cycle even when Cdc7 is overproduced (Figure 1) and the viability of cells overproducing Cdc7 (Yoon and Campbell, 1991).

Phosphatase Treated Cdc7 Immune Complexes Are Inactive as Histone H1 Kinase

The positive correlation between the extracts in which Cdc7 protein is maximally phosphorylated and in which it is active predicted that phosphorylation might be essential for activity of the Cdc7 kinase. The histone H1 kinase activity of Cdc7 was therefore

monitored after incubating Cdc7 immune complexes in the presence or absence of phosphatase (Figure 4A). For these experiments we used immune complexes prepared from cells overproducing native Cdc7 protein by incubation with Cdc7 antibody (Figure 4A, lanes 1-3). We also prepared a Cdc7-hemagglutinin fusion protein and overproduced it in yeast as described in Experimental Procedures. Cdc7-containing immune complexes were then prepared with the hemagglutinin monoclonal antibody, 12-CA5, which recognizes the hemagglutinin epitope (Figure 4A, lanes 4-7). The Cdc7 protein immunoprecipitated by the two antibodies was active as a histone H1 kinase (Figure 4A, lanes 1 and 4). Upon phosphatase treatment, however, Cdc7 was inactivated as a kinase, as shown in Figure 4A, lanes 2, 3, and 5. The amount of Cdc7 protein remained constant under the conditions of the phosphatase treatment (Figure 4B), assuring that the inactivation was not due to proteolysis. Although phosphatase was removed by extensive washing before assaying for kinase, two experiments were performed to ensure that there was no residual phosphatase bound tightly to the protein A-Sepharose beads, which might interfere with the Cdc7 kinase. In Figure 4A, lane 6, a combined sample of both phosphatase-treated (1/2) and untreated (1/2) immune complexes is seen to contain about one half of the kinase activity of that shown in Figure 4A, lane 4, confirming that phosphatase was not dephosphorylating histone H1 during the kinase reaction. In a separate experiment, okadaic acid was added to the kinase reaction following phosphatase removal to inhibit any residual phosphatase. Again, the Cdc7 kinase remained inactive (Figure 4A, lane 7). Thus, we conclude that the absence of histone H1 kinase activity in the phosphatase treated Cdc7 protein is due to removal of phosphate(s) important for kinase activity.

Cdc28 Protein Can Phosphorylate Cdc7 Protein Directly

The preceding experiments suggested that Cdc7 protein could be a substrate of the Cdc28 kinase. In order to directly test this, the Cdc7 protein had to be purified so that it could be used as a substrate. Cdc7 protein was expressed in *E. coli* as a fusion protein

with the hemagglutinin epitope (see Experimental Procedures for details). Although a number of different conditions were tested, the bacterially produced fusion protein, designated HA-Cdc7 protein, was not active as a kinase, in contrast to that produced in yeast (see Figure 4). In addition, Cdc7 protein expressed in *E. coli* was not phosphorylated (our unpublished data). The unphosphorylated Cdc7 protein was therefore ideally useful as a substrate to test the effects of phosphorylation by Cdc28 immune complexes.

Antibodies raised against the N-terminal 11 amino acids of Cdc28 protein (gift of M. Tyers and A. B. Futcher, Cold Spring Harbor, NY) were used to prepare Cdc28 immune complexes. As shown in Figure 5, the Cdc7 fusion protein was phosphorylated by the Cdc28 kinase. Two controls were performed to ensure that Cdc28 was responsible for the phosphorylation. First, extracts from a wild-type strain and from strain *cdc28-13*, extracts of which contain a thermolabile Cdc28 kinase (Reed et al., 1985; Wittenberg and Reed, 1988), were compared as a source of Cdc28 kinase. The protein kinase reaction was carried out at the permissive (25°C) and nonpermissive temperature (38°C) for the *cdc28-13* mutation. Comparison of lanes 1 and 2 in Figure 5 demonstrates that there was less Cdc7 phosphorylating activity in the immune complexes prepared from *cdc28-13* strain at 38°C than at 25°C. In contrast, immune complexes from the wild-type strain were more active at 38°C than at 25°C (lanes 7 and 8). As a second control, the 11 amino acid, Cdc28-derived peptide employed as immunogen was used as a competitor in the immunoprecipitation. Cdc7 was not phosphorylated when the Cdc28 antibody was preincubated with the Cdc28-peptide (Figure 5, lanes 5, 6, 11, and 12). Thus, phosphorylation is most likely due to the Cdc28 protein.

Additional proteins were phosphorylated by the Cdc28 immune complexes as shown in Figure 5. None of these proteins appeared to be phosphorylated in a Cdc28-specific way, since the level of phosphorylation was the same at either temperature or the kinase activity was thermolabile even in the wild-type strain. However, the ~62 kDa band

present above the HA-Cdc7 protein could represent a coprecipitating cyclin. We have been unable to increase the level of phosphorylation of HA-Cdc7 fusion protein as compared to that of other proteins, due to difficulties thus far in obtaining concentrated HA-Cdc7 protein in soluble form. The fusion protein precipitates at concentrations higher than 50 ng/μl under reaction conditions tested thus far.

Similar results have been obtained using p13 beads (gift of W. G. Dunphy; Dunphy and Newport 1989) and Cks1 antibodies (gift of C. Wittenberg) (data not shown). *CKS1* encodes the *S. cerevisiae* p13 analog and Cks1 antibodies have been shown to coimmunoprecipitate active Cdc28 kinase along with Cks1 protein (Hadwiger et al., 1989a). Since the enzymological specificity of Cdc28 present in Cdc28 kinases prepared with these different reagents has not been characterized, we cannot yet derive any information about which of the postulated forms of Cdc28 kinase is active in our experiments.

Phosphorylation of Cdc7 Protein by Cdc28 Activates the Cdc7 Kinase

To test whether phosphorylation of Cdc7 by Cdc28 is sufficient to activate the inactive bacterially produced Cdc7 protein, we incubated the Cdc7 protein with Cdc28 immune complexes and ATP. The Cdc7 protein was then purified and assayed for ability to phosphorylate histone H1. As shown in Figure 6, Cdc7 kinase is active after treatment with Cdc28 kinase. Inclusion of the Cdc28 peptide during preparation of Cdc28 immune complexes competed out the activation and activation did not occur at high temperature when *cdc28-13* was used as source of the Cdc28 protein. [A report that Cdc7 was active as a kinase when prepared in an in vitro translation system could be explained by the presence of a Cdc28-like kinase in the translation kit (Bahman et al., 1988).]

***CDC7* Hyperexpression Suppresses the Lethality of *cdc28-13* Mutants**

These biochemical experiments suggest that Cdc7 protein is an important physiological substrate of Cdc28. If so, then the two proteins must interact, at least transiently as

enzyme and substrate, in vivo. Overproduction of one interacting partner often stabilizes a thermolabile form of the other partner, a situation deduced in part from the fact that various temperature sensitive mutants can be suppressed by introduction of the gene encoding an interacting protein on a high copy number expression vector. The *cdc28* alleles have proven particularly amenable to this kind of suppression, allowing identification of both G1 and G2 cyclins in other studies (Hadwiger et al., 1989b; Surana et al., 1991). In order to see if increasing the intracellular level of Cdc7 protein could suppress the lethality of the *cdc28* mutation, we introduced a high copy number plasmid carrying *CDC7* under control of the inducible *GAL1,10* promoter into the *cdc28-13* strain. We estimate that Cdc7 protein is overproduced at least 10-fold in strains carrying this plasmid, as determined by comparison of protein kinase levels in the presence and absence of plasmid (Yoon and Campbell, 1991). For the experiment shown in Figure 7, plasmids pSYC758, pSYC7-158, and pSEY18-Gal were transformed into both *cdc7-1* and *cdc28-13* strains. pSYC758 carries wild-type *CDC7* under the *GAL1,10* promoter. pSYC7-158 contains the temperature sensitive *cdc7-1* gene, and pSEY18-Gal is the vector without a *CDC7* allele. As shown in Figure 7, only wild-type *CDC7* (pSYC758) complements the growth defect in *cdc7-1* cells at the nonpermissive temperature (36°C) and that complementation occurs even on glucose plates (Figure 7C and 7D, 1). This is most likely due to the leakage of glucose repression and the fact that very low levels of Cdc7 protein are required for cell cycle progression. The amounts of Cdc7 protein produced in the absence of galactose induction are not sufficient for detection by immunoblot (Yoon and Campbell, 1991). We then looked at the *cdc28* strains. None of the plasmids were able to suppress *cdc28-13* allele at 36°C (7C and 7D, 4-6). However, *cdc28-13* cells containing pSYC758 grew and formed single colonies at 33°C (7A and 7B, 6). pSYC7-158 also complemented *cdc28-13* mutation at 33°C but only on galactose plates (7A and 7B, 5). Similarly, pSYC7-158 complemented the *cdc7-1* mutation at 33°C after galactose induction (7A and 7B, 2). Since the *cdc7-1* sequence encodes a thermolabile protein (see Experimental Procedures), it is

reasonable that higher dosage of thermolabile protein is necessary and sufficient to suppress the mutations.

These results show that elevating the intracellular level of Cdc7 protein can rescue cells carrying the *cdc28-13* mutation. One explanation for this could be that Cdc7 can phosphorylate an essential Cdc28 substrate when Cdc7 is overproduced, thus bypassing the need for Cdc28. The fact that rescue does not occur at 36°C, suggests that Cdc7 cannot bypass Cdc28 and favors the interpretation that Cdc7 protein is interacting at some level with Cdc28. As established above, Cdc7 can act as a substrate of Cdc28. In further support of an interaction between Cdc28 and Cdc7, aside from the enzyme-substrate relationship, we find that Cdc7 protein coprecipitates with Cdc28 in the presence of p13 beads, as evidenced by Cdc7 crossreacting material in the p13 precipitates (Figure 8). The material must be Cdc7 protein, not only because it has the correct molecular weight (58 kDa) but because it is detectable only after Cdc7 overproduction (Figure 8, lanes 1 and 2).

Yeast Replication Protein-A, RP-A as a Substrate of Cdc7 and Cdc28

Since we have shown that Cdc7 kinase is maximally active at the G1/S boundary and since the *CDC7* gene is required for entry into S phase, we wished to test the idea that activated Cdc7 may function in the initiation of mitotic DNA synthesis by phosphorylating replication proteins. Several yeast replication proteins were available in purified form in this laboratory. Cdc7 kinase assays were performed using these proteins as substrates. In order to detect Cdc7-specific phosphorylation, we overproduced Cdc7 protein in the *cdc7-1* mutant background. Cdc7 immune complexes prepared from the mutant strain containing either *CDC7*-overproducing plasmid pSYC758, or control vector pSEY18-Gal were preincubated at 37°C for 30 min before kinase assay. Under this condition, the endogenous, thermolabile Cdc7 kinase is inactive and therefore the kinase activity in the immune complexes is most likely due to the Cdc7 protein originating from plasmid pSYC758. When DNA polymerase α (pol α), DNA polymerase ε (pol ε), pol ε-stimulatory factor I (SFI),

ARS-binding factor 1 (ABF1), or primase were tested, none of them served as a substrate of Cdc7 kinase (data not shown).

Recently, Brill and Stillman (1989) described the yeast analog of the human replication protein RP-A that is required for SV40 DNA synthesis. Further, they showed that the 36 kDa subunit was phosphorylated only during S phase and that phosphorylation depended on both Cdc28 and Cdc7 functions in vivo (Din et al., 1990). In cells blocked at the *cdc7* step, RP-A was not phosphorylated. However, if extracts of *cdc7* blocked cells were not processed carefully, RP-A did get phosphorylated, suggesting that Cdc7 renatured during extract preparation and directly phosphorylated RP-A (Din et al., 1990). To study RP-A phosphorylation, we purified RP-A from yeast and tested it as a substrate for Cdc7 kinase. We first used the monoclonal antibody 12-CA5 to immunoprecipitate HA-Cdc7 fusion protein from wild-type yeast cells containing plasmid pSYCHA7, and found that p36 RP-A was strongly labeled by the Cdc7 immune complexes (Figure 9A, lanes 4 and 5). The HA-Cdc7 fusion protein was also phosphorylated in this reaction (lanes 4 and 5), consistent with our previous report that native Cdc7 protein contains an autophosphorylating activity (Yoon and Campbell, 1991). Phosphorylation of both HA-Cdc7 and p36 was competed by preincubating the antibody with the hemagglutinin epitope peptide (Figure 9A, lanes 2 and 3). Immunoprecipitates prepared from control vector pSEY18-Gal containing cells phosphorylated p36, however, at a 4-fold lower level than in HA-Cdc7 overproducing cells as shown in Figure 9A, lanes 6 and 7. This suggested that Cdc7 might not be the only protein kinase responsible for phosphorylation of RP-A in cells containing vector alone. Another likely protein kinase may be Cdc28 (see below). We therefore overproduced both wild-type Cdc7 protein and thermolabile cdc7 protein in the *cdc28-13* mutant background and carried out kinase assays at the permissive (25°C) and nonpermissive temperature (38°C) (Figure 9B). When Cdc7 immunoprecipitates were prepared from the *cdc28-13* strain that overproduced wild-type Cdc7 protein, phosphorylation of

p36 RP-A was increased at high temperature (Figure 9B, lanes 1 and 2). In contrast to this, the clone expressing the thermolabile cdc7 protein contained less kinase activity at high temperature (Figure 9B, lanes 3 and 4). Thus, the p36 subunit of RP-A was phosphorylated in a Cdc7-dependent manner. (The lower affinity of the polyclonal antibody used for the experiment in panel B, perhaps combined with a greater inhibitory effect on Cdc7 autophosphorylating activity and the absence of the *GALA*-expression plasmid from this host, did not allow detection of Cdc7 phosphorylation in this experiment.)

Din et al. (1990) showed that RP-A was not phosphorylated in *cdc28* mutants. Although this could be explained by the fact that Cdc7 phosphorylates RP-A (Figure 9) and that Cdc7 is not active in *cdc28* mutants (Figure 2), we were interested in seeing whether Cdc28 might also phosphorylate p36 RP-A directly. Indeed, as shown in Figure 10, Cdc28 immunoprecipitates of yeast specifically phosphorylated the p36 subunit of RP-A. The kinase activity in Cdc28 immune complexes was thermolabile in *cdc28-13* strain compared to wild-type and competed by Cdc28-peptide (Figure 10). Since the extent of p36 phosphorylation did not increase in Cdc28 immunoprecipitates prepared from HA-Cdc7 overproducing cells, when compared to cells with a genomic copy of *CDC7*, the activity was not due to Cdc7 kinase that adventitiously coprecipitated (Figure 10B, lanes 5 and 6). Thus, both Cdc28 and Cdc7 phosphorylate the p36 subunit of yeast RP-A in vitro.

Discussion

Our studies were initiated in an attempt to define the molecular basis of the role of Cdc7 in the initiation of DNA replication inferred from earlier genetic studies. Demonstration that *CDC7* encodes a protein kinase (Patterson et al., 1986; Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991) led us to test the hypothesis that the essential function of Cdc7 was to phosphorylate a replication protein at a critical point late in G1. Biochemical experiments showed that Cdc7 kinase activity fluctuates in the cell cycle with a

periodicity coinciding with the in vivo function of Cdc7, supporting the prediction that it is the kinase activity of Cdc7 that is responsible for its essential role and that it is activation rather than abundance that accounts for cell cycle variation. In addition, we showed that Cdc7 kinase activity is undetectable in extracts of cells arrested by a *cdc28* mutation, that phosphorylation of Cdc7 is required for its activity as a protein kinase, that Cdc7 protein is hypophosphorylated in *cdc28* extracts, and that phosphorylation of Cdc7 protein by Cdc28 immune complexes is sufficient to activate Cdc7 kinase on a histone H1 substrate. Overproduction of Cdc7 kinase in vivo could suppress the lethality of a temperature sensitive *cdc28* mutation, providing genetic evidence of an in vivo relationship to complement the biochemical data demonstrating that the two proteins may interact. The prediction that Cdc7 phosphorylates a replication protein was verified by demonstrating that RP-A, whose phosphorylation is known to require Cdc7 in vivo (Din et al., 1990), was a substrate of Cdc7 kinase and led to the further observation that Cdc28 also phosphorylates RP-A.

Timing of Cdc7 function in the cell cycle

Early work from the Hartwell laboratory defined three genes necessary for the initiation of DNA replication, *CDC28, CDC4* and *CDC7*, as discussed in the Introduction. *CDC28* functions at START, and after return of *cdc28* mutants from the nonpermissive temperature the cells can still adopt the alternative pathway of conjugation. Several more genes that have this phenotype have been identified, but as yet have not been well studied. Three additional genes that affect START, *CLN1*, *CLN2*, and *CLN3*, are apparently involved in a positive feedback loop controlling the activation of the *CDC28* gene product (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). These genes were identified either as suppressors of *cdc28* mutations (*CLN1*, *CLN2*) or as dominant gain of function mutations (*CLN3*) (Cross, 1988; Hadwiger et al., 1989b; Nash et al., 1988; Sudbery et al., 1980) and have at least partially redundant functions, in that one *CLN* gene can compensate for loss of the other, but loss of all three is lethal (Richardson et al., 1989). The *CLN* genes encode proteins that are distantly related to the cyclins that activate the

p34/cdc2+/MPF kinase for induction of mitosis in *Xenopus*, *S. pombe* and other organisms. By analogy, it has been proposed that *CLN1*, *CLN2* and *CLN3* interact with and activate the Cdc28 kinase subunit and passage through START (Richardson et al., 1989; Wittenberg et al., 1990; see also Cross, 1988; Nash et al., 1988 and for review Nasmyth, 1990). The molecular nature of the events set in motion by these START genes, however, has been more difficult to define.

Morphological analysis of single and double mutants and reciprocal shift studies suggest *CDC7* acts subsequent to *CDC28*/START (Hereford and Hartwell, 1974). In addition to *CDC4* and *CDC7*, three other genes, *CDC34, DBF4*, and possibly *DBF2* also function in the post-START/pre-DNA synthesis period (Goebl et al., 1988; Johnston and Thomas, 1982; Chapman and Johnston, 1989; Johnston et al., 1990). These dependent genetic relationships predicted that activation of the Cdc7 protein should be dependent on completion of START. In keeping with this, we found that Cdc7 kinase was inactive in extracts of cells arrested at START - either by α-factor or by a *cdc28* arrest. We then found that after release from a block and passage through START the kinase activity increased, decreased abruptly late in the cell cycle and then rose again in G1 in the second cell cycle. This correlation of appearance of kinase activity and in vivo function is the best evidence to date that the kinase activity of Cdc7 is related to its essential and periodic function.

Genetic studies predicted that Cdc7 activity might be dependent on *CDC4*, also. The results in Figure 2 suggest that, on the contrary, the Cdc7 kinase is already active even before *CDC4* completes its function. Why does *CDC7* appear to depend upon the completion of *CDC4* in vivo? One possibility is that the substrates of Cdc7 kinase may not be available during the *cdc4* block. According to the results of Hereford and Hartwell (1974), *cdc4* mutants blocked and released in the presence of the protein synthesis inhibitor cycloheximide do not enter S phase. Further protein synthesis is thus necessary after the *CDC4* step. Proteins newly synthesized after the *CDC4* step may include replication proteins that require the Cdc7 kinase for post-translational activation. Alternatively, substrates of Cdc7

kinase may exist during the *cdc4* block, but somehow may be inaccessible to Cdc7 protein until after *CDC4* has functioned. A third possibility is that Cdc7 kinase, like many other kinases, actually consists of two subunits, a catalytic subunit and a regulatory subunit whose abundance or modification is dependent on *CDC4* function. We feel that it is likely that Cdc7 does have a regulatory subunit that enhances activity and is encoded by a second yeast gene, since we have recently been able to purify an active Cdc7 kinase from yeast and have found that Cdc7 copurifies with an 80 kDa protein, though we have not yet determined whether this 80 kDa protein represents another substrate or an essential cofactor for Cdc7 activity (Loo and Campbell, unpublished). We also observe an 80 kDa protein in Cdc7 immunoprecipitates from yeast (see Figure 3, Yoon and Campbell, 1991).

How is the timing of Cdc7 action controlled? One component of the regulation seems to be phosphorylation of Cdc7 protein. Phosphatase treatment of Cdc7 kinase abolishes its histone H1 kinase activity (Figure 4). In addition, its phosphorylation state correlates well in the cell cycle with the time when it is active (Figures 2 and 3). It appears likely from our studies that Cdc28 kinase provides at least one essential phosphate modification (Figures 5 and 6). We also know that Cdc7 is phosphorylated by at least one other kinase, in that Cdc7 appears to autophosphorylate (Figure 3 in Yoon and Campbell, 1991; and this work Figure 9).

Is *CDC7* a physiological substrate of *CDC28*?

Recently, three criteria have been set for establishing a protein as the physiological substrate of a particular kinase (Lewin, 1990). First, the amino acid phosphorylated by the kinase in vitro should be occupied in vivo at the point in the cell cycle when it is needed. Second, a mutation in the kinase should block phosphorylation in vivo. Third, some function of the protein should be altered by phosphorylation. We have thus far met only the second and third criteria in our studies of the Cdc28/Cdc7 interaction, namely that Cdc7 is not phosphorylated in *cdc28* mutants and it is not active when it is not phosphorylated. Given the nature of cell cycle mutants, one could still argue that the effect of the *cdc28*

mutation is indirect and that another intermediate kinase is required. While this is certainly possible, it seems unlikely. For one thing, though many other kinases have been identified in yeast, none has been shown to be essential for the *CDC28-CDC4-CDC7* pathway as yet. A more convincing argument is our demonstration that phosphorylation of Cdc7 by Cdc28 is sufficient to activate Cdc7 kinase. Thus, the kinase active in vivo must have the same specificity as Cdc28. A third argument is that, as shown in Figure 7, *CDC7* expressed from an inducible *GAL* promoter on a high copy number plasmid rescues the inviability of *cdc28-13* cells. Why wasn't *CDC7* previously identified as one of the high copy plasmid suppressors of *cdc28* mutations? Originally, such suppressors were isolated based upon their ability to rescue the growth defect of *cdc28* mutations at 36°C. In addition, there may be allele specificity. Among the dosage-dependent suppressors of *cdc28* mutations, both *CKS1* and *CLN* genes were isolated using a *cdc28-4* allele (Hadwiger et al., 1989a; 1989b). The ability to suppress the lethality of *cdc28-1N* allele but not that of a *cdc28-4* allele led to the discovery of *CLB* genes (Surana et al., 1991). In the study described here, we used a *cdc28-13* allele. Finally, in our experiments, *CDC7* did not suppress simply by virtue of being present on a high copy plasmid but required induction from a *GAL* promoter, which led to higher intracellular concentrations of *CDC7* than can be expected from simple increase in gene dosage of *CDC7* under its own, perhaps stringently regulated promoter.

It is essential that we now fulfill the first criteria for correspondence of in vitro and in vivo substrates mentioned above. The consensus motif for a cdc2^{+}/Cdc28 phosphorylation site has been reported to be S/T-P-X-Z (where X is a polar amino acid, and Z is generally a basic amino acid) (for reviews, see Lewin, 1990; Moreno and Nurse, 1990 and Pines and Hunter, 1990). The primary amino acid sequence of *CDC7* contains one such motif, TSS^{83}PQR, near the N-terminus, adjacent to the ATP binding site. Mutation of S^{83} to A^{83} results in failure to complement a *cdc7-1* or a *cdc7Δ* mutant (Loo, Yoon and Campbell, unpublished), consistent with but not proving that S^{83} is the site of a crucial

phosphorylation. In some cases, even the simple sequence S/T-P is recognized by cdc2+ kinases (see references in Roach, 1991). Cdc7 contains three SP or TP sites which lie in the C terminal half of Cdc7. Mutations of the respective serines and threonines in these positions have no effect on in vivo function (Loo, Yoon and Campbell, unpublished).

Which form of Cdc28 might be involved in the Cdc7 activation? Either by using the *cdc28-1N* allele or by modifying the restrictive conditions and using *cdc28-4 and cdc28-13,* alleles that were previously used to define the *CDC28-CDC4-CDC7* pathway, it was shown that *CDC28* has an execution point in G2 as well as G1(Piggott et al., 1982; Reed and Wittenberg, 1990; Surana et al., 1991). Results presented in this paper suggest that *CDC28* may be required for at least two more post-START events closely linked to exit from G1 — to phosphorylate Cdc7 and to phosphorylate RP-A. *CDC28*, *CDC7* and the initiation of DNA synthesis do not appear to be interdependent based on previous assays of cell cycle function. Previous definitions of START were based on morphological landmarks — bud size, spindle development, nuclear migration. The addition of biochemical landmarks — *CLN* transcription, Cdc7 phosphorylation and RP-A phosphorylation, expand the range of assays for *CDC28* function and may allow a finer dissection and enzymological characterization of the multiple activities of Cdc28 and its complexes. For instance, it may be possible to use these phenomena as assays to differentiate the functions of the various cyclins. The various phosphorylation states of Cdc28, which do not appear to change with passage through START (Hadwiger and Reed, 1988) may be different for Cdc7 and RP-A phosphorylation. Since the relationship of the *CDC7* step and START is not clear, it will be of particular interest to see if phosphorylation of Cdc28 is required for the kinase to phosphorylate the Cdc7 protein. In *Schizosaccharomyces pombe*, it has been shown that T^{167} phosphorylation of cdc2+ is involved in the association of cdc2+ with cdc13+ (Gould et al., 1991). In sum, while the precise relationship of the post-START functions to START is not yet clear, there is accumulating evidence that the transitions in

the cell cycle are monitored by Cdc28, or one of its homologs, at more discrete stages than just the two previously defined commitment points-START and G2/M.

Exit from G1 and Entry into S, What is the G1/S Transition?

Presumably the signals integrated at START that commit the cell to growth and replication must eventually be communicated to the replication apparatus itself. In the case of SV40 DNA replication, $cdc2^+$ kinase has been shown to phosphorylate T^{124} of T antigen, which is essential for T antigen's function in DNA replication (McVey et al., 1989). D'Urso et al. (1990) have also presented evidence that some component of the replication apparatus is modified by a cyclin A dependent, $cdc2^+$ kinase in human cells late in G1. The initiator protein for chromosomal DNA replication has not been isolated yet and so the host protein analogous to T antigen is not available to test as a substrate of the G1 kinases. Previous results by Din et al. (1990), however, showed that the p36 subunits of both human and yeast RP-A were phosphorylated at a specific point in the cell cycle, late in G1 or early in S phase. Not only did this coincide with the timing of Cdc7 function, but also the p36 subunit of yeast RP-A was not phosphorylated in *cdc7*-ts cells arrested at the nonpermissive temperature. Interestingly, it was also unphosphorylated in *cdc28*-ts cells. In the process of searching for substrates of Cdc7 kinase, we identified RP-A as an in vitro substrate of both Cdc7 and Cdc28 (Figures 9 and 10). We have thus added biochemical evidence to the implied result of the previous work by Din et al. (1990) that replication protein RP-A is a substrate of Cdc28 and Cdc7.

This biochemistry raises several obvious questions. Is phosphorylation of RP-A by either or both kinases essential in vivo? Do they modify the same or different amino acids? Is RP-A the only substrate of Cdc7 and Cdc28 that participates in chromosomal DNA synthesis? At present we do not have sufficient information to decide if either kinase is the physiological kinase and whether phosphorylation of RP-A is either necessary or sufficient to trigger DNA replication. Perhaps both kinases must act on this substrate for

yeast cells to enter S phase. Previous phosphorylations are often necessary for secondary ones to occur (Roach et al., 1991). It is very important to emphasize that the phosphorylated doublet of p36 RP-A reported by Din et al. (1990) does not appear until after Cdc7 has functioned. It does not appear directly after the *CDC28* or even after the *CDC4* step, where the Cdc7 kinase is active, at least in vitro. It is also noteworthy that both Cdc7 and both the phosphorylated and unphosphorylated forms of RP-A are localized in the yeast nuclei (Yoon and Campbell, 1991; our unpublished data). *CDC28* could be required only to activate Cdc7, or alternatively, since Cdc28 directly phosphorylates RP-A in vitro, Cdc28 could use RP-A as a direct substrate in vivo as well. The idea that Cdc28 may act both as a master regulator of START and a direct activator has been raised previously, and our results may constitute evidence for such a dual role in the G1 phase of the yeast cell cycle. Experiments are under way to map the sites of phosphorylation by Cdc28 and Cdc7 in p36.

In conclusion, our studies provide biochemical evidence supporting, as well as refining, the existing *S. cerevisiae* genetic cell cycle map. Future studies will be aimed at obtaining additional in vivo data.

Experimental Procedures

Strains, Media and Plasmids

The genotypes and sources of the yeast strains used were SEY6210, α *his3 leu2 lys2 suc2 trp1 ura3* (S. D. Emr, California Institute of Technology); YC7379, **a** *ade1 his7 lys2 tyr1 ura3 cdc7-1* (Yoon and Campbell, 1991); CU4-1, **a** *ade1 ura3 cdc4-1* (A. Y. Jong, University of Southern California); USC6-5, **a** *leu2 ura3 cdc6-1* (A. Y. Jong, University of Southern California); YC488m, α *his1 leu2 pol1-17 trp1 ura3 can1* (M. Budd, California Institute of Technology); YC28, **a** *ade1 his3 ura3 cdc28-13* (this work); G1906c, **a** *bar1-1 leu1 rme1 trp5 ura3 can1 cir*$^+$ (A. Y. Jong, University of

Southern California). Cells were routinely grown in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) or synthetic minimal medium supplemented with amino acids and adenine but lacking uracil. Either 2% glucose or 2% raffinose plus 2% galactose were used as the carbon source. For in vivo $^{32}P_i$ labeling, cells were grown in LPSM medium (Reneke et al., 1988). *E. coli* cells were grown in LB medium containing ampicillin (50 μg/ml). In order to overexpress wild-type *CDC7*, thermolabile *cdc7-1*, and the *CDC7*-hemagglutinin fusion gene in yeast, we used a high copy number, 2 μm containing vector pSEY18-Gal (S. D. Emr). pSEY18-Gal also contains the *URA3* gene and the inducible *GAL1,10* promoter. pSYC758 carries wild-type *CDC7* under the *GAL10* promoter of pSEY18-Gal (Yoon and Campbell, 1991). pSYC7-158, carries a temperature sensitive *cdc7-1* gene under the *GAL1,10* promoter, and was constructed for this work as explained in detail below. pSYCHA7 carries the *CDC7*-hemagglutinin epitope fusion gene under the control of the *GAL1,10* promoter (see below for details). The T7 promoter containing vector, pT7-7 (Tabor and Richardson, 1985), was used to express the Cdc7-hemagglutinin epitope fusion protein in *E. coli*.

Buffers and Inhibitors

The buffers and inhibitors used were as follows: IP buffer (immunoprecipitation buffer), 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.2 mM EDTA, and protease inhibitors; K buffer (kinase buffer), 25 mM Tris-HCl at pH 7.5, 5 mM NaF, 15 mM $MgCl_2$, 5 mM EGTA, and protease inhibitors; L buffer (lysis buffer), 100 mM Hepes at pH 7.9, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, 0.2 mM EGTA, 2.5 mM dithiothreitol, protease inhibitors, and phosphatase inhibitors; AP buffer (acid phosphatase buffer), 10 mM Pipes at pH 6.0 and protease inhibitors; CIP buffer (calf intestinal alkaline phosphatase buffer), 50 mM Tris-HCl at pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, and protease inhibitors; protease inhibitors, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 10

μg/ml soybean trypsin inhibitor; phosphatase inhibitors, 10 mM sodium azide, 10 mM sodium fluoride, 10 mM sodium molybdate, 10 mM sodium pyrophosphate, 10 mM potassium phosphate, 1 mM EDTA, and 1 mM EGTA. Okadaic acid was used at 5 μM.

Antisera

The production of polyclonal Cdc7 antibody is described in Yoon and Campbell (1991). To purify the Cdc7 antibody, an affinity column was made by linking 5 mg of bacterially produced Cdc7 protein to 1 g of CNBr-activated Sepharose as recommended by Pharmacia. Using this Cdc7 affinity column, the antibody was purified according to the method detailed in Harlow and Lane (1988). The monoclonal antibody 12-CA5, a subclone of H26D08 (mouse IgG 2b) was raised against the influenza hemagglutinin peptide (HA; YPYDVPDYA) as previously described by Field et al. (1988). The hybridoma was kindly provided by D. J. Anderson (California Institute of Technology), and was cultured in the Caltech Monoclonal Antibody Facility. The anti-HA peptide antibody was purified from ascites fluid using a protein A-Sepharose column according to manufacturer's instructions (Sigma). Antibodies to the 11 N-terminal amino acids of Cdc28 as well as the N-terminal peptide immunogen were the gift of M. Tyers and A. B. Futcher, Cold Spring Harbor, NY. p13 beads were the gift of W. G. Dunphy, California Institute of Technology, Pasadena, CA.

Immunoprecipitations and Protein Kinase Assays

Either whole cell or nuclear extracts were used to prepare immune complexes. The amounts of extracts and antisera used are indicated specifically in each Figure legend. Immune complexes were precipitated with 20 μl of protein A-Sepharose beads (100 mg/ml; Sigma), washed four times in IP buffer, twice in K buffer, and resuspended in 25 μl of K buffer. After 5 min preincubation at 25°C or 38°C, 0.1 mM ATP, 10 μCi [γ-^{32}P] ATP (3000 Ci/mmol, Amersham PB10168) and substrate were added to a final vol of 36 μl.

Incubation was continued at the same temperature for 20 min and reaction products were analyzed on 10% SDS-polyacrylamide gels.

Purification of HA-Cdc7 Fusion Protein from *E. coli*

Since we had previously shown that the N-terminal 20 amino acids were dispensable for *CDC7* function in vivo (Yoon and Campbell, 1991), we chose to add the hemagglutinin epitope (HA) to the N-terminus of the Cdc7 protein. Plasmid pTHA-*CDC7* encoding the hemagglutinin epitope-Cdc7 fusion protein, called HA-Cdc7 in the text, was constructed from the plasmid pT7-*CDC7*. pT7-*CDC7* carries the complete *CDC7* gene cloned into the T7 promoter based vector pT7-7, and has a unique NdeI site which marks the initiation codon for *CDC7*. To construct pTHA-*CDC7*, the synthetic oligonucleotide 5'-T ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CC-3' encoding the HA epitope, YPYDVPDYA, and the linker sequence, SLGGP, was inserted into the NdeI site of pT7-*CDC7*. The linker GGP is used to connect the HA peptide to the *CDC7* gene because it is expected to cause the HA peptide to protrude from the rest of the protein. Then, the junction sites of recombinant clones were sequenced to confirm the cloning procedures yielded an in-frame fusion gene.

For expression of the HA-Cdc7 fusion protein in *E. coli*, pTHA-*CDC7* was transformed into the bacterial strain BL21 (DE3) (Studier and Moffat, 1986). This strain carries the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Cells carrying pTHA-*CDC7* were grown in 500 ml of LB medium supplemented with ampicillin (50 μg/ml) to an OD_{600} of 0.5. The polymerase, and hence the HA-*CDC7* fusion gene, were induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM, and the culture was allowed to grow for another 3 hrs. Cells were harvested, resuspended in 20 ml of ice-cold 10 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10 μg/ml lysozyme, protease inhibitors, and disrupted by sonication with four 30 sec pulses. The inclusion bodies containing the insoluble HA-Cdc7 fusion protein were

isolated by centrifuging the bacterial extracts at 10,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor. The pellet was resuspended in 200 mM Tris-HCl at pH 8.2, 500 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, sonicated once for 30 sec, and centrifuged again at 10,000 rpm for 10 min. The final pellet was dissolved in 50 ml of buffer A (25 mM Tris-HCl at pH 8.0, 1 mM dithiothreitol, and 6 M urea).

To purify HA-Cdc7 fusion protein, protein extracts were loaded onto 20 ml of DEAE-Cellulose (Whatman) column that had been equilibrated with buffer A containing 25 mM NaCl. After washing the column with two column vol of buffer A with 50 mM NaCl, proteins were eluted by a linear gradient of 50-500 mM NaCl in buffer A. The HA-Cdc7 fusion protein was eluted at about 250 mM NaCl, as determined by protein blotting of the fractions with Cdc7 antibody. The fractions containing the fusion protein were pooled, diluted to a protein concentration of about 50 ng/μl with buffer B (200 mM Tris-HCl at pH 8.0, 500 mM NaCl, and 1 mM dithiothreitol) containing 6 M urea, and dialyzed against buffer B with 4 M urea for more than 8 hrs. The concentration of urea in the dialysis buffer was gradually reduced from 4 M to 2 M, 1 M, and finally 0 M. The length of dialysis in each urea concentration was for more than 8 hrs each. The fusion protein was judged to be more than 90% pure by Coomassie blue staining following SDS-polyacrylamide gel electrophoresis.

Expression of HA-*CDC7* Fusion Gene in Yeast

To produce HA-Cdc7 fusion protein in yeast, pTHA-*CDC7* was first digested with NdeI and SphI. The 1.8 kb DNA containing the HA-*CDC7* fusion gene was then treated with T4 DNA polymerase, EcoRI linkers were attached and the fragment was ligated into the EcoRI site of the yeast expression vector, pSEY18-Gal. The resulting plasmid, pSYCHA7, carries HA-*CDC7* gene under the control of the inducible *GAL1 ,10* promoter and is capable of efficiently suppressing the temperature sensitive phenotype of a *cdc7-1*

allele at the nonpermissive temperature, 36°C. Thus the epitope does not seem to interfere with Cdc7 function in vivo when fused to the Cdc7 protein.

Cloning of the *cdc7-1* allele and Overproduction of Thermolabile cdc7-1 Protein in Yeast

The mutation in *cdc7-1* has been mapped between the SacI and SphI sites (Patterson et al., 1986). The polymerase chain reaction was performed in a Perkin Elmer Cetus DNA Thermal Cycler using ~150 ng of yeast genomic DNA isolated from strain *cdc7-1* and 5 μg each of the oligonucleotide primers (5' primer, 5'-TAT AAT GAG CTC AAC CTG CTG TAC ATA ATG ACG-3', where the SacI site is underlined; 3' primer, 5'-CTT AAG CGC ATG CCA CCA ATT ATG CTA AAC CGT-3' in which the SphI site is underlined). The plasmid containing the temperature sensitive allele, pSYC7-158, was constructed by ligation of the 1,393 basepair SacI/SphI PCR fragment of the *cdc7-1* allele into the SacI and SphI sites of plasmid pSYC758, which expresses wild-type Cdc7 protein from the *GAL10* promoter (Yoon and Campbell, 1991). This creates an in-frame fusion of the 3'-1,393 basepairs of the *cdc7-1* allele to the 5'-282 basepairs of the *CDC7* gene. Clones were transformed into a heterozygous diploid, *CDC7/cdc7*::Tn3, carrying a disrupted *cdc7* allele, which was constructed for this test. After sporulation, the spores containing plasmids and disruptions grew but were temperature sensitive. Thus, the clone complements a gene disruption and gives a temperature sensitive phenotype. As expected, the clone does not complement *cdc7-1* strains at 36°C. Protein extracts were prepared from a *cdc7-1* strain containing either pSYC758 (wild-type *CDC7*) or pSYC7-158 (ts *cdc7-1*). Immunoblot analysis of these extracts confirmed that the cdc7-1 protein was overproduced at the permissive temperature (data not shown).

Acknowledgements

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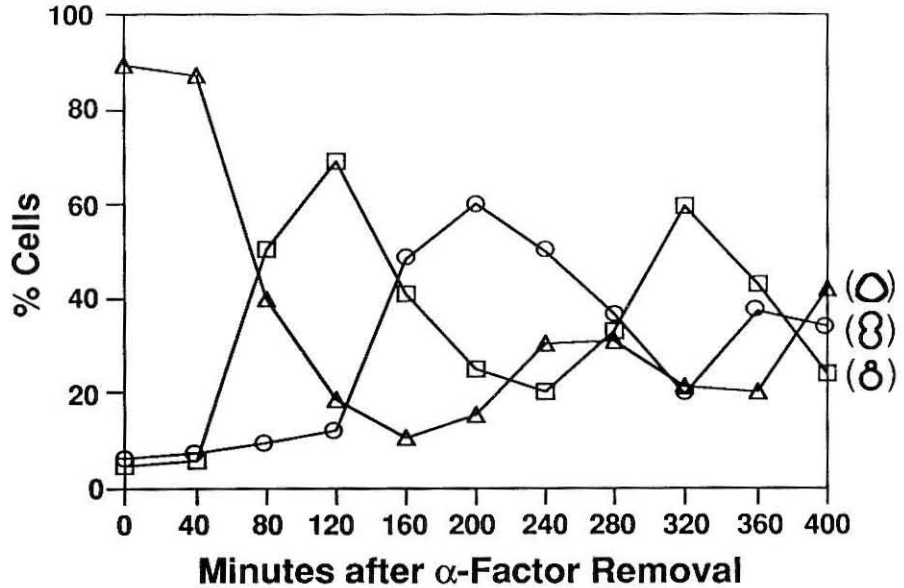
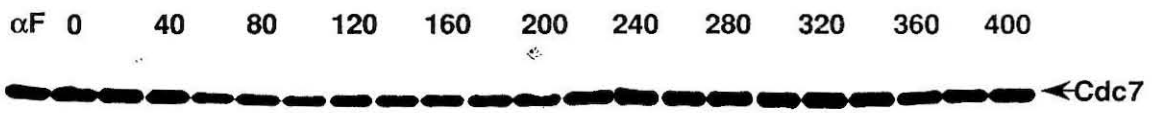
Figure 1. The Activity of Cdc7 Kinase Accumulates Periodically During the Cell Cycle.

Strain G1906c *bar1* carrying the *CDC7*-overproducing plasmid, pSYC758, was grown at 30°C in 2 liters of 2% raffinose synthetic complete (SC) medium (minus uracil). At OD_{595} = 0.5, cells were treated with 2% galactose and synchronized in G1 with 300 ng/ml α-factor for 7 hr. Cells were washed twice with fresh SC medium (minus uracil) containing both 2% raffinose and 2% galactose, and resuspended in 1.5 liters of the same medium to release cells from mating pheromone arrest. Samples (50 ml) were taken at 20 min intervals for 400 min. Whole yeast lysates were prepared from each sample by vortexing in the presence of glass beads and clarified by centrifugation. The supernatants, which contain no detectable Cdc7 protein, were discarded and the pellets were resuspended in L buffer, incubated for 30 min on ice, and centrifuged twice to remove insoluble materials. Protein extracts were analyzed by immunoblotting for Cdc7 quantitation and by histone H1 kinase assay for Cdc7 activity. Protein (40 μg each) blots were stained with Cdc7 antibody (1:250 dilution) and the Bio-Rad ImmunBlot assay kit (goat anti-rabbit IgG alkaline phosphatase conjugate). For histone H1 kinase assay, Cdc7 immune complexes were prepared as described in Experimental Procedures using 120 μg of protein and 20 μl of Cdc7 antibody. The reaction was carried out at 38°C for 20 min with 5 μg of histone H1 (Boehringer Mannheim) and the products were visualized by autoradiography after separation on 10% SDS-polyacrylamide gels. For parameters of cell cycle synchrony, samples taken at each time point were fixed with 3.7% formaldehyde and scored for cell morphology by phase-contrast microscopy. Symbols: Triangle, unbudded cells; Square, cells with small buds; Circle, cells with large buds.


100
80
60
40
20
0
% Cells
0 40 80 120 160 200 240 280 320 360 400
Minutes after α-Factor Removal
(○)
(□)
(△)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Cdc7

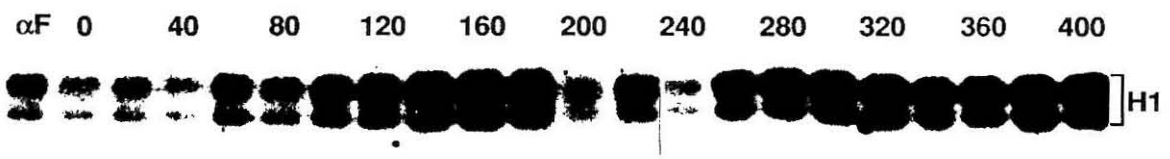

αF 0 40 80 120 160 200 240 280 320 360 400
H1

Figure 2. Cdc7 Kinase Is Not Active in Cells Arrested at START.

cdc strains (*cdc28-13*, *cdc4-1*, and *pol1-17*) were grown at 25°C and a *bar1* strain at 30°C in YPD media. At OD_{595} = 0.7, *cdc* strains were shifted to 38°C and held there until approximately 90% of the cells were arrested with the appropriate terminal phenotype (lanes 6-8). *bar1* cells were treated with α-factor (αF; 300 ng/ml final concentration), hydroxyurea (HU; 0.2 M final concentration), or nocodazole (NC; 20 μg/ml final concentration) for 4 hr at 30°C (lanes 2-4). Nuclear protein extracts (500 μg) prepared from these strains were immunoprecipitated with affinity purified Cdc7 antibody (20 μl) and assayed for histone H1 kinase activity. The reaction was carried out at 38°C for 20 min with 5 μg of histone H1. As a control, histone H1 was incubated in the absence of Cdc7 immune complexes (lane 1). For the *cdc28-13* strain, nuclear extracts were also prepared from cells grown constantly at 25°C and tested for the histone H1 kinase activity at 25°C for 20 min (lane 5). The reaction products were visualized by autoradiography after separation on SDS-polyacrylamide gels.

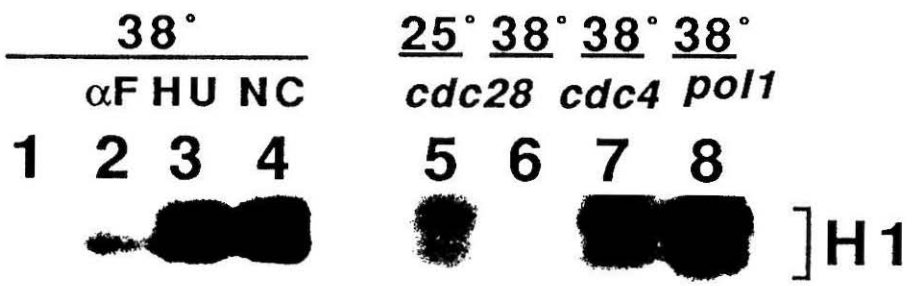

38°
αF HU NC
25° 38° 38° 38°
cdc28 cdc4 pol1
1 2 3 4 5 6 7 8
]H1

Figure 3. Labeling of Cdc7 Protein with $^{32}P_i$ in Cells Arrested at Different Points in the Cell Cycle.

Yeast strains (*cdc28-13*, *cdc4-1*, *cdc6-1*, *pol1-17*, and wild-type) carrying the *CDC7*-over-producing plasmid, pSYC758, were grown at 25°C in 20 ml of LPSM medium. Both 2% raffinose and 2% galactose were used as a carbon source. At $OD_{595} = 0.5$, the temperature was shifted to 38°C for 3 hr. Cells were then labeled with 0.7-1.0 mCi of $^{32}P_i$ for 2 hr at 38°C (lanes 2-6). For the *cdc28-13* strain, cells grown constantly at 25°C were also labeled with $^{32}P_i$ for 2 hr at 25°C (lane 1). Whole yeast lysates were prepared as described in the legend to Figure 1. Proteins were immunoprecipitated with affinity purified Cdc7 antibody (20 µl) and analyzed by electrophoresis in a 10% SDS-polyacrylamide gel. The gel was run until the 30 kDa colored marker (Rainbow Marker, Amersham) reached the bottom.

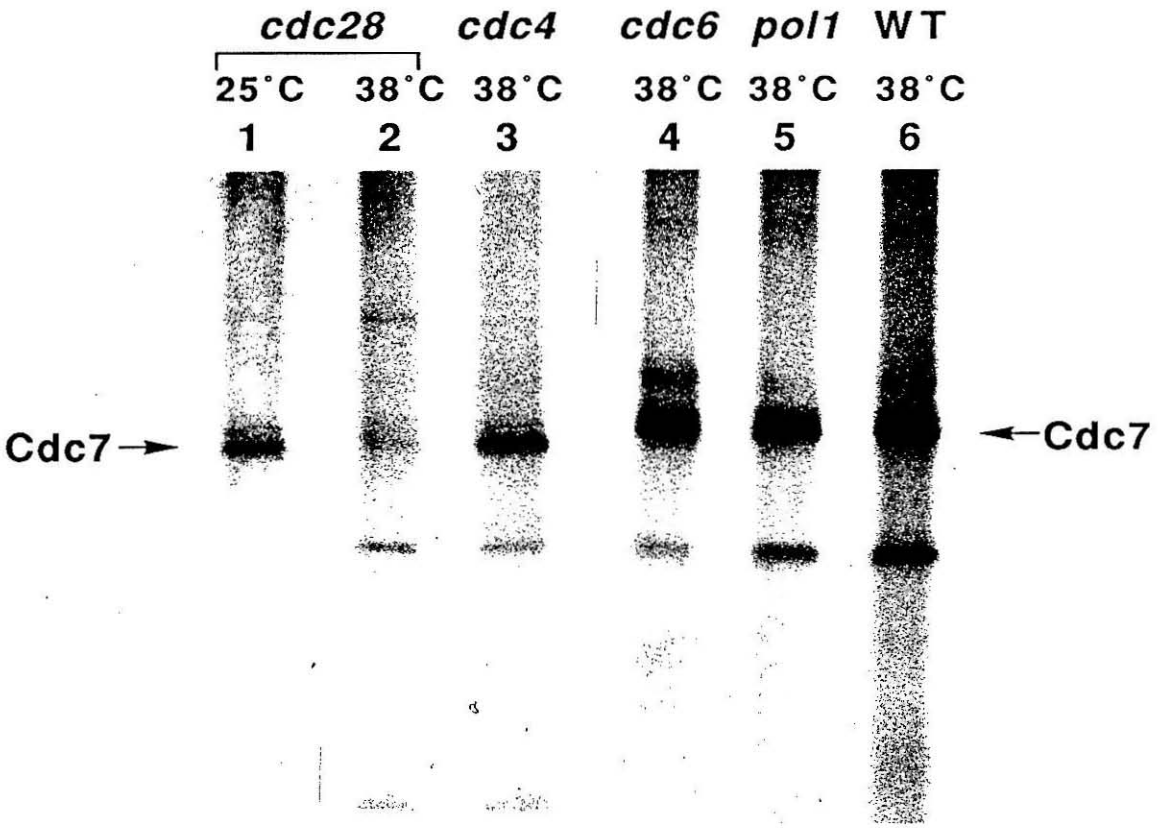

cdc28
cdc4
cdc6
pol1
WT
25°C
38°C
38°C
38°C
38°C
38°C
1
2
3
4
5
6
Cdc7
Cdc7

Figure 4. In Vitro Inactivation of Cdc7 Kinase with Phosphatase.

(A) Three separate Cdc7 immune complexes were prepared from nuclear extracts (500 μg each) of Cdc7-overproducing cells using affinity purified Cdc7 antibody (20 μl each; lanes 1-3). After washing four times in IP buffer, two of them were washed twice in CIP buffer and incubated for 15 min at 37°C in the presence (+) or absence (-) of 3 U of CIP (lanes 1 and 2). The third immune complex was treated with 3 U of AP after washing twice in AP buffer (lane 3). Each immune complex was then washed three times in IP buffer and twice in K buffer, and assayed for histone H1 kinase activity (15 min at 37°C) (lanes 1-3).

Whole cell lysates were prepared from cells overproducing the hemagglutinin-Cdc7 fusion protein (HA-Cdc7). Extracts were made from cells (1 liter) harvested at OD_{595}=2 by extensive manual grinding under liquid nitrogen as described by Schultz et al. (1991). The powder of broken cells were resuspended in L buffer and centrifuged at 20,000 rpm for 30 min. The supernatant was frozen in aliquots at -70°C until needed. The monoclonal antibody 12-CA5 (20 μl each) was used to immunoprecipitate HA-Cdc7 fusion protein from these extracts (400 μg each; lanes 4-7). Immune complexes were washed as above and incubated with (+) or without (-) 3 U of AP before assay for H1 kinase activity (lanes 4 and 5). In lane 6, immune complexes of AP-treated (1/2) and untreated (1/2) samples were combined and tested for kinase activity. Lane 7 contained immune complexes treated in the same way as those in lane 5 except okadaic acid (5 μM) was added in the kinase reaction. Reaction products were analyzed on an SDS-polyacrylamide gel, stained with Coomassie blue, dried, and autoradiographed.

(B) Three separate Cdc7 immune complexes were prepared from Cdc7-overproducing cells using affinity purified Cdc7 antibody. After washing four times in IP buffer, two samples were washed twice in CIP buffer and incubated for 15 min at 37°C in the presence (+) or absence (-) of 3U of CIP (lanes 1 and 2). The third immune complex was treated with 3U of AP after washing twice in AP buffer (lane 3). Each immune complex was then subjected to immunoblot analysis as described in the legend to Figure 1.

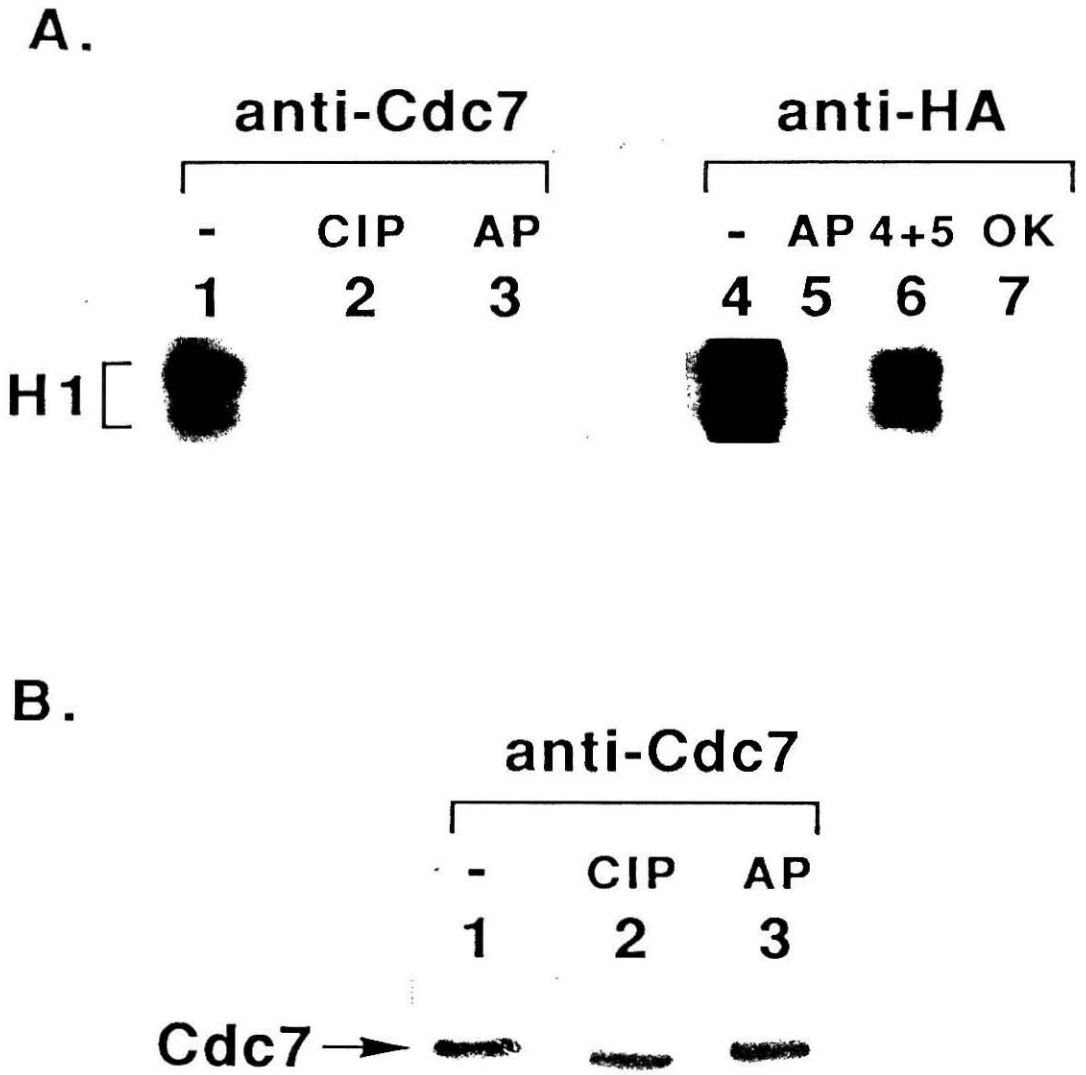

A.
anti-Cdc7
anti-HA
-
CIP
AP
-
AP
4+5
OK
1
2
3
4
5
6
7
H1
B.
anti-Cdc7
-
CIP
AP
1
2
3
Cdc7

Figure 5. Phosphorylation of Cdc7 Protein by Cdc28 Immune Complexes. Whole yeast lysates were prepared from a *cdc28-13* strain and a wild-type strain as described in the legend to Figure 4. The Cdc28 kinase was then immunoprecipitated from these extracts (100 μg each) with excess antibody (2 μl each) raised against the N-terminal 11 amino acids of Cdc28 protein (lanes 1-4 and 7-10), or with the same antibody preincubated with 30 μg of the N-terminal peptide (lanes 5, 6, 11, and 12). Cdc28 immune complexes were prepared from *cdc28-13* strain in lanes 1-6 and from wild-type strain in lanes 7-12. Kinase reactions were carried out at 25°C (lanes 1, 3, 5, 7, 9, and 11) or 38°C (lanes 2, 4, 6, 8, 10, and 12) using 500 ng of HA-Cdc7 fusion protein as substrate. The fusion protein was purified out of *E. coli* cells as explained in Experimental Procedures. In lanes 3, 4, 9, and 10, Cdc28 immune complexes were incubated in the absence of the fusion protein. Products were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel, which was run until the 30 kDa marker protein reached the bottom.

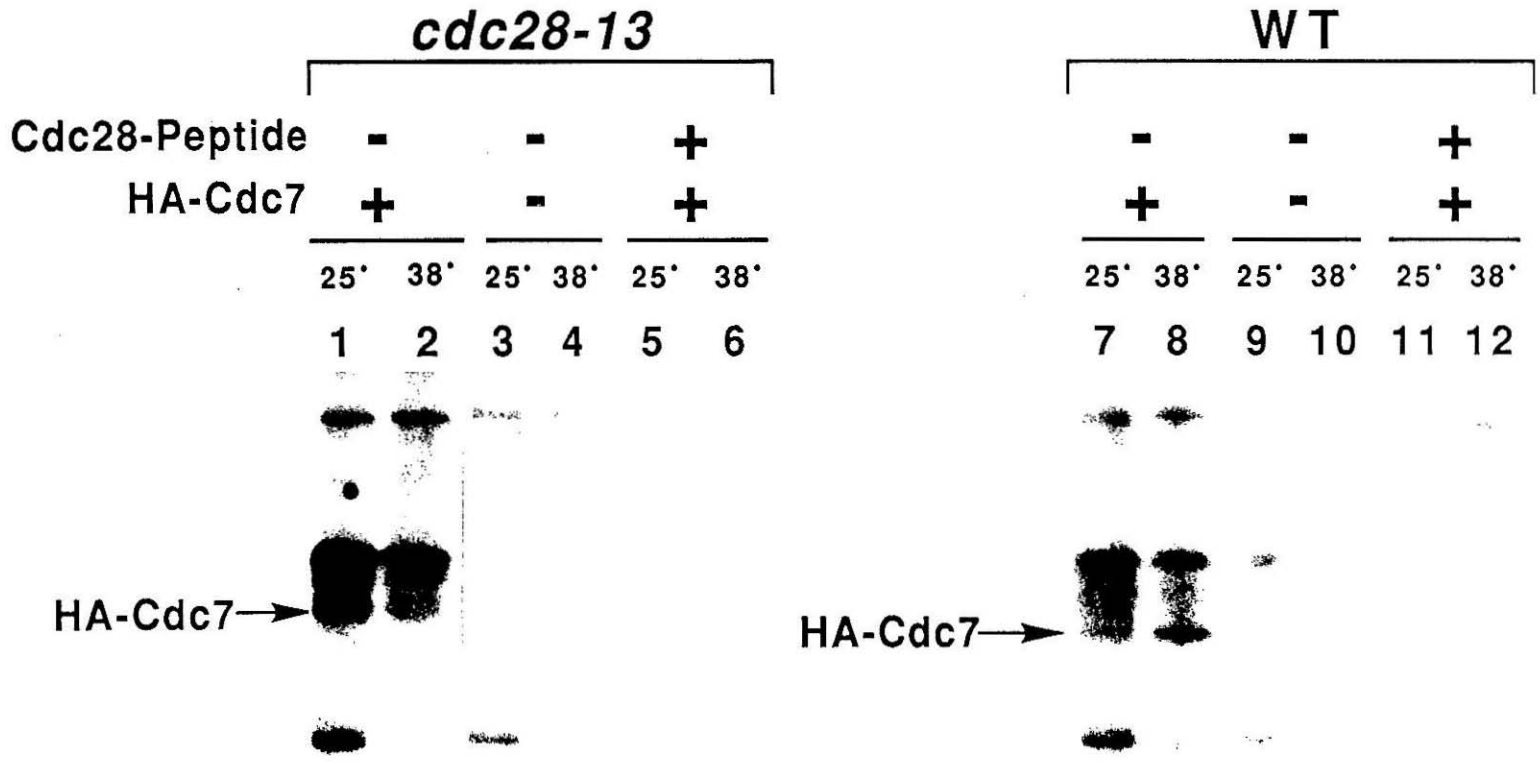

cdc28-13
WT
Cdc28-Peptide - - + - - +
HA-Cdc7 + - + + - +
25° 38° 25° 38° 25° 38° 25° 38° 25° 38° 25° 38°
1 2 3 4 5 6 7 8 9 10 11 12
HA-Cdc7
HA-Cdc7

Figure 6. Cdc28 Kinase Activates Cdc7 Kinase In Vitro.

Cdc28 immune complexes were prepared from a *cdc28-13* strain as described in the legend to Figure 5. In lane 3, the Cdc28 antibody was preincubated with 30 μg of the Cdc28 peptide. After 5 min at 25°C (lanes 1-3) or 38°C (lane 4), either 20 μl of HA-Cdc7 fusion protein in buffer B (1 μg each; lanes 2-4) or 20 μl of buffer B alone (lane 1) were added together with 1 mM ATP. The fusion protein purified out of *E. coli* cells were resuspended in buffer B at a concentration of about 50 ng/μl (see Experimental Procedures for details). Incubation continued at the same temperature for 10 min and reaction mixtures were centrifuged for 1 min to remove Cdc28 immunoprecipitates. The supernatants that contained either HA-Cdc7 protein or buffer alone were preincubated at 38°C for 5 min to inactivate any residual Cdc28-dependent kinase. Then, kinase reactions were carried out at 38°C for 10 min using 0.1 mM ATP, 10 μCi [γ-^{32}P] ATP, and 5 μg of histone H1. Reaction products were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

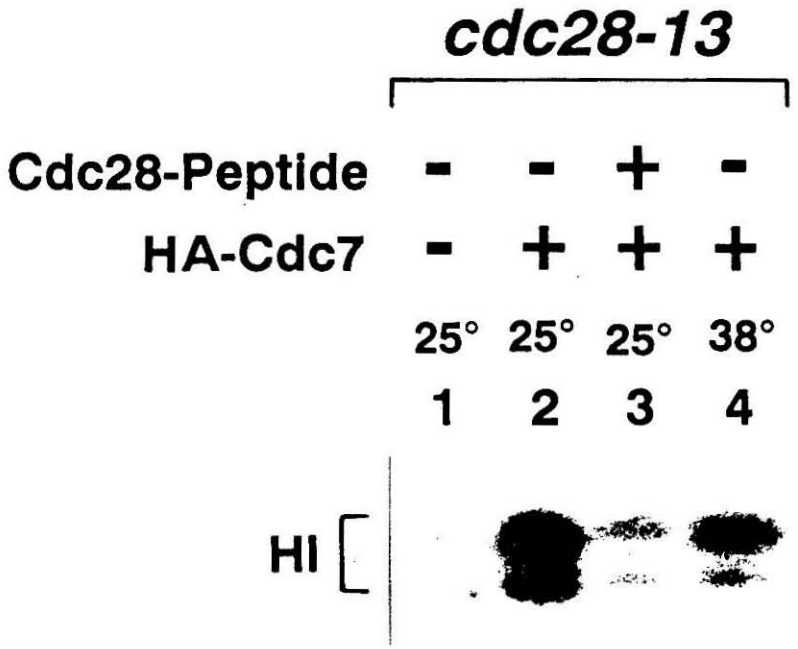

cdc28-13
Cdc28-Peptide - - + -
HA-Cdc7 - + + +
25° 25° 25° 38°
1 2 3 4
HI

Figure 7. The Temperature-Sensitive Phenotype of the *cdc28-13* Mutation Can Be Complemented by *CDC7* Overexpression.

Yeast strains *cdc7-1* (1-3) and *cdc28-13* (4-6) were transformed with plasmid pSYC758 (1 and 6), pSYC7-158 (2 and 5), or pSEY18-Gal (3 and 4). Transformants were isolated on uracil-deficient, glucose plates at the room temperature (25°C). To determine the ability of plasmids to suppress the mutations, single colonies were streaked out on both glucose and galactose plates (minus uracil) and incubated at either 33°C or 36°C.

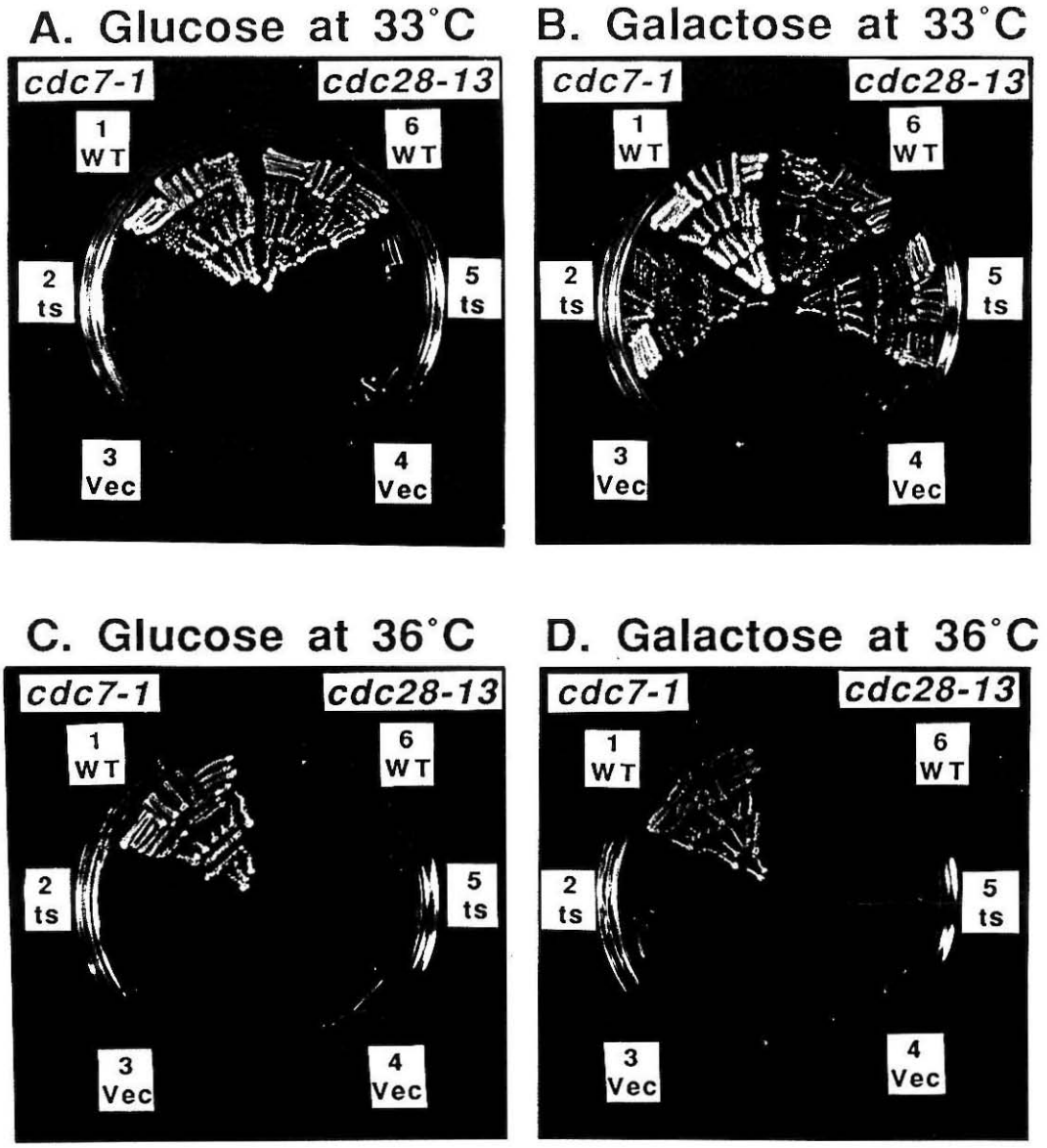

A. Glucose at 33°C
cdc7-1
cdc28-13
1 WT
6 WT
2 ts
5 ts
3 Vec
4 Vec
B. Galactose at 33°C
cdc7-1
cdc28-13
1 WT
6 WT
2 ts
5 ts
3 Vec
4 Vec
C. Glucose at 36°C
cdc7-1
cdc28-13
1 WT
6 WT
2 ts
5 ts
3 Vec
4 Vec
D. Galactose at 36°C
cdc7-1
cdc28-13
1 WT
6 WT
2 ts
5 ts
3 Vec
4 Vec

Figure 8. Cdc7 Protein Is Precipitated by p13 Beads.

Protein extracts (4 mg in lane 1; 2 mg each in lanes 2 and 3) prepared from a wild-type yeast strain carrying either *CDC7*-containing pSYC758 (lanes 1 and 2) or vector pSEY18-Gal (lane 3) were precipitated with p13 beads (60 μl in lane 1; 30 μl each in lanes 2 and 3). After washing 5 times with K buffer (200 μl for each time), proteins bound to the p13 beads were subjected to immunoblot analysis using polyclonal Cdc7 antibody as probe. Supernatant contained 90% of the Cdc7 protein (not shown).

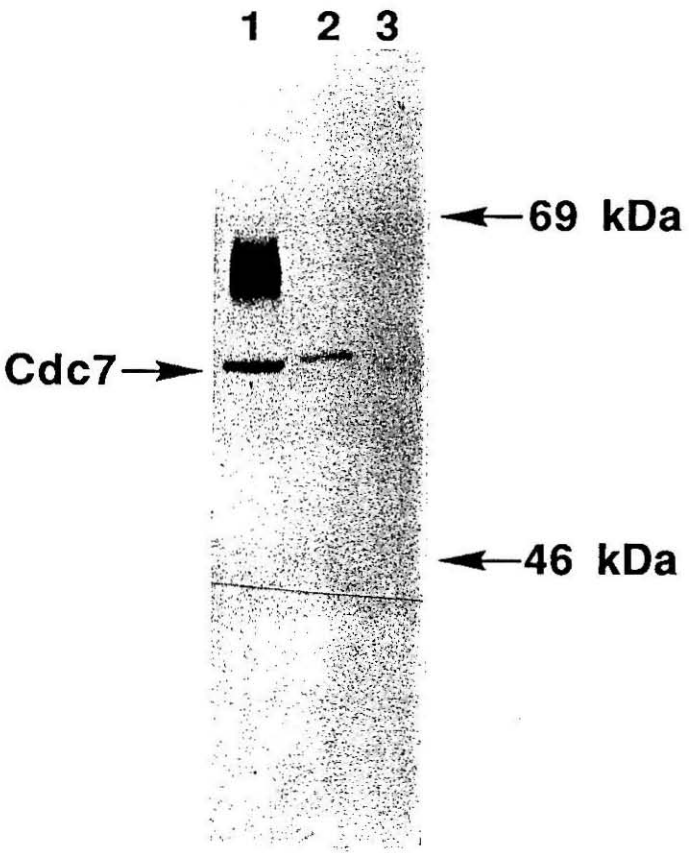

1
2
3
69 kDa
Cdc7
46 kDa

Figure 9. Cdc7 Protein Phosphorylates p36 RP-A.

(A) Yeast extracts (200 μg) prepared from cells containing either pSYCHA7 (HA-Cdc7 fusion protein overproducing plasmid; lanes 2-5) or control vector pSEY18-Gal (lanes 6 and 7) were immunoprecipitated using the monoclonal antibody 12-CA5 (20 μl). In lanes 2 and 3, the antibody was preincubated with the hemagglutinin peptide (50 μg). Kinase assays were performed at either 25°C (lanes 2, 4, and 6) or 38°C (lanes 3, 5, and 7) with 2 μg of yeast RP-A (~700 ng p36) as substrate. In lane 1, RP-A was incubated in the kinase assay in the absence of immunoprecipitates at 38°C. Yeast RP-A was purified according to the published protocol (Brill and Stillman, 1988).

(B) The polyclonal Cdc7 antibody (20 μl each) was used to prepare immune complexes from *cdc28-13* mutant cells (200 μg proteins), which overproduced either wild-type Cdc7 protein (lanes 1 and 2) or thermolabile cdc7 protein (lanes 3 and 4). Cells were grown at the permissive temperature (25°C). Protein kinase assays were carried out as above.

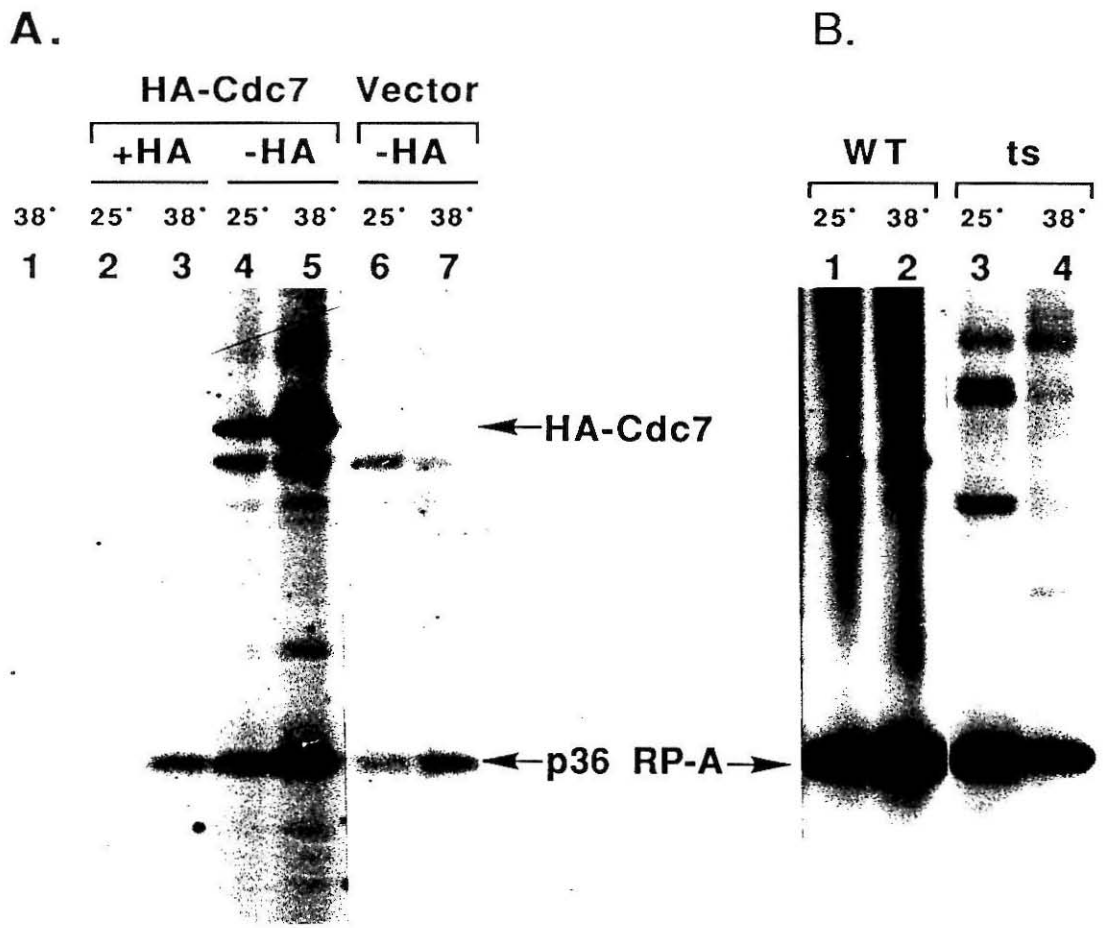
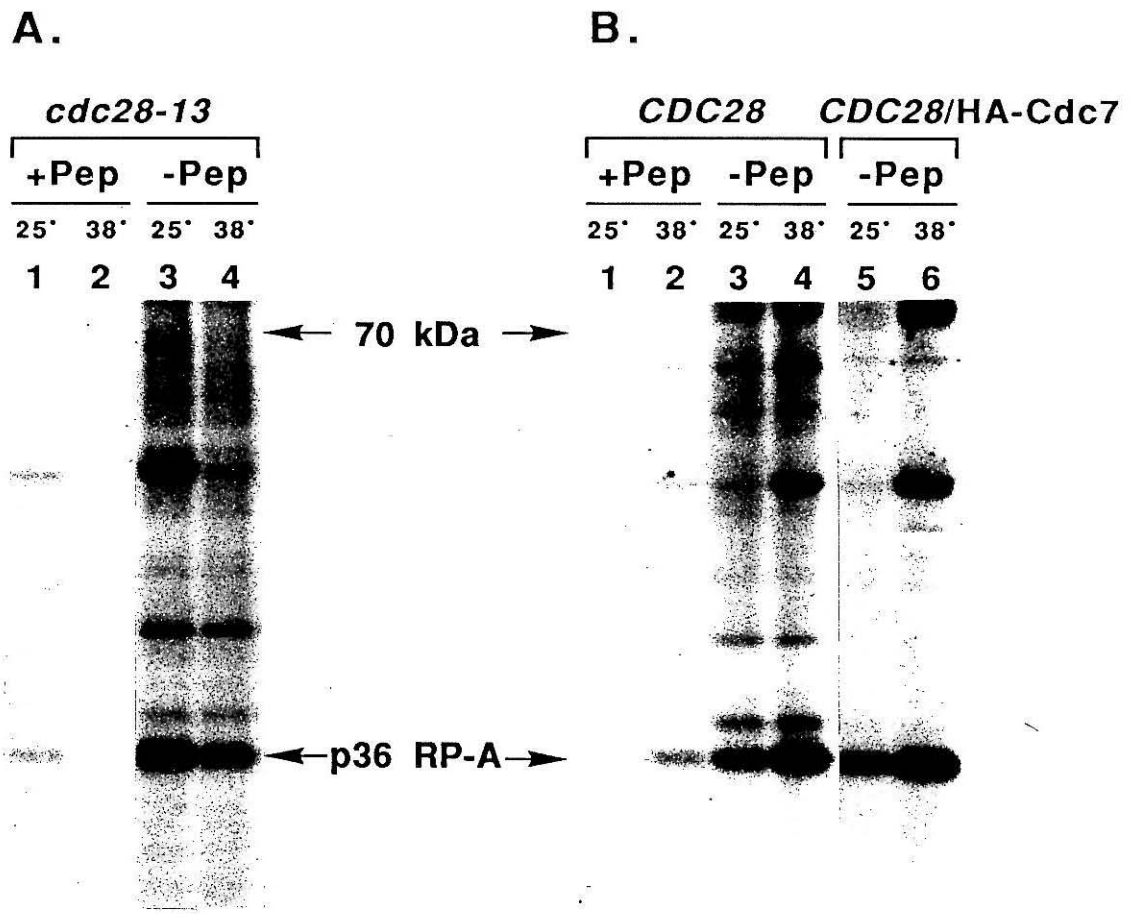

A.
HA-Cdc7
Vector
+HA
-HA
-HA
38°
25°
38°
25°
38°
25°
38°
1
2
3
4
5
6
7
HA-Cdc7
p36
RP-A
B.
WT
ts
25°
38°
25°
38°
1
2
3
4

Figure 10. Phosphorylation of RP-A by Cdc28 Kinase.

(A) The Cdc28 kinase was immunoprecipitated from *cdc28-13* extracts (200 μg each) with the polyclonal antibody raised against the N-terminal peptide (4 μl each; lanes 3 and 4), or with the same antibody preincubated with the epitope peptide (50 μg; lanes 1 and 2). Kinase reactions were done as described in the legend to Figure 9.

(B) The ability to phosphorylate yeast RP-A was measured as in (A) except that Cdc28 immune complexes were prepared from wild-type yeast cells carrying either pSEY18-Gal (lanes 1-4) or pSYCHA7 (lanes 5 and 6).


A.
cdc28-13
+Pep
-Pep
25°
38°
25°
38°
1
2
3
4
70 kDa
p36 RP-A
B.
CDC28
CDC28/HA-Cdc7
+Pep
-Pep
-Pep
25°
38°
25°
38°
25°
38°
1
2
3
4
5
6

APPENDIX

Expression of Double-Stranded-RNA-Specific RNase III of *Escherichia coli* Is Lethal to *Saccharomyces cerevisiae*

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Expression of Double-Stranded-RNA-Specific RNase III of *Escherichia coli* Is Lethal to *Saccharomyces cerevisiae*

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Received 1 December 1987/Accepted 6 April 1988

The gene for the double-stranded RNA (dsRNA)-specific RNase III of *Escherichia coli* was expressed in *Saccharomyces cerevisiae* to examine the effects of this RNase activity on the yeast. Induction of the RNase III gene was found to cause abnormal cell morphology and cell death. Whereas double-stranded killer RNA is degraded by RNase III in vitro, killer RNA, rRNA, and some mRNAs were found to be stable in vivo after induction of RNase III. Variants selected for resistance to RNase III induction were isolated at a frequency of 4×10^{-5} to 5×10^{-5}. Ten percent of these resistant strains had concomitantly lost the capacity to produce killer toxin and M dsRNA while retaining L dsRNA. The genetic alteration leading to RNase resistance was localized within the RNase III-coding region but not in the yeast chromosome. These results indicate that *S. cerevisiae* contains some essential RNA which is susceptible to *E. coli* RNase III.

It is known that double-stranded RNA (dsRNA) plays various important roles in procaryotes, as well as in eucaryotes. For example, formation of dsRNA between mRNA and targeted antisense RNA blocks expression of a specific gene (for a review, see reference 8); formation of dsRNA is an important step during virus infection (e.g., see reference 24), RNA maturation (19), and interferon induction (14). *Escherichia coli* is known to contain a dsRNA-specific RNase called RNase III. This enzyme introduces specific cleavages in a number of RNA (rRNA and mRNA) precursors in *E. coli* as part of the RNA maturation process (6). In contrast to various single-stranded RNases, RNase III nonspecifically degrades dsRNA (21). RNase III is able to cleave RNA-RNA duplexes greater than 20 base pairs long, producing fragments an average size of 15 base pairs long (20). The *E. coli* RNase III gene has been cloned (25), and its DNA sequence has been determined (16, 18). It is intriguing to examine whether RNase III expression in eucaryotes results in specific effects on cell growth, gene regulation, cell physiology, cell morphology, or cell viability.

In this study, we examined expression of the *E. coli* RNase III gene in *Saccharomyces cerevisiae* and its effects on cell growth and cell RNA. We found that induction of expression of the RNase III gene resulted in abnormal cell morphology and cell death. We were able to isolate cells that became resistant to RNase III induction at a frequency of 4×10^{-5} to 5×10^{-5}. Among those resistant strains, approximately 10% were found to be cured of the killer factor M dsRNA. Analysis of the resistant strains revealed that the mutation was localized within the RNase III-coding region. These results indicate that yeast cells possess essential RNAs containing a double-stranded structure(s). Degradation of this double-stranded structure is considered to result in cell death.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strain DMM1-15A (*leu2 ura3 ade2 his5*) and the plasmid YEp51 (2) were kindly provided by J. Broach of Princeton University, New Jersey. Strain S228 and the assay for yeast killer toxin were described by Sherman et al. (22). The growth medium (SD) used in all the experiments contained 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) and 2% (wt/vol) glucose, galactose, or raffinose and was supplemented with the appropriate amino acids. Cultures for growth experiments, RNA preparations, and labeling experiments were pregrown to the stationary phase (12 to 24 h) in SD-glucose medium at 30°C. These cultures were then inoculated into fresh SD medium supplemented with the appropriate carbon source. In growth experiments, culture turbidity was monitored by measuring optical density at 660 nm, and cell viability was monitored by plating appropriate dilutions on SD-glucose plates (2% agar). Lithium acetate DNA-mediated transformation was used for *S. cerevisiae* (11). *E. coli* SB221 and standard cloning techniques have been previously described (15). Variant strains resistant to RNase III induction were isolated by plating culture of DMM1-15A::pPY2 on SD-galactose plates.

Cell labeling and immunoprecipitation. *S. cerevisiae* DMM1-15A cells containing the appropriate plasmid were grown to 2×10^7 cells per ml, harvested, and resuspended in the same SD medium lacking methionine. After the cells were grown for 30 min at 30°C, 100 μCi of [^{35}S]methionine was added to a 5-ml culture and the cells were incubated for 1 to 2 h. The labeled cells were harvested, washed once with fresh SD medium, and suspended in 300 μl of TE (10 mM Tris, pH 8, 1 mM EDTA). Cells were broken with glass beads and centrifuged to recover a clear supernatant. This extract was then immunoprecipitated by RNase III antiserum and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (12).

Southern blot analysis. Yeast DNA was prepared as described by Sherman et al. (22). DNA was transferred to nitrocellulose essentially as described by Southern (23). An 850-base-pair fragment containing the RNase III gene was nick translated and used as the probe. Hybridization was performed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M

sodium citrate)–3× Denhardt solution (4)–0.5% SDS at 65°C for 12 to 16 h. The filters were washed in 3× SSC–0.5% SDS at 65°C for 2 h, dried, and analyzed by autoradiography.

Total yeast RNA isolation and RNA blot analysis. For preparation of total RNA from *S. cerevisiae*, 200-ml log-phase cultures (2×10^7 cells per ml) were harvested and suspended in 2.5 ml of LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris hydrochloride [pH 7.4], 0.2% SDS). Cells were then broken with glass beads in 3 ml of phenol equilibrated with LETS buffer. LETS buffer (5 ml) was added, and the aqueous phase was extracted twice with 5 ml of phenol-chloroform-isoamyl alcohol (25:25:1) and once with chloroform. Finally, total yeast RNA was precipitated with 0.1 volume of 5 M LiCl and 2 volumes of ethanol.

Nick translation of DNA probes and transfer of total RNA were performed essentially as described by Maniatis et al. (15). Filters were prehybridized at 42°C for 2 to 4 h in 5× SSC–50% formamide–5× Denhardt solution–0.5 mg of calf thymus DNA per ml–0.1% SDS. Hybridization was performed under the same conditions, except that the concentration of calf thymus DNA was 0.1 mg/ml and a denatured probe (2×10^7 to 3×10^7 cpm) was included. After hybridization for 16 to 20 h, filters were washed with a solution containing 50% formamide and 5× SSC at 42°C and then washed four times at 42°C with a solution containing 5 mM Tris hydrochloride (pH 7.0), 25 mM NaCl, 1 mM EDTA, and 0.1% SDS. The dried filters were then autoradiographed.

Radioactively labeled RNA was prepared by adding 1 mCi of $^{32}PO_4$ to cultures at 8 h after induction. The cells were labeled for 5 h and then extracted as described above. The amount of label in L dsRNA, 25S rRNA, and 18S RNA was determined in appropriate gel slices from a 0.5% agarose gel.

Assays for RNase III activity. The standard mixture (50 μl) for assaying RNase III contained 0.13 M NH_4Cl, 0.01 M magnesium acetate, 5% sucrose, and 0.02 M Tris hydrochloride (pH 7.9) as described by Robertson et al. (21). The [^{3}H] poly(A · U) copolymer substrate (21) was kindly provided by H. Robertson of Rockefeller University, and the reaction was performed at 37°C for 30 min and terminated by addition of 5% trichloroacetic acid. Bovine serum albumin carrier (0.2 mg) was added, and the precipitate was collected on Whatman GF/A glass fiber filter pads. The pads were dried and counted. An alternative substrate used a total yeast RNA preparation, described above, which was subjected to electrophoresis on a 0.5% agarose gel buffered with 40 mM Tris (pH 7.8)–20 mM sodium acetate–2 mM EDTA. RNase III activity was detected by specific degradation of L dsRNA at various RNase III concentrations (see Fig. 4). Purified RNase III was provided by J. Ahnn (unpublished data).

DNase I and RNase A hydrolysis. Total RNA preparations were incubated at 37°C for 30 min with DNase I (40 μg/ml) and RNase A (4 μg/ml) in a buffer containing 20 mM K_2HPO_4 (pH 7.6), 4 mM $MgCl_2$, and 0.6 M NaCl. To detect both L and M dsRNA species, total RNA samples were applied to a 3.5% polyacrylamide gel after DNase I and RNase A digestion. Electrophoresis was performed with 40 mM Tris hydrochloride (pH 7.8)–20 mM sodium acetate–2 mM EDTA.

RESULTS AND DISCUSSION

Expression of RNase III. To express RNase III in *S. cerevisiae*, a GAL10 expression system (2) was used which allows strong and rapid induction of transcription of the cloned gene when galactose is provided as a carbon source.

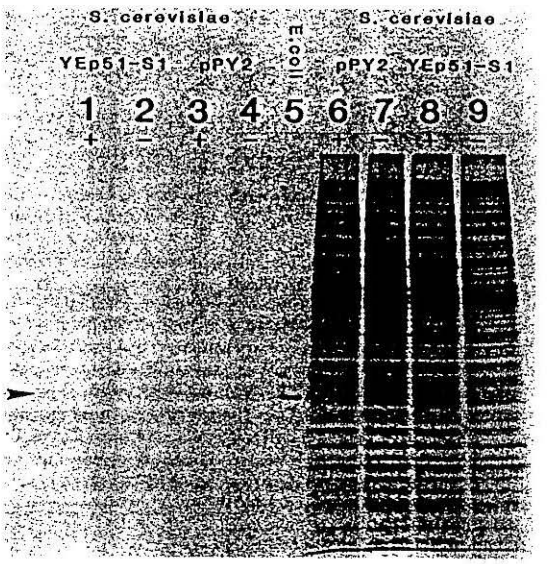

FIG. 1. Production of RNase III in *S. cerevisiae*. *S. cerevisiae* DMM1-15A cells harboring plasmid pPY2 or YEp51-S1 were grown to 10^7 cells per ml with raffinose (uninduced cultures) or galactose (induced cultures) as the carbon source and labeled for 1 h with [^{35}S]methionine. Samples of extracts prepared from the labeled cells were immunoprecipitated with antiserum to RNase III and then analyzed by SDS-polyacrylamide (17.5%) gel electrophoresis. Immunoprecipitated (lanes 1 to 4) and total (lanes 6 to 9) extracts of induced (+) and uninduced (−) cultures harboring either pPY2 (lanes 3, 4, 6, and 7) or YEp51-S1 (lanes 1, 2, 8, and 9) are presented. Lane 5 contained an immunoprecipitated extract of *E. coli* containing ^{35}S-labeled RNase III. The arrow shows the position of RNase III.

The shuttle vector YEp51 contains, in addition to the GAL10 promoter, the 2μm high-copy-number origin of replication and the yeast *LEU2* gene. An 850-base-pair *Hin*cII-*Bam*HI fragment from pTD101 (3) containing the RNase III gene was inserted into the *Sal*I and *Bam*HI sites of YEp51, thus placing the protein-coding sequence between the appropriate promoter and termination sites. The cloned fragment contains 24 base pairs upstream of the initiating ATG from the *E. coli* gene, in which there are no other initiation or termination codons.

To examine the expression of *E. coli* RNase III in *S. cerevisiae*, cells carrying YEp51-RNase III (designated pPY2) were labeled with [^{35}S]methionine and the total cell protein was analyzed by SDS-polyacrylamide gel electrophoresis. As a control throughout this study, the same strain (DMM1-15A) carrying a YEp51 derivative carrying the gene for protein S of *Myxococcus xanthus* (10) was used. A band with a mobility identical to that of *E. coli* RNase III was found in the extract from induced cultures harboring pPY2 (Fig. 1, lanes 6 and 7). A corresponding band was absent from extracts of cultures harboring YEp51-S1 which were induced under similar conditions (Fig. 1, lane 8). Immunoprecipitation of the above-described extracts with RNase III antiserum demonstrated that the suspected band was retained and thus contained material that cross-reacts with RNase III (compare lanes 3 and 4 in Fig. 1).

Strains containing either pPY2 or YEp51-S1 exhibited the

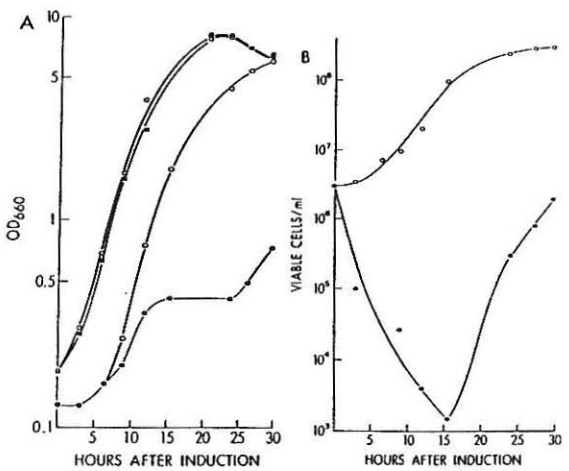

FIG. 2. Growth of *S. cerevisiae* DMM1-15A after induction of RNase III. DMM1-15A harboring pPY2 or YEp51-S1 was first grown to stationary phase in glucose-containing medium and then inoculated into either galactose- or glucose-containing medium. (A) Culture turbidity was monitored by optical density at 660 nm. (B) Cell growth was monitored by a viable count on glucose-containing plates. Symbols of respective plasmid and growth medium: ●, pPY2-galactose; □, pPY2-glucose; ○, YEp51-S1-galactose; ■, YEp51-S1-glucose.

same growth kinetics as monitored by culture turbidity when glucose was used as the carbon source (Fig. 2A). In contrast, when galactose was used as the carbon source, the strain harboring pPY2 grew more slowly than the strain harboring YEp51-S1 (Fig. 2A). The RNase III-induced culture stopped growing after 16 to 18 h at only about 20% of the turbidity of the control culture. The turbidity of the RNase III-induced culture eventually started to increase again because of the growth of a fraction of the culture which survived the lethal effect of RNase III induction. A striking effect on cell viability was observed after induction of RNase III (Fig. 2B). Within 3 h, the viability of induced cultures harboring pPY2 dropped 10-fold, and after 12 to 15 h the frequency of surviving cells was 4×10^{-5} to 5×10^{-5}. It was of interest that in the initial period after RNase III induction there was no change in cell morphology and the cells appeared to bud normally, as observed by phase-contrast light microscopy. However, after 10 to 12 h, the RNase III-induced cells appeared slightly larger than the control, and at 18 to 20 h, extremely large cells were observed (Fig. 3B), in contrast to control cells harboring YEp51-S1 (Fig. 3A). A yeast strain harboring one copy of the RNase III gene integrated into the chromosome displayed the same lethal effect and cell morphology in response to induction as the 2μm-derived pPY2 plasmid described (data not shown).

Activity of RNase III. Two assays were used to determine RNase III activity in yeast lysates. Initially we used the method of Robertson et al. (21), in which a ^{3}H-labeled double-stranded poly(A · U) copolymer is the RNase III substrate. Significant reduction in ^{3}H-labeled trichloroacetic acid-precipitable counts was found only in the lysate of the galactose-induced strain harboring pPY2 (data not shown). A second activity assay used naturally existing dsRNA from *S. cerevisiae*. Many *S. cerevisiae* strains contain dsRNA which is encapsidated in viruslike particles (27). Two types

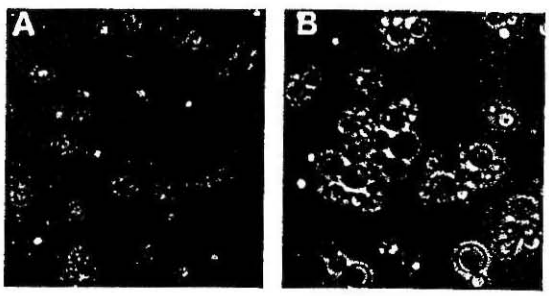

FIG. 3. Cell morphology after induction of RNase III. Cultures induced for 18 h (described in the legend to Fig. 2) harboring YEp51-S1 (A) or pPY2 (B) were subjected to phase-contrast light microscopy (magnification, ×640).

of dsRNA molecule are usually found in viruslike particles of *S. cerevisiae*. The larger, designated L, encodes the capsid protein (9), whereas the smaller dsRNA, designated M, encodes a toxin (1) which is secreted and can kill other *S. cerevisiae* strains. L dsRNA from a preparation of total RNA of *S. cerevisiae* was degraded by purified RNase III (Fig. 4). The dose-dependent degradation of L dsRNA appears to be specific, since rRNA (25S and 18S) was unaffected. Figure 4B presents the effects of crude yeast lysates on L dsRNA. Only lysates from cultures harboring pPY2 which were induced by galactose caused specific, dose-dependent digestion of L dsRNA (lanes 5 and 6). Lysates from uninduced cultures harboring pPY2 (lane 4) or lysates from strains harboring the control plasmid YEp51-S1 (lanes 2 and 3) exhibited no RNase III activity by this assay.

The lethality of RNase III induction in *S. cerevisiae* raises the question of the target of this enzyme in vivo. One possibility is that RNase III causes a general disruption of

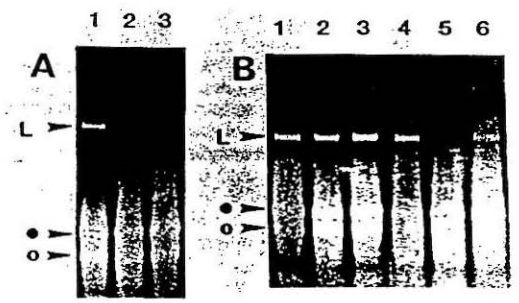

FIG. 4. Assay of RNase III activity in yeast lysates. (A) Total yeast RNA (20 μg) was treated with 0, 0.4, or 10 μg (lanes 1, 2, and 3, respectively) of purified RNase III at 37°C for 30 min, subjected to 0.5% agarose gel electrophoresis, and stained with ethidium bromide. (B) Strain DMM1-15A harboring pPY2 or YEp51-S1 was grown in either galactose- or glucose-containing medium as described in the legend to Fig. 2. Cultures (5 ml) were harvested at an optical density of 1 at 660 nm, and cell extracts were prepared. Samples of these extracts were incubated with total yeast RNA and subjected to gel electrophoresis as described above. The gel order in panel B with respect to the culture extract used (plasmid harbored-growth medium) was as follows: 1, no extract added; 2, 5 μl of YEp51-S1-glucose; 3, 5 μg of YEp51-S1-galactose; 4, 5 μl of pPY2-glucose; 5, 5 μl of pPY2-galactose; 6, 1 μl of pPY2-galactose. Symbols: L, L dsRNA; ●, 25S rRNA; ○, 18S rRNA.

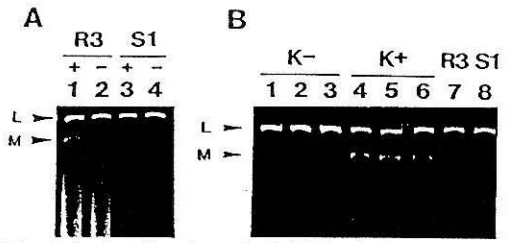

FIG. 5. (A) Stability of L and M dsRNAs in *S. cerevisiae* after induction. Total cell RNAs from cultures harboring pPY2 or YEp51-S1 grown for 6 h in either galactose (+)- or glucose (−)-containing medium were treated with RNase A and DNase I (as described in Materials and Methods), subjected to 3.5% polyacrylamide gel electrophoresis, and stained with ethidium bromide. Lanes: 1 and 2, RNA from DMM1-15A::pPY2; 3 and 4, RNA from DMM1-15A::YEp51-S1. (B) L and M dsRNAs in variants resistant to RNase III induction. Total cell RNAs from variants resistant to RNase III induction were analyzed as described for panel A. RNA was prepared from induced cultures of toxin-negative variants (K^-; lanes 1 to 3), toxin-positive variants (K^+; lanes 4 to 6), DMM1-15A::pPY2 (R3; lane 7), and DMM1-15A::YEp51-S1 (S1; lane 8).

RNA stability or synthesis or both. The double-stranded killer RNA was considered to be useful for probing RNase III in vivo since it is an RNase III substrate in vitro (Fig. 4). In contrast to the in vitro result, however, killer dsRNA appears to be stable after induction of RNase III in vivo. The polyacrylamide gel pattern for both L and M dsRNAs was the same with all four culture extracts examined (Fig. 5A). Furthermore, RNA preparations from cells that were labeled 8 h after induction incorporated the same proportion of $^{32}PO_4$ into L killer RNA relative to its incorporation into rRNA (data not shown). According to these results, RNase III does not cause specific degradation of L RNA in vivo, and we could not detect any effect on newly synthesized double-stranded L RNA.

A reasonable explanation for the stability of killer RNA in vivo may be the protection of dsRNA by proteins associated with the viruslike particle (27). In this regard, eucaryotic RNAs exist in vivo in the form of ribonucleoproteins which may render these RNAs unavailable for digestion by nonspecific RNases. RNAs involved in protein synthesis, such as rRNA, mRNA, and tRNA, do not seem to be the primary target of RNase III, since protein synthesis was not shut off after induction. Furthermore, at 12 h after induction, incorporation of [^{35}S]methionine into a culture harboring pPY2 was still 35% of the incorporation into a culture harboring YEp51-S1. The total RNA extracted from RNase III-induced cells exhibited an unchanged pattern of rRNA on ethidium bromide-stained gels. RNA blot analysis of actin, *LEU2*, and RNase III mRNA from *S. cerevisiae* exhibited no significant differences in the amounts of these mRNAs because of RNase III induction. In addition, at 12 h after induction of RNase III, incorporation of [^{32}P]phosphate and [^{35}S]methionine appeared to be affected to the same degree (total incorporation was about 35% when compared with that of a control culture). These results do not support the hypothesis that RNA, in general, is destabilized by expression of RNase III in *S. cerevisiae*. Instead, the effect seems to be on a specific target, presumably a cytoplasmic dsRNA moiety.

Resistance to RNase III induction. As described earlier, 12 to 15 h after induction of cultures harboring pPY2, variants resistant to RNase III induction were isolated at a frequency of 4×10^{-5} to 5×10^{-5}. We examined the ability of RNase III-resistant variants to produce toxin encoded by the M dsRNA. Two hundred independently isolated variant colonies were spotted onto a lawn of a toxin-sensitive strain. We found that 10% of these RNase III-resistant variants did not produce detectable amounts of toxin by this assay. All these killer-negative RNase III-resistant strains were found to lose M dsRNA concomitantly (Fig. 5B, lanes 1 to 3). In both killer-positive and killer-negative strains, the L dsRNA appeared to be intact (Fig. 5B, lanes 1 to 6). In a similar sample of induced cultures harboring YEp51-S1, no toxin-negative strains were isolated. As mentioned earlier, neither the stability nor the synthesis of dsRNA appeared to be affected by RNase III induction; thus, it is not clear how M dsRNA was cured in some RNase III-resistant variants. It is possible that RNase III has a subtle effect on stability or segregation of this dsRNA. Since it is known that cycloheximide (7), 5-fluorouracil (17), and yeast growth at high temperatures (26) can cure the cell of M dsRNA, it is also possible that M dsRNA was cured as a result of some metabolic changes caused by RNase III induction.

It was of interest to examine these RNase III-resistant variants further to determine whether the mutation occurred on the chromosome or on the plasmid. Southern blot analysis of K^+ and K^- strains (data not shown) revealed that both the plasmid and its 850-base-pair RNase III gene insertion appeared to be unaltered. Surprisingly, it was found that, by densitometric scanning of SDS-polyacrylamide-protein gels, similar amounts of RNase III protein were produced after induction of pPY2 in both the wild-type and variant strains (data not shown). The variants tested at different times after induction also exhibited the same level of RNase III activity as the wild type, as shown by two different assay systems described earlier (data not shown).

RNase III resistance due to a mutation in the RNase III gene. To determine whether the genetic change accompanying RNase III resistance was chromosomal or occurred within the plasmid pPY2, one of the K^+ RNase III-resistant variants was analyzed further. This variant was cured of its plasmid, and conversely the plasmid itself was isolated from the variant cells. When wild-type cells transformed with either pPY2 or the variant plasmid (designated SP1) were compared, only cells containing the original plasmid, pPY2, displayed the lethal phenotype. When the variant strain (designated BK3) harboring these same plasmids was checked, cells harboring pPY2 displayed the lethal phenotype, whereas cells harboring the variant plasmid were resistant to induction. These results indicate that RNase III resistance was due to the variant plasmid. Finally, the 850-base-pair RNase III-containing fragment from the variant plasmid was cloned into the original YEp51 vector. Cells harboring this plasmid did not exhibit the lethal phenotype, confirming that the genetic alteration leading to RNase III resistance had occurred within the 850-base-pair RNase III gene-containing insert.

The results described above indicate that the RNase III resistance mutation occurred in the structural gene of RNase III. The production of the altered RNase III appeared to be at the same level as that of the wild-type RNase III (Fig. 6). Furthermore, the mutated RNase was fully active when tested with dsRNA as a substrate. Therefore, the mutation in RNase III possibly changes the specificity of the enzyme in vivo in such a way that it does not recognize its lethal target anymore. In this regard it should be noted that RNase III specifically cleaves only certain single-stranded RNAs of *E. coli* in vivo (6). Alternatively, RNase III may require a yeast

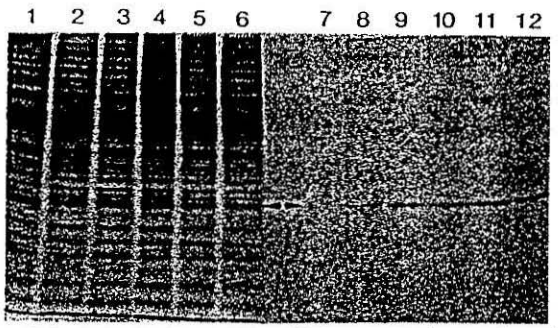

FIG. 6. Production of RNase III in yeast strains transformed with original and variant plasmids. Cell labeling, immunoprecipitation, and electrophoresis were as described in the legend to Fig. 1. Extracts of induced cultures of DMM1-15A::YEp51-S1 (lane 1), BK3::pPY2 (lane 2), BK3::SP1 (lane 3), DMM1-15A::pPY2 (lane 4), DMM1-15A::SP1 (lane 5), and an original RNase III-resistant variant, K^+2 (lane 6), were used. Lanes 7 to 12 contained immunoprecipitated samples of extracts shown in lanes 1 to 6, respectively. pPY2, original RNase III-containing plasmid; SP1, variant plasmid; DMM1-15A, wild-type strain; BK3, a resistant strain cured of its plasmid.

component for its activity in vivo. A mutation in RNase III blocking the interaction between these two components would result in loss of the lethal activity in vivo. Since the native RNase III enzyme has been shown to be a dimer of two homologous polypeptides (5), it is also possible that a mutation rendering the protein unable to form dimers would affect its activity in vivo. In this respect, it is interesting to examine whether the mutated RNase III gene from *S. cerevisiae* can complement a chromosomal RNase III mutation in *E. coli* (13).

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