The yeast *Saccharomyces cerevisiae* Cdc7p/Dbf4p protein kinase complex was purified to near homogeneity from insect cells. The complex efficiently phosphorylated yeast Mcm2p and less efficiently the remaining Mcm proteins or other replication proteins. Significantly, when pretreated with alkaline phosphatase, Mcm2p became completely inactive as a substrate, suggesting that it must be phosphorylated by other protein kinase(s) to be a substrate for the Cdc7p/Dbf4p complex. Mutant Cdc7p/Dbf4p complexes containing either Cdc7-1p or Dbf4-1p5p were also partially purified from insect cells and characterized in vitro. Furthermore, the autonomously replicating sequence binding activity of various *dbf4* mutants was also analyzed. These studies suggest that the autonomously replicating sequence-binding and Cdc7p protein kinase activation domains of Dbf4p collaborate to form an active Cdc7p/Dbf4p complex and function during S phase in *S. cerevisiae*. It is shown that Rad53p phosphorylates the Cdc7p/Dbf4p complex in vitro and that this phosphorylation greatly inhibits the kinase activity of Cdc7p/Dbf4p. This result suggests that Rad53p controls the initiation of chromosomal DNA replication by regulating the protein kinase activity associated with the Cdc7p/Dbf4p complex.

Initiation of chromosomal DNA replication and cell cycle progression are tightly regulated in eukaryotes. In the yeast *Saccharomyces cerevisiae*, several *cdc* (cell division cycle) mutants that block initiation of chromosomal DNA replication have been isolated and characterized (1, 2, for example, *cdc28*, *cdc4*, *cdc6*, and *cdc7*. The stepwise assembly of proteins at origins of DNA replication is a crucial part of regulating entry into S phase. Two key factors that mediate such cell cycle regulation are Cdc6 protein level and availability and the presence of an active cyclin-dependent kinase (Cdk) (see Ref. 3 for review). The origin recognition complex is bound to origins of DNA replication at all stages of the cell cycle in *S. cerevisiae* (4–6). However, Cdc6 is not recruited to origins until late in M phase and is required for the association of the Mcm2–7 family proteins at origins to form a prereplicative complex (6–8).

The Cdc6 protein-dependent stage of the assembly reaction is inhibited by active Clb-Cdks (6, 9, 10). Because Cdc6 protein is synthesized only from late M phase until late G1 (11), prereplicative complexes can only be assembled during this period of the cell cycle. S phase cyclin-Cdk (Cdc28p/Clb5p or Cdc28p/Clb6p) activity is required for the chromatin association of Cdc45p just before the initiation of chromosomal DNA replication (12). The previous results demonstrated that Cdc28-protein-Cdk kinase is required throughout S phase to activate origins when they are scheduled to fire (13).

The Cdc7/Dbf4 complex is a Cdk-like protein kinase (see Refs. 14 and 15 for review) that is also required for entry into S phase at a very late stage. *CDC7* transcript levels are constant throughout the cell cycle, whereas *DBF4* transcription is cell cycle regulated and the transcript abundance peaks near the G1 to S phase transition. The *DBF4* gene was first identified as a gene that could suppress *cdc7* mutants when present in multiple copies (16), and Dbf4p is now known to be required for Cdc7p-mediated kinase activity. It has been shown that a Cdc7p-associated H1 kinase activity is cell cycle regulated (17). It is not clear, however, whether this H1 kinase activity reflects the normal activity of the Cdc7p kinase. Recently, homologues of Cdc7p have been identified in *Schizosaccharomyces pombe* (18), mouse (19), *Xenopus* , and humans (20–22), and a Dbf4p homologue has also been found in *S. pombe* (23, 24) and human (25). The presence of these homologues in evolutionarily distant species suggests that Cdc7p/Dbf4p plays a conserved role in the initiation of DNA replication in eukaryotes.

Several lines of evidence suggest that Cdc7p/Dbf4p acts directly at origins of DNA replication and that the Mcm proteins may be one of the targets of the kinase. *DBF4* was isolated in a one-hybrid screen for autonomously replicating sequence (ARS)-interacting factors (26). Interestingly, its interaction with origins was found to be independent of its ability to bind to Cdc7p. Two recent reports (27, 28) suggest that Cdc7p activity is required to activate individual origins rather than for a global activation of S phase, because temperature-sensitive *cdc7* mutants demonstrate an inability to activate late rather than early replication origins at a semi-permissive temperature. Potential target(s) of Cdc7p that are required for the initiation of DNA replication were revealed through genetic

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To whom correspondence should be addressed. Tel.: 81-6-6879-8331; Fax: 81-6-6877-3584; E-mail: asugino@biken.osaka-u.ac.jp.

1 The abbreviations used are: ARS, autonomously replicating sequence; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; BAP, alkaline phosphatase; HU, hydroxyurea.
analysis of a mutant in *S. cerevisiae* that could bypass the requirement for CDC7 or DBF4 (17). The mutation was found to reside in *MCM5/CDC46* (29), a member of the Mcm family of six sequence-related proteins (30). Since then, a number of reports have suggested that some Mmc proteins such as Mmc2 and Mmc3 are in *vitro* substrates of Cdc7p kinases from various sources (21, 23, 31). Another study recently reported that all yeast Mmc proteins, except for Mmc5 expressed and prepared from insect cells, are substrates for the Cdc7p/Dbf4p protein kinase complex (32).

**RAD53** encodes a dual specificity protein kinase that is required for all three DNA damage checkpoints at G1, S phase, and G2 (33). It is thought to be part of the transducer class (34). Several lines of circumstantial evidence suggest that in addition to its checkpoint function, Rad53p could be also involved in the regulation of temporal order origin firing during chromosomal DNA replication (35).

In this report, we describe the expression and the purification of recombinant yeast Cdc7p/Dbf4p to near homogeneity and biochemical analysis of the Cdc7p/Dbf4p complex-associated protein kinase activity. We show that both subunits of the recombinant enzyme are auto-phosphorylated and that this reaction requires active Dbf4p and Cdc7p. The recombinant protein efficiently phosphorylates Mmc2 and also phosphorylates with somewhat lower efficiency the remaining Mmc proteins as well as the largest subunit of DNA polymerase α-primase and Rpa. However, when Mmc2 protein was pretreated with an alkaline phosphatase, it was not phosphorylated by the Cdc7p/Dbf4p kinase, suggesting that Mmc2 protein must be prephosphorylated by an unknown kinase(s) to be a substrate for Cdc7/Dbf4 protein kinase activity. Expression of mutant Cdc7/Dbf4 protein kinases suggests that ARS-binding and Cdc7 protein kinase activation domains of Dbf4p collaborate to form an active Cdc7/Dbf4 complex that functions in chromosomal DNA replication. Finally, inhibition of the Cdc7p/Dbf4p complex kinase activity by Rad53p-mediated phosphorylation is demonstrated. Based on these results, it is proposed that Rad53p regulates the temporal activation of chromosomal DNA replication origins in yeast by regulating the activity of Cdc7p/Dbf4p protein kinase.

**MATERIALS AND METHODS**

**Yeast Strains**—The following yeast strains were used in this study: *S. cerevisiae* 208 (MATa cdc7-1 ura3-52 leu2-3, 112), L128-2D (MATa cdc7-2 ura3-52 leu2-3, 1, 5), L202-1A (MATa cdc7-4 ura3 leu2 trp1), KKY74 (MATa cdc7-4 ura3 leu2 trp1), KKY74a (MATa cdc7-4 ura3 leu2 trp1), and KKY745 (MATa cdc7-4 ura3 leu2 trp1), L202-1A (MATa cdc7-4 ura3 leu2 trp1), KKY74 (MATa cdc7-4 ura3 leu2 trp1). KKY74a (MATa cdc7-4 ura3 leu2 trp1), and KKY745 (MATa cdc7-4 ura3 leu2 trp1) were initially described previously (16, 16).

**Yeast DNA and Plasmid DNA**—Standard DNA manipulations were carried out according to Sambrook et al. (38). Oligonucleotides 5'-GGGC-CAGATCTCATATAGCAAGAACAAAGA-3', 5'-GGGCCAGATCTTGTC-CTATTAGGATATAG-3', 5'-GGGCCGATCTGCAGAAATGTTTCC-TCCCAAGAACAAC-3', and 5'-GGGCCGATCTGTTATTTGAATCTGAGATT-3' (underlined nucleotides represent either BglII or BamHI sites, and bold nucleotides are the initiation or termination codons) were synthesized on an automated DNA synthesizer (Beckman) and were used for polymerase chain reaction amplification of the DNA fragments containing CDC7 and DBF4 on the plasmid pKK709 and pKK616 DNA (16). Amplified DNA was digested with either BglII (for CDC7) or BamHI enzyme (for DBF4) and inserted into either BglII- or BamHI-digested baculovirus vector pBacPAK. The resulting constructs, pBacCdc7p and pBacDbf4p, were co-transfected with Bsl2/3I-linearized wild type virus BacPAK6 viral DNA into SF9 cells using cationic liposome-mediated transfection according to the Invitrogen protocol, and recombinant virus was plaque purified. The presence of an insert in a putative recombinant virus was verified by polymerase chain reaction using primers complementary to the polyhedron locus. Recombinant virus pBac-Cdc7 17 and pBac-Dbf4 32 were amplified in SF9 cells to a titer of 10^9 plaque-forming units/ml. Recombinant virus containing mutant CDC7-1 or dbf4-1–5 were constructed as wild type CDC7 and DBF4 genes.

**Expression of Recombinant Protein**—SF9 cells were grown to confluence in T-75 Falcon flasks at 27 °C in Grace’s medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, and 0.1% pleurocidin to a cell density of 5–10 × 10^6 cells/ml. The infected SF9 cells were then harvested at 72 h post-infection by centrifugation at 1,200 × g and washed twice with serum-free Grace’s medium.

**Purification of the Recombinant Cdc7p/Dbf4p Protein Kinase Complex**—Recombinant virus pBac-Cdc7 17 and pBac-Dbf4 32 were used to infect 2 L of SF9 cells (2 × 10^6 cells/ml) at a multiplicity of infection of 10. After 72 h the cells were harvested, washed with serum-free Grace’s medium, resuspended in 60 ml of ice-cold HS1 buffer (50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, and 500 mM NaCl) containing a mixture of protease inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml bacitracin, 250 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine), and subjected to sonication by using an Ultrasonic disruptor (Tomy, Japan). To the lysate (52 ml), obtained by centrifugation at 27,000 × g for 20 min, 16.3 g of solid (NH₄)₂SO₄ (50% saturation) was gradually added with stirring. After 30 min stirring, the extract was centrifuged at 27,000 × g for 20 min. The precipitated protein was dissolved in 30 ml of buffer A (50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol (v/v), and 1 mM phenylmethylsulfonyl fluoride) and dialyzed against 2 liters of buffer A containing 50 mM NaCl for 4 h at 0 °C. After centrifugation at 27,000 × g for 20 min, the suspension was loaded onto an S-Sepharose column (80 ml) equilibrated in buffer A containing 100 mM NaCl. The column was washed with three bed volumes of the same buffer, and the protein was eluted with a 320-mM linear gradient of 100–600 mM NaCl in buffer A. Fractions containing Cdc7p and Dbf4p, as identified by activity assays and Western blot analysis (0.2–0.3 M NaCl), were pooled, dialyzed twice against 1 liter of buffer A containing 100 mM NaCl for 3 h, and loaded onto an Hi Trap Heparin column (5 ml) equilibrated with buffer A containing 200 mM NaCl. After washing with three bed volumes of equilibrium buffer, the protein was eluted with a 120-mM linear gradient of 0.2–1 M NaCl in buffer A. The peak fractions (0.5–0.65 M NaCl) were pooled, dialyzed twice against 1 liter of buffer A containing 100 mM NaCl for 3 h, and loaded onto a Mono-Q column (HR/5, 1 ml) prestained with buffer A containing 100 mM NaCl. Protein fractions were eluted with a 15-mL linear gradient from 100 to 700 mM NaCl in buffer A. Active fractions (0.3–0.4 M NaCl) were pooled together, dialyzed against 50% glycerol, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptopethanol, and 1 mM phenylmethylsulfonyl fluoride for 2 h and stored at –80 °C.

**Purification of Yeast 2HA-tagged Rad53p Protein Kinase**—*S. cerevisiae* CB001 cells expressing 2HA-tagged Rad53p were grown at 30 °C in 24L YPD medium to an 1 = 1–2 and harvested by centrifugation at 8,000 rpm for 20 min. Cells were resuspended in buffer A (30) to 1 g/ml and disrupted by passage through a Gaulin homogenizer at 10,000 psi. To cell extracts, NaCl was added to a final concentration of 0.5 M. After stirring at 0 °C for 30 min, cell debris was removed by centrifugation in a Beckman JLA10.5 rotor at 8,000 rpm for 20 min, and the supernatant was saved. To the supernatant, 10% (w/v) Polymin P (pH 8) solution was slowly added to 0.5% (final concentration), mixed for 15 min, and centrifuged in a Beckman JLA 10.5 rotor at 8000 rpm for 20 min. 0.313 g of ammonium sulfate was added per ml of supernatant, and the mixture was stored at 30 °C. The protein precipitate was collected by centrifugation in a Beckman JLA 10.5 rotor at 8000 rpm for 30 min. The pellet was stored at –80 °C. The precipitate was resuspended in 20 ml of buffer A and dialyzed against 2 liters of buffer A for 2 h and 2 liters of 0.1 M NaCl in buffer A for 2 h. Insoluble material was removed from the dialysate by centrifugation in a Beckman JLA20.0 rotor at 10,000 rpm for 10 min. The supernatant was applied on an S-Sepharose column (75 ml) equilibrated with 0.1 M NaCl in buffer A, the column was washed with three column volumes of 0.1 M NaCl in buffer A, and 10 mM medium in 0.5 M NaCl in buffer A. The protein was eluted at 0.5 M NaCl. Fractions were checked by Western blot analysis followed by immunostaining. The fractions containing the protein were pooled together (Fraction 1). Fraction 1 was dialyzed twice against 2 liters of 0.05 M NaCl in buffer A for 2 h and centrifuged in a Beckman JLA20.0 rotor at 10,000 rpm for 10 min to remove any precipitates. The supernatant was applied on a Mono Q
column (HR 10/10) equilibrated with 0.05 M NaCl in buffer A. The column was washed with three column volumes of 0.05 M NaCl in buffer A, and then protein was eluted with 20 column volumes of linear gradient from 0.05 to 0.5 M NaCl in buffer A. Fractions containing the Rad53 protein were identified by Western blot analysis and pooled (Fraction I). Fraction II was dialyzed against 2 liters of 0.1 M NaCl in buffer A for 2 h and applied on a Mono S column (HR 5/5) equilibrated with 0.1 M NaCl in buffer A. The column was washed with three column volumes of 0.1 M NaCl in buffer A and then eluted with a 30-ml linear gradient of 0.1–0.5 M NaCl in buffer A. Fractions containing Rad53 protein were identified by protein kinase activity assay using Histone H1 and Western blot analysis, and the active fractions were pooled (Fraction III). Fraction III was dialyzed against 1 liter of 0.1 M NaCl in buffer A for 3 h and applied on a Hi-Trap Heparin column (HR5/5) equilibrated with 0.1 M NaCl in buffer A. The column was washed with three column volumes of 0.1 M NaCl in buffer A and then eluted with 20 ml of linear gradient of 0.1–0.5 M NaCl in buffer A. Fractions containing Rad53 protein were identified by protein kinase activity assays and Western blot analysis, and the active fractions were pooled (Fraction IV).

HU-activated Rad53p was also purified as described above from CB001 cells expressing 2HA-tagged Rad53p after treating cells with 0.2 M hydroxyurea for 3 h at 30 °C. More detailed characterization of the purified 2HA-tagged Rad53p will be published elsewhere.

**SDS Polyacrylamide Gel Electrophoresis and Protein Determination**—4–20% gradient SDS-PAGE was performed by the method of Laemmli (38). Protein concentration was determined by the method of Bradford with bovine serum albumin as standard as described previously (31, 36).

**Immunoblot Analysis**—Following SDS-PAGE, proteins were electroblotted onto an Immobilon-P (Millipore) membrane that was incubated for 30 min with blocking buffer, Tris-buffered saline (50 mM Tris- HCl, pH 7.4, and 100 mM NaCl) containing 2% nonfat dry milk, and incubated overnight with rabbit polyclonal antipeptide antiserum to Dbf4p (provided by J. Difflay) or with rabbit polyclonal antiserum to Cdc7p expressed in *Escherichia coli* (provided by H. Masai). After washing three times with blocking buffer, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 1.5 h at room temperature, washed three times with blocking buffer, and washed twice with Tris-buffered saline. Color was developed as described previously (31).

**Protein Kinase Assay**—Protein kinase activity was assayed with GST-Mcm2p purified from *S. cerevisiae* as a substrate. Reaction mixtures (20 μl) contained 20 mM Hepes, pH 7.5, 10 mM MgCl{	extsubscript{2}}, 1 mM dithiothreitol, 10% (v/v) glycerol, 300 pmol of [γ-32P]ATP (specific activity, 10,000 cpm/pmol), 10–30 pmol of GST-Mcm2p, and Cdc7p/Dbf4p protein kinase complex or cell extracts. After 5 min at 30 °C, reactions were stopped by the addition of 5 μl of stop solution, heated at 95 °C for 3 min, and subjected to 4–20% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, destained, dried, and autoradiographed (31). To measure radioactivity incorporated into the protein, the corresponding protein bands were excised from the gel and quantitated in the presence of scintillation fluid using a scintillation spectrometer.

**Other Materials and Methods**—The purification of yeast GST-Mcm2-Mcm7 from *S. cerevisiae* was described previously (31), and their purity was more than 95% (data not shown). Calf thymus histone H1 (more than 95% pure) was obtained from Nacalai Tesque, Japan. Other methods and materials used in this study were previously described (31, 36, 39).

**RESULTS**

**Expression of Cdc7p/Dbf4p Protein Kinase Complex in Insect Cells**—The goal of this study was to characterize the Cdc7p/Dbf4p protein kinase and its role in chromosomal DNA replication initiation. Initially, attempts were made to purify the Cdc7p/Dbf4p complex from *S. cerevisiae* cell extracts. However, because of its low abundance and the cell cycle-dependent expression of its activity, it was not possible to purify the complex to homogeneity. Thus, Cdc7p or Dbf4p were expressed in insect cells using the baculovirus system. Although insect cells expressed each protein, the majority of the protein was insoluble (data not shown). Attempts to reconstitute a soluble Cdc7/Dbf4 complex by mixing two cell extracts containing Cdc7p and Dbf4p did not produce any significant amount of soluble, active Cdc7p/Dbf4p complex. Therefore, Cdc7p and Dbf4p were co-expressed in insect cells by mixed infection using the baculovirus system. When soluble extracts were prepared from these cells and precipitated with rabbit antiserum against Cdc7p, both Cdc7p and Dbf4p were precipitated efficiently (see below). Furthermore, the immunoprecipitates incorporated a significant amount of [γ-32P]ATP when co-incubated with the substrate and [γ-32P]ATP.

**Purification of Cdc7p/Dbf4p Protein Kinase Complex**—The Cdc7p/Dbf4p protein kinase complex was purified to near homogeneity from insect cells expressing both Cdc7p and Dbf4p as described under “Materials and Methods.” Fig. 1 shows the elution profile of the partially purified protein complex when applied to a Hi Trap Heparin column, eluted from the column with a 0.2–1 M NaCl linear gradient, and assayed for protein kinase activity using GST-Mcm2p as a substrate. Two peaks of kinase activity, which were not detected in uninfected insect cells (data not shown), are observed; the first peak coincides with peaks of both Cdc7p and Dbf4p (as detected by immunoblotting), suggesting that this peak represent the Cdc7p/Dbf4p protein kinase complex. On the other hand, the second activity peak did not include a significant amount of Dbf4p polypeptide, although it did include some Cdc7p polypeptide (Fig. 1). One explanation for this result is that the second activity peak might coincide with the elution position of an insect cell Dbf4 homologue. Alternatively, Cdc7p might be activated by unknown factor(s).

For the purpose of this study, the fractions in the first peak of kinase activity were pooled and further purified by chromatography using several columns (see “Materials and Methods”). The product of the purification is nearly homogeneous and includes two major polypeptides (85 and 58 kDa, respectively) in nearly equimolar quantities. These polypeptides match the product of the purification is nearly homogeneous and includes two major polypeptides (85 and 58 kDa, respectively) in nearly equimolar quantities. These polypeptides match the well-known factor(s).

As found for most other protein kinases, the activity requires Mg{	extsuperscript{2+}} or Ca{	extsuperscript{2+}} depending on the experiment (1). Furthermore, the Km for the substrate could not be determined because only 0.2 mol of phosphate was incorporated per mole of substrate, which is most likely because the substrate is not 100% active.

As found for most other protein kinases, the activity requires 5–10 mM Mg{	extsuperscript{2+}}; 1–5 mM Zn{	extsuperscript{2+}} or Ca{	extsuperscript{2+}} partially substitutes for Mg{	extsuperscript{2+}} giving 40 and 55% maximal activity, respectively. As shown in Fig. 3A, an addition of 0.2 M NaCl severely inhibited phosphorylation of the GST-Mcm2p substrate. In contrast, NaCl up to 0.8 M did not inhibit the autophosphorylation of Dbf4p. Similar results were observed with KCl (data not shown) for the Cdc7p/Dbf4p complex with nearly equal activity (Fig. 3).

**Characterization of the Purified Cdc7p/Dbf4p** Protein Kinase—The untagged and soluble form of the Cdc7p/Dbf4p protein kinase (Fig. 1) was used for extensive biochemical characterization. Previous work (31) demonstrated that the best substrate for the Cdc7p/Dbf4p protein kinase is GST-Mcm2p purified from yeast cells, so this protein was used as a substrate in many of the following experiments. As shown in Fig. 2, the Cdc7p/Dbf4p protein kinase quickly phosphorylated GST-Mcm2p (Fig. 2, closed circles) and autophosphorylated both Dbf4p (Fig. 2, open squares) and Cdc7p (data not shown) in 10 min at 30 °C. These data were used to determine that the V_{max} of the kinase activity is 14.5 mol of phosphate/mole of Cdc7/Dbf4p complex/min at 30 °C. However, the K_{m} for the substrate could not be determined because only 0.2 mol of phosphate was incorporated per mole of substrate, which is most likely because the substrate is not 100% active.

As found for most other protein kinases, the activity requires 5–10 mM Mg{	extsuperscript{2+}}; 1–5 mM Zn{	extsuperscript{2+}} or Ca{	extsuperscript{2+}} partially substitutes for Mg{	extsuperscript{2+}} giving 40 and 55% maximal activity, respectively. As shown in Fig. 3A, an addition of 0.2 M NaCl severely inhibited phosphorylation of the GST-Mcm2p substrate. In contrast, NaCl up to 0.8 M did not inhibit the autophosphorylation of Dbf4p. Similar results were observed with KCl (data not shown) for the Cdc7p/Dbf4p complex with nearly equal activity (Fig. 3).

2 M. Kihara, W. Nakai, S. Asano, A. Suzuki, K. Kitada, Y. Kawasaki, L. H. Johnston, and A. Sugino, unpublished results.
Therefore, these data strongly suggest that the Cdc7p/Dbf4p kinase complex is very stable in vitro and may not dissociate even at a high monovalent salt concentration. As described above and shown in Figs. 2 and 3A, the Cdc7p/Dbf4p kinase complex, especially Dbf4p, undergoes autophosphorylation and phosphorylates exogenously added GST-Mcm2p. When the complex was preincubated with unlabeled ATP, the mobility of Dbf4p during SDS-PAGE decreased significantly as the reaction proceeded (Fig. 3B). Similar results were observed with Cdc7p (data not shown), although its autophosphorylation was to a lesser extent than that of Dbf4p. Nevertheless, preincubation with unlabeled ATP did not change the activity of the protein kinase toward the GST-Mcm2p substrate (Fig. 3B). This result suggests that autophosphorylation of Dbf4p/Cdc7p does not influence its kinase activity.

Many proteins are required for the initiation of DNA replication in S. cerevisiae, and these proteins are potential substrates for the Cdc7p/Dbf4p protein kinase complex. Therefore, several replication proteins were tested for their ability to act as a substrate for Cdc7p/Dbf4p in vitro. The proteins tested included origin recognition complex (six subunits) (provided by John Diffley) (40), Cdc6p (provided by Ambrose Jong) (41), Pol2* (four subunits, Pol2p, Dpb2, Dpb3, and Dpb4) (36), Pol3 complex (three subunits, Cdc2p, Hys2p, and Pol32) (42), Pol32 (provided by Naomi Nakashima), and proliferating cell nuclear antigen (42). None of those proteins were efficiently phosphorylated in vitro by the kinase (data not shown). However, as shown in Fig. 4, members of the Mcm protein family purified from yeast cells were phosphorylated in vitro by the Cdc7p/Dbf4p kinase complex. Mcm2p was the best substrate, as reported previously (31). However, these data are different from those obtained with yeast Mcm proteins expressed and purified from insect cells (32). Although other proteins such as RFA (RPA) complex, the 180-kDa catalytic subunit of DNA polymerase α-primase complex (36), and Mcm10 were also phosphorylated by the kinase, they were phosphorylated less efficiently than GST-Mcm2p.

The amino acid residues of GST-Mcm2p phosphorylated by the Cdc7p/Dbf4p kinase complex were analyzed, demonstrating that more than 95% of the incorporated $^{32}$P was in serine and less than 5% was in threonine (data not shown). This confirms that Cdc7p/Dbf4p is a serine/threonine kinase (17).

3 N. Nakashima, K. Hashimoto, and A. Sagino, unpublished results.
Prephosphorylated Mcm2p Is a Substrate for the Cdc7p/Dbf4p* Protein Kinase Complex—

It was previously shown that Mcm2p is phosphorylated by other protein kinases during the cell cycle in addition to Cdc7p/Dbf4p (31). Because the GST-Mcm2p substrate was prepared from unsynchronized yeast, it was likely that it was isolated in a phosphorylated form. Therefore, Mcm2p was dephosphorylated using E. coli alkaline phosphatase (BAP), repurified by MonoQ column chromatography, and tested as a substrate for Cdc7p/Dbf4p* protein kinase. As shown in Fig. 5A, BAP treatment increased the mobility of GST-Mcm2p during SDS-PAGE, suggesting that GST-Mcm2p purified from yeast cells is already phosphorylated. Unexpectedly, the dephosphorylated GST-Mcm2p was a poor substrate for Cdc7p/Dbf4p*; virtually no 32P was incorporated into the dephosphorylated protein (Fig. 5, B and C). It is unlikely that this is due to an inhibition of the kinase, because efficient autophosphorylation of Cdc7p/Dbf4p* is observed in the presence of dephosphorylated GST-Mcm2p. Furthermore, if dephosphorylated GST-Mcm2p was mixed with native GST-Mcm2p, the same amount of 32P as without dephosphorylated GST-Mcm2p was incorporated into GST-Mcm2p (Fig. 5B, lanes m–p). Thus, GST-Mcm2p phosphorylated in vivo is a substrate for Cdc7p/Dbf4p* protein kinase in vitro, but dephosphorylated GST-Mcm2p is not. These results are consistent with earlier results indicating that Mcm2p is sequentially phosphorylated during the cell cycle and that the last phosphorylation of Mcm2p is blocked at the restrictive temperature in cdc7 mutant cells (31).

Previous studies indicate that bob1 mutants bypass the requirement for CDC7/DBF4 in the yeast cell cycle (29). This suggests that GST-Mcm2p purified from bob1-ΔCDC7 yeast cells should not be phosphorylated at the Cdc7p/Dbf4p sites and could therefore be a better substrate for the Cdc7p/Dbf4p* protein kinase in vitro. This prediction was tested, and the results are shown in Fig. 6. Contrary to our prediction, GST-Mcm2p purified from bob1-ΔCDC7 cells was two to three times less active as a protein kinase substrate than GST-Mcm2p from wild type cells (Fig. 6). There are several possible explanations for this result. For example, there may be another protein...
kinase in bob1-∆CD7 yeast that partially phosphorylates GST-Mcm2p at the same sites phosphorylated by Cdc7p/Dbf4p.

Alternatively, in bob1-∆CD7 cells, GST-Mcm2p may undergo a post-translational modification that causes a conformational change inhibiting its subsequent phosphorylation by the Cdc7p/Dbf4p kinase complex. Another possibility is that the bob1 mutation blocks an upstream modification of GST-Mcm2p that is required prior to its phosphorylation by Cdc7p/Dbf4p. Nonetheless, GST-Mcm2p from bob1-∆CD7 cells could be phosphorylated by Cdc7p/Dbf4p* (Fig. 6B) to a greater extent than dephosphorylated GST-Mcm2p (Fig. 5C).

S. cerevisiae Mcm2p was also expressed in and purified from insect cells (provided by Y. Ishimi) and was tested as a substrate for the Cdc7p/Dbf4p* kinase; the activity was two to three times less than that of GST-Mcm2p from yeast cells (data not shown). These results are also consistent with the conclusion that an upstream phosphorylation of Mcm2p is required prior to its phosphorylation by the Cdc7p/Dbf4p* protein kinase.

**Molecular Characterization of Dbf4 Mutants**—Several temperature-sensitive dbf4 mutants were previously isolated and characterized (16, 43, 44). This work extends those studies and determines the nucleotide sequence changes in several mutant alleles of the DBF4 gene. In dbf4-1 and dbf4-2, there is a single-base pair change in the coding region of DBF4 resulting in a single amino acid change from Pro to Leu and from Pro to Ser, respectively (Fig. 7A). In dbf4-3, there are two base changes resulting in a single amino acid change from Pro to Ser in the coding region. On the other hand, in dbf4-4, there is a single-base change in the coding region that creates a stop codon at amino acid 596 (from Glu to stop (TAG)). Therefore, dbf4-4 mutant may produce a truncated Dbf4p. Finally, there are three base pair changes in dbf4-5. Two of those base pair changes result in a stop codon at amino acid 112 (from TGG (Trp) to TAA (Ter)) and the remaining base pair change creates a GAA (Glu) to AAA (Lys) substitution.

Thus, the dbf4-5 mutant may generate two polypeptides, one with 111 amino acid residues and another with 584 amino acids (assuming that Met is used as an initiation codon). This expectation was confirmed by the experiments described below (Fig. 7C).

To find which mutation site(s) in dbf4-5 is responsible for temperature-sensitive cell growth phenotype, we generated three additional dbf4-5-derivative mutant genes. These were dbf4-5′- that contains the dbf4-5 gene, whose first 112 amino acid residues were deleted, dbf4-5″, which is the same as dbf4-5′, but the second mutation site (AAA (Lys 414)) in dbf4-5 was reversed to a wild type sequence (GAA(Glu 414)), and dbf4-5‴, which contains only the second mutation (AAA (Lys 414) of dbf4-5, respectively (Fig. 7A). Those genes were tested to be able to complement lethality of ∆dbf4 at 25 and 37°C. The results showed that the dbf4-5 and dbf4-5′- mutant genes complemented lethality of ∆dbf4 at 25°C, but not at 37°C, while the dbf4-5″ gene complemented lethality of ∆dbf4 at 25°C and 37°C. Surprisingly, the dbf4-5‴ gene did not complement lethality of ∆dbf4 mutation at 25°C. These results indicate that either the first mutation site (TGG (Trp 112) to TAA (Ter)) or the second mutation site (GAA(Glu 414) to AAA (Lys) substitution) is not sufficient to reconstitute temperature sensitivity in the dbf4-5 mutant. Therefore, it was concluded that the two mutation sites are required for making the dbf4-5 mutant temperature-sensitive.

A yeast strain containing an ARS1-lacZ reporter construct was tested for expressing β-galactosidase after transformation with Dbf4 fused to GAD (26). As shown in Fig. 7B, all mutant genes, except for dbf4-4 and its derivatives, showed a more or less wild type level of ARS1 binding activity at all temperatures tested. Interestingly, the dbf4* mutant had neither complementation activity of ∆dbf4 lethality at 25°C (Fig. 7A) nor ARS1 binding activity at all temperatures, whereas the dbf4-5 and other dbf4-5 derivatives, which complemented ∆dbf4 lethality at 25°C, did not exhibit ARS1 binding activity (Fig. 7B). Therefore, if the ARS binding activity of Dbf4p is essential for yeast cell growth as shown previously (26), Dbf4-5p and Dbf4-5′p should retain the activity, which could not be detected under our assay conditions. Alternatively, the ARS binding activity missing in Dbf4-5p or Dbf4-5′p may be
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Cdc7p/Dbf4p, Cdc7p/Dbf4-4p, Cdc7p/Dbf4-5p, mutant Dbf4p complexes (Cdc7p/Dbf4-1p, Cdc7p/Dbf4-3p, As shown in Fig. 7, co-expression of the mutant Dbf4p with wild type Cdc7p in temperature sensitivity of the kinase activity was tested by GST-Mcm2p. Lanes 2–7, with 0.01, 0.1, 0.2, 0.3, 0.5 and 1.0 µg of GST-Mcm2p from wild type yeast, respectively. Lanes 9–12, with 0.1, 0.1, 0.3, 0.5, and 1.0 µg of GST-Mcm2p from bob1-ΔCDC7 yeast, respectively. B, protein kinase activity was quantified as described above. substituted with the conformational change of the protein by the second mutation (GAA (Glu\(^{412}\) to AAA (Lys\(^{412}\) change) in dbf4-5. From the results with dbf4-5\(^{\ast}\), the latter is more likely possibility.

The dbf4-1–dbf4-5 mutants described above are reportedly to be temperature-sensitive in growth in vivo (16, 43, 44). Thus, temperature sensitivity of the kinase activity was tested by co-expression of the mutant Dbf4p with wild type Cdc7p in insect cells followed by partial purification and kinase assay. As shown in Fig. 7C, this analysis was carried out for six mutant Dbf4p complexes (Cdc7p/Dbf4-1p, Cdc7p/Dbf4-3p, Cdc7p/Dbf4-4p, Cdc7p/Dbf4-5p, Cdc7p/Dbf4-5\(^{\ast}\), and Cdc7p/Dbf4-5\(^{\ast}\) (the dbf4-5\(^{\ast}\) mutant gene did not complement Δdbf4 lethality) as well as for the mutant Cdc7-1p/Dbf4p complex. In all cases, the Cdc7p antiserum precipitated both kinase subunits Cdc7p and Dbf4p (Fig. 7C and data not shown for Cdc7p/Dbf4-2p and Cdc7p/Dbf4-5p), indicating that the mutant protein complexes were stably formed in insect cells in vivo. Interestingly, the bands corresponding to Dbf4p, Dbf4-4p, Dbf4-5, Dbf4-5\(^{\ast}\), and Dbf4-5\(^{\ast}\) were diffuse in character when detected on a Western blot. These diffuse bands were not observed when cell extracts were pretreated with BAP (data not shown), suggesting that the diffuse quality of these bands results from protein phosphorylation. This phosphorylation could be due to autophosphorylation of the complex, because in the mutant complexes (Cdc7-1p-Dbf4p (Fig. 7C, lane f), Cdc7p/Dbf4-1p (Fig. 7C, lane g), and Cdc7p/Dbf4-3p (Fig. 7C, lane h)), Dbf4p migrated as a single band in SDS-PAGE and the immunoprecipitates with Cdc7p antibodies did not have any significant protein kinase (data not shown). Thus, these data suggest that Cdc7p/Dbf4p-4p, Cdc7p/Dbf4-5p, Cdc7p/Dbf4-5\(^{\ast}\), and Cdc7p/Dbf4-5\(^{\ast}\) complexes retain a significant amount of the protein kinase activity at permissive temperatures, whereas other mutant complexes do not.

The temperature sensitivity of the protein kinase activity of the Cdc7p/Dbf4p mutants was tested using partially purified protein complexes. As shown in Fig. 8A, although the mutant Cdc7-1p/Dbf4p protein complex could be partially purified, only a small amount of protein kinase activity was detected at 25 °C. Therefore, we could not convincingly demonstrate that this activity is temperature-sensitive. Similar results were also obtained for Cdc7p/Dbf4-1p and Cdc7p/Dbf4-3p (the kinase activity of Cdc7p/Dbf4-2p was not tested but is expected to be the same as Cdc7p/Dbf4-3p). In the cases of Cdc7p/Dbf4-4p and Cdc7p/Dbf4-5p, a significant amount of protein kinase activity co-eluted with the mutant protein complex on Hi Trap Heparin column chromatography (data not shown), thus letting us to measure their temperature sensitivities. Those kinase activities were more quickly inactivated at 35 °C than the wild type protein kinase (Fig. 8, B and C, and data not shown), indicating that those mutations confer temperature sensitivity on the protein kinase of Cdc7p/Dbf4p complex. The dbf4-4 mutation generates a termination in the second Cdc7p-binding domain (Fig. 7A). This domain may not be essential for Cdc7 activation at 25 °C (26). However, the cell growth of the mutant and the Cdc7p/Dbf4p kinase are both temperature-sensitive, thus the domain also plays an important role for the kinase activity.

Genetic Interaction between DBF4 and RAD53—To understand better the function of Dbf4p, mutations were identified that suppress the temperature-sensitive growth phenotype of dbf4-1 after treating dbf4-1 mutant cells with ethyl methane sulfonate as published (16). More than 50 mutants were obtained that separated into two complementation groups. One of those complementation groups, dbf4-1 sup1, was further characterized in this study. Genetic mapping experiments indicated that dbf4-1 sup1 mutation resides at 17.0 cM from the CDC60 locus on chromosome XVI, where RAD53 and PEP4 are closely located (data not shown). Thus, a single copy plasmid of either RAD53 or PEP4 was introduced into the dbf4-1 sup1 mutant. Only the mutant cell harboring the RAD53 gene on a single-copy plasmid became temperature-sensitive. Thus, we concluded that dbf4-1 sup1 is an allele of rad53 mutations. This result suggests that RAD53, which encodes a serine/threonine protein kinase and plays a role in both S phase checkpoint and DNA damage checkpoint in yeast (34), interacts with Dbf4p and regulates the function of Dbf4p during S phase.

Rad53 Phosphorylates Cdc7p/Dbf4p and Inhibits the Mcm2p Protein Kinase Activity—Previous studies also indicate that RAD53 interacts with DBF4 genetically and physically (32, 46). These prompted us to test in vitro the ability of Rad53p to directly phosphorylate the Cdc7p/Dbf4p\(^{\ast}\) protein kinase complex. Rad53p was purified to near homogeneity from S. cerevisiae cells expressing 2HA-tagged Rad53p (Fig. 9A, panel a). Although cells were not treated with either HU or methyl methane sulfonate to activate Rad53p, Rad53p had a significant autophosphorylation activity as well as histone H1 kinase activity, indicating that it was an active protein kinase (Fig. 9A, panel c). It was noted that autophosphorylation of Rad53p is stimulated in the presence of a substrate histone H1. Because the Cdc7p/Dbf4p\(^{\ast}\) complex also exhibits autophosphorylation (Figs. 2 and 3), the Cdc7p/Dbf4p\(^{\ast}\) complex was preincubated with a cold ATP to mask its autophosphorylation. Then the Cdc7p/Dbf4p\(^{\ast}\) complex was incubated with the purified 2HA-Rad53p. As shown in Fig. 10A, \(^{32}\)P was incorporated into Dbf4p, and the band of the protein were shifted upward in SDS-PAGE in Rad53p-dependent manner. Similarly, \(^{32}\)P were incorporated into Cdc7p, but to a considerably lesser extent (Fig. 10A). If lane 2 in Fig. 9B (1.2 pmol of \(^{32}\)P was incorporated into 60 pmol of histone H1) is compared with lane 6 in Fig. 10A (1.0 pmol of \(^{32}\)P was incorporated into 0.1 pmol of Cdc7p/Dbf4p\(^{\ast}\)), the Cdc7p/Dbf4p\(^{\ast}\) purified from insect cells was at least 100 times more active as substrate for Rad53p protein kinase than calf thymus histone H1. Thus, this result strongly suggests that the Cdc7p/Dbf4p\(^{\ast}\) complex, particularly Dbf4p, may be one of in vivo targets of the Rad53p protein kinase. In addition, phosphorylation of the Cdc7p/Dbf4p\(^{\ast}\) complex by
Rad53p altered the protein kinase activity of the complex; when 2HA-Rad53p was co-incubated with Cdc7p/Dbf4p* and GST-Mcm2p, the phosphorylation of GST-Mcm2p by Cdc7p/Dbf4p* was strongly inhibited (Fig. 10, A and B). Furthermore, 2HA-Rad53p purified from yeast cells treated with 0.2 M HU was more efficient in inhibiting the Cdc7p/Dbf4p* complex than nonactivated 2HA-Rad53p; 10-fold less amount of 2HA-Rad53p purified from HU-treated yeast cells inhibited the Cdc7p/Dbf4p* kinase activity as much or more than 2HA-Rad53p from yeast cells (Fig. 10).
Rad53p from HU-treated yeast cells, and the phosphorylation of GST-Mcm2p by Cdc7p/Dbf4p* was inhibited by time-dependent manner. Taken together, these results and those of Dohrmann et al. (46) suggest that Rad53p directly interacts with and regulates the Cdc7p/Dbf4p protein kinase activity in vivo during S phase.

DISCUSSION

This study reports the biochemical characterization of Cdc7p/Dbf4p protein kinase that was purified to near homogeneity from insect cells co-expressing Cdc7p and Dbf4p. Because an active, untagged, soluble and pure Cdc7p/Dbf4p protein kinase from yeast cells has not been characterized previously, this report becomes the first paper to describe a thorough biochemical characterization of Cdc7p/Dbf4p complex, which plays an important function during the initiation of chromosomal DNA replication in yeast. To distinguish between Cdc7p/Dbf4p complex from yeast cells and Cdc7p/Dbf4p complex purified from insect cells, we designate the Cdc7p/Dbf4p complex from yeast cells and Cdc7p/Dbf4p complex purified from insect cells co-expressing Cdc7p and Dbf4p. Because Cdc7p/Dbf4p protein kinase that was phosphorylated with Cdc7p/Dbf4p* was completely digested with the Lys-C endopeptidase, we could determine the stoichiometry of the enzyme-substrate complex with Cdc7p/Dbf4p*. Alternatively, it is possible that Cdc7p/Dbf4p* purified from insect cells may be phosphorylated at specific sites by Cdc7p/Dbf4p*. The reason for this discrepancy is not clear at present, but it may reflect the higher purity of the protein kinase complex used in this study.

Although GST-Mcm2p is a good in vitro substrate for Cdc7p/Dbf4p*, less than 0.2 mol of phosphate/mol of GST-Mcm2p was incorporated by the protein kinase (Fig. 2). When the GST-Mcm2p that was phosphorylated with Cdc7p/Dbf4p* was completely digested with the Lys-C endopeptidase, we detected at least five different 32P-labeled oligopeptides. Thus, it is estimated that as few as 4% of the GST-Mcm2p molecules may be phosphorylated at specific sites by Cdc7p/Dbf4p*. There are several factors that may contribute to this result. First, data presented here (Figs. 5 and 6) and elsewhere (31) indicate that sequential phosphorylation events target Mcm2p during the cell cycle in S. cerevisiae, and the ability of Cdc7p/Dbf4p to utilize Mcm2p as a substrate is dependent on proper prephosphorylation at specific protein sites of Mcm2p. For example, BAP treatment of Mcm2p after purification from yeast cells completely blocked its phosphorylation in vitro by Cdc7p/Dbf4p* (Fig. 5), and Mcm2p from bob1/ΔCDC7 cells (Fig. 6), insect cells, or E. coli (data not shown) were much poorer substrates for the Cdc7p/Dbf4p* protein kinase than Mcm2p from yeast cells. Furthermore, it was previously shown that only a portion of the phosphorylated Mcm2p is further phosphorylated during S phase in vivo and that various mcm2 mutations affect the Cdc7p/Dbf4p phosphorylation activity (31). These results suggest that protein conformation or post-translational modification plays a role in the recognition of a substrate by Cdc7p/Dbf4p protein kinase. Alternatively, it is still possible that Cdc7p/Dbf4p* purified from insect cells may not be properly activated and is not active as Cdc7p/Dbf4p from yeast cells.

It has been shown that the nuclear and subnuclear localization of the Mcm2p and Mcm3p are temporally regulated with respect to the cell cycle. The nuclear concentration of these

4 M. Kihara and A. Sugino, unpublished results.
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proteins increases at the end of mitosis, remains elevated throughout G2 phase, and decreases at the beginning of S phase. Once inside the nucleus, a fraction of the total Mcm2p and Mcm3p becomes tightly associated with DNA (32, 47). The association of these proteins with chromatin presumably leads to the initiation of DNA synthesis, and their subsequent disappearance from the nucleus presumably prevents reinitiation of DNA synthesis at replication origins. This temporally and spatially restricted localization of Mcm2p and Mcm3p in the nucleus may serve to ensure that DNA replication occurs once and only once per cell. However, Mcm2p and Mcm3p are abundant in the cell (4 × 10^6/cell and 2 × 10^6/cell, respectively), yet Mcm2 is limiting for DNA replication (48). If we assume that the binding of Mcm2p at replication origins plays an important in vivo function, and subsequently Cdc7p/Dbf4p protein kinase specifically phosphorylates the origin-bound Mcm2p through an ARS binding activity of Dbf4p, it can be argued that less than 1,000 molecules of Mcm2p (about 2–3% of the total pool of Mcm2p) might be a substrate for the kinase (there are less than 1,000 replication origins in yeast). Interestingly, this calculation roughly matches with the calculation of the fraction of GST-Mcm2p phosphorylated by Cdc7p/Dbf4p* in vitro (4%). Therefore, the difference between chromatin-bound and unbound Mcm2p could be the phosphorylated state of the protein, because the BAP-pretreated GST-Mcm2p was inert as a substrate for Cdc7p/Dbf4p* protein kinase complex. Thus, we further speculate that if chromatin-bound Mcm2p could be specifically purified from the cell, it might be a better substrate for Cdc7p/Dbf4p* protein kinase in vivo than the pool of total nuclear Mcm2p.

It has been recognized that Mcm2–7 family proteins form a complex and play an essential role in the initiation of chromosomal DNA replication in several eukaryotes (see Ref. 30 for review). Thus, it is reasonable to suggest that a Mcm2–7 family protein complex might be the best substrate for the Cdc7p/Dbf4p protein kinase. In S. cerevisiae, Mcm2–7 family proteins form a complex in vivo and bind to chromatin during the cell cycle, as in other eukaryotes. However, unlike for other eukaryotes (49–51), a reasonable quantity of the Mcm2–7p complex from yeast has not yet been successfully isolated; thus it has not been possible to directly test its potential as a substrate for Cdc7p/Dbf4p* protein kinase. Nonetheless, the substrate specificity that we observe in this study is very similar to that of S. pombe Hsk1p-Dbf4p using the Mcm2–7 complex as a substrate (23).

Mutant protein kinase complexes including Dbf4-1p, Dbf4-2p, Dbf4-3p, Dbf4-4p, and Dbf4-5p, and Dbf4-5p derivatives were also characterized in this study. Complexes that included Cdc7p-1p/Dbf4p, Cdc7p/Dbf4-1p, and Cdc7p/Dbf4-3p had no significant protein kinase activity in vitro, although these complexes could be isolated and partially purified (Fig. 8A). Therefore, it is possible that these mutant complexes might be extremely heat labile and readily inactivated in insect cells during or prior to purification. Alternatively, those mutant complexes were not properly formed in insect cells; thus those might be artifacts of the protein expression in insect cells. However, Cdc7p/Dbf4-4p and Cdc7p/Dbf4-5p complexes expressed in insect cells exhibited a temperature-sensitive protein kinase activity. Thus, we rather consider another possibility that a small amount of the protein kinase activity detected in the mutant Cdc7p/Dbf4p complexes may be directly related to the nature of their mutations rather than protein expression artifact. This may explain why a multicopy plasmid harboring GLC8, which encodes the regulatory subunit of Glc7 protein phosphatase,

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5 K. Kitada, M. Shinohara, and A. Sugino, unpublished results.
specifically suppresses temperature sensitivity of mutants cdc7-1, dbf4-1, dbf4-2, and dbf4-3 but not dbf4-4 and dbf4-5.5

The characterization of complexes containing Dbf4-5p, Dbf4-5′p, Dbf4-5″p, or Dbf4-5‴p was particularly informative and interesting. Previous studies showed that Dbf4p has separate ARS-binding and Cdc7p-binding domains (26, 46). Its interaction with ARSs was found to be independent of its ability to bind to Cdc7p (26). The dbf4-5 allele has sequence changes at two sites; one change is near or in the ARS-binding domain and the other change is in the Cdc7p-binding region (Fig. 7A), and two distinct polypeptides are produced from this dbf4 allele. As shown in Fig. 7, Dbf4-5p, the larger polypeptide produced by dbf4-5, had no ARS binding activity, although it has activation of Cdc7p protein kinase activity. More importantly, the Cdc7p/Dbf4-5p and Cdc7p/Dbf4-5′p complexes exhibited a temperature-sensitive protein kinase activity (Fig. 8C and data not shown), and the dbf4-5 and dbf4-5′′ genes complement the lethality of the Δdbf4 mutation and support temperature-sensitive cell growth of Δdbf4 (Fig. 7A). These data clearly demonstrate that each single mutation site of dbf4-5 (either TGG (Trp112) to TAA (Term) or GAA (Glu412) to AAA (Lys412) change) is not sufficient to reconstitute temperature sensitivity of the dbf4-5 mutation. As published previously (26), deletion of a portion of the ARS-binding domain of DBF4 (i.e., the dbf4-5′ allele) abrogated the ability of the gene to complement Δdbf4 (Fig. 7A). However, the truncated gene product formed a complex with Cdc7p and exhibited a protein kinase activity on GST-Mcm2p (data not shown). Thus, these results suggest that a defect of the first mutation site (deletion of a portion of the ARS-binding domain) is suppressed by the second mutation of dbf4-5 and therefore suggest that both the ARS-binding domain and Cdc7p protein kinase activation domains collaborate to form an active Cdc7p/Dbf4p complex that functions in the mutant cells. Nevertheless, it should be emphasized that these results are the first demonstration that the Mcm2 phosphorylation activity of the mutant Cdc7p/Dbf4p complex is temperature-sensitive in vitro, because the previous studies always used immunoprecipitates from cell extracts and histone H1 as a substrate to assay the kinase activity (17).

In previous studies, Jackson et al. (17) showed that mutant extracts from cdc7-1 and dbf4-1 demonstrate temperature-sensitive Cdc7p/Dbf4p kinase activity but that temperature-resistant kinase activity is restored when the two extracts are mixed. In contrast, mixed extracts from insect cells expressing Cdc7p-Dbf4p or Cdc7p and Dbf4-1–Dbf4-3p were inactive as protein kinases, regardless of the temperature, when we performed a similar experiment. This might be due to tight association of the mutant complex in our samples. Furthermore, Jackson et al. (17) detected a temperature-resistant protein kinase activity in the immunoprecipitates from the mixture of dbf4-1 and cdc7-1 mutant cell extracts; therefore, it is possible that exchange of the polypeptides in their experiments is achieved in immunoprecipitates. However, this possibility is considered less likely, because temperature-resistant protein kinase activity was not detected in our hands in similarly prepared immunoprecipitates from yeast cells as well as insect cells expressing the mutant Cdc7p/Dbf4p (data not shown). Therefore, the difference in these results may derive from the fact that Jackson et al. (17) measured histone H1 kinase activity and our experiments measured Mcm2p kinase activity. Nevertheless, it is equally possible that the mutant Cdc7p/Dbf4p is not properly formed and/or phosphorylated in insect cells. Therefore, further characterization of the wild type and mutant Cdc7p/Dbf4p complexes expressed and purified from both yeast cells and other eukaryotic cells is needed.

The purified Cdc7p/Dbf4p complex showed an autophosphorylation of both Cdc7p and Dbf4p. Especially, Dbf4p was highly phosphorylated. Apparently, this autophosphorylation did not alter its protein kinase activity (Fig. 3B). Interestingly, Dbf4p is hyperphosphorylated, presumably by Rad53p, upon treatment of cells with HU or upon inactivation of DNA replication enzymes, and this hyperphosphorylated kinase is less active than unmodified Cdc7p/Dbf4p kinase (32). Because the checkpoint regulator Rad53p is required for modification of Dbf4p in response to HU, Cdc7p/Dbf4p kinase may play a role in the response of yeast cell to DNA replication inhibitors (32). Recently, Dohrmann et al. (46) showed that dbf4-1 is a synthetic lethal with rad53–31 and Dbf4p physically interacts with Rad53p by a two-hybrid assay. Furthermore, we show here that an extragenic suppressor screen of dbf4-1 yielded a new temperature-sensitive allele of rad53. All these results strongly suggest that Rad53p directly interacts with and regulates Cdc7p/Dbf4p protein kinase activity in vivo. Thus, this possibility was directly tested in vitro in this study using the highly purified Cdc7p/Dbf4p complex and 2HA-tagged Rad53p. As shown in Fig. 10, a small amount of 2HA-tagged Rad53p significantly phosphorylated both Dbf4p and Cdc7p. But they were not equally phosphorylated. Although we do not know at the present time which protein phosphorylation has an effect, these phosphorylations consequently inhibited the GST-Mcm2p protein kinase activity of Cdc7p/Dbf4p*. This result further strengthens the possibility that Rad53p directly regulates the protein kinase activity of Cdc7p/Dbf4p by phosphorylation.

Weinreich and Stillman (32) recently showed that a Cdc7p monomer was bound to chromatin at all times during the cell cycle, whereas Dbf4p was only associated with chromatin at certain times. From these results they proposed that the binding of Dbf4p to chromatin might determine which origins become activated. In addition, the binding of Dbf4p to the chromatin-bound Cdc7p might form an active Cdc7p/Dbf4p kinase complex that could promote initiation at that origin. However, we argue against this possibility, because we completely failed to reconstitute Cdc7p/Dbf4p in vitro by mixing two extracts containing Cdc7p and Dbf4p. Thus, if the binding of Dbf4p to the chromatin-bound Cdc7p may form an active Cdc7p/Dbf4p kinase complex, we speculate that another factor(s) might play a role for its association.

Two recent reports (27, 28) suggest that Cdc7p activity is required to activate individual origins rather than being required for a global activation of S phase. Also, it is well known that in the presence of HU, which activates Rad53p, only early firing origins are activated in S phase (35). How can these results be explained by phosphorylation and inactivation of Cdc7/Dbf4p protein kinase by Rad53p protein kinase? We propose here that Rad53 protein kinase might control the total levels of Cdc7p/Dbf4p protein kinase activity during S phase, which is sufficient to activate a small number of origins, such that an active Cdc7p/Dbf4p complex always binds at the first available origins (which might be determined by their chromatin structure) and activates these origin firings. Once origin firing occurs, the Cdc7p/Dbf4p no longer binds to the same origin because the activity associated with the Cdc7p/Dbf4p complex phosphorylates Mcm2p, changing the phosphorylation state of the Mcm2–7 family protein complex. Thus, the active Cdc7p/Dbf4p protein kinase binds to the next available origin(s), and so on. Finally, the same activated protein kinase complex would bind to the late firing origins as the early firing origins. This model is very attractive and provides a mechanism to explain how a temporal order of origin firing might be controlled by Rad53p during S phase. In addition, this model could explain why only certain origins are fired in the presence
of HU, because HU activates Rad53p protein kinase and inhibits the majority of the Cdc7p/Dbf4p protein kinase activity, and the active Cdc7p/Dbf4p protein kinase is no longer available for activation of both early and late firing origins. Furthermore, this model can easily explain why rad53 sup1 mutation suppresses dbf4-1; a small amount of Cdc7p/Dbf4p protein kinase in dbf4-1 mutant is sufficient for the initiation of chromosomal DNA replication in the absence of Rad53 kinase, which is an inhibitor for Cdc7p/Dbf4p protein kinase.

In summary, this study provides detailed characterization of a nearly homogeneous Cdc7p/Dbf4p protein kinase complex. The availability of large amounts of active Cdc7p/Dbf4p will support additional biochemical characterization of this important kinase in future studies, such as the determination of a consensus substrate phosphorylation site. Furthermore, the availability of large amounts of Cdc7p/Dbf4p and Rad53p protein kinases, DNA polymerases α, δ, and ε (36, 42), and other initiation proteins will facilitate reconstitution of eukaryotic DNA replication in vitro, including the processes of initiation, subsequent loading of DNA replication complexes, and elongation of the replication fork.

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