

Mammalian Cdc7–Dbf4 protein kinase complex is essential for initiation of DNA replication

Wei Jiang^{1,2}, David McDonald,
Thomas J.Hope and Tony Hunter

The Salk Institute, 10010 North Torrey Pines Road, La Jolla,
CA 92037, USA

²Present address: Department of Biochemistry, New York University
Medical Center, 550 First Avenue, New York, NY 10016, USA

¹Corresponding author
e-mail: wjiang@salk.edu

The Cdc7–Dbf4 kinase is essential for regulating initiation of DNA replication in *Saccharomyces cerevisiae*. Previously, we identified a human Cdc7 homolog, HsCdc7. In this study, we report the identification of a human Dbf4 homolog, HsDbf4. We show that HsDbf4 binds to HsCdc7 and activates HsCdc7 kinase activity when HsDbf4 and HsCdc7 are coexpressed in insect and mammalian cells. HsDbf4 protein levels are regulated during the cell cycle with a pattern that matches that of HsCdc7 protein kinase activity. They are low in G₁, increase during G₁–S, and remain high during S and G₂–M. Purified baculovirus-expressed HsCdc7–HsDbf4 selectively phosphorylates the MCM2 subunit of the minichromosome maintenance (MCM) protein complex isolated by immunoprecipitation with MCM7 antibodies *in vitro*. Two-dimensional tryptic phosphopeptide-mapping analysis of *in vivo* ³²P-labeled MCM2 from HeLa cells reveals that several major tryptic phosphopeptides of MCM2 comigrate with those of MCM2 phosphorylated by HsCdc7–HsDbf4 *in vitro*, suggesting that MCM2 is a physiological HsCdc7–HsDbf4 substrate. Immunoneutralization of HsCdc7–HsDbf4 activity by microinjection of anti-HsCdc7 antibodies into HeLa cells blocks initiation of DNA replication. These results indicate that the HsCdc7–HsDbf4 kinase is directly involved in regulating the initiation of DNA replication by targeting MCM2 protein in mammalian cells.

Keywords: DNA replication/HsCdc7/HsDbf4/kinase/
MCM

Introduction

Eukaryotic chromosomal replication is a tightly regulated process, which must be strictly coordinated with other cell-cycle events, such as cell division, to ensure that the daughter cells maintain the same ploidy as the parental cell. Replication of each segment of DNA occurs once and once only during the S phase of the cell cycle. Genetic and biochemical evidence has now emerged to support a model in which the initiation of DNA replication is regulated through two mutually exclusive cellular states (Dutta and Bell, 1997). The first occurs in the G₁ phase

of the cell cycle and involves the regulated assembly of prereplication complexes (pre-RCs) at DNA replication origins. Assembly of the pre-RCs is sequential, with the origin recognition complex (ORC) recruiting the Cdc6 protein, which in turn promotes loading of minichromosome maintenance (MCM) proteins (Coleman *et al.*, 1996; Donovan *et al.*, 1997; Tanaka *et al.*, 1997). Although necessary, the assembly of pre-RCs during G₁ is not sufficient to initiate DNA replication. During the G₁–S transition, cells must enter the second state in which DNA replication can be initiated. This process requires the activation of two S-phase-promoting kinases: cyclin-dependent kinases, Cdks, and the Dbf4-dependent kinase, Cdc7 (Stillman, 1996; Dutta and Bell, 1997).

In budding yeast, the Cdc28 Cdk, complexed with specific cyclin subunits, controls the assembly, as well as activation, of pre-RCs and is required for initiation of DNA replication in budding yeast (Stillman, 1996; Dutta and Bell, 1997), but a second S-phase-promoting kinase, Cdc7, also plays an essential role in promoting DNA replication. *cdc7* mutant yeast cells arrest at the restrictive temperature with a 1C DNA content with high activities of Cdks, indicating that Cdc7 function is required for the onset of DNA synthesis (Amon *et al.*, 1992). Like the Cdks, whose activities require association with their regulatory subunit cyclins, Cdc7 kinase activity requires its own regulatory subunit, the Dbf4 protein (Sclafani and Jackson, 1994). However, Cdc7p is not in the Cdk subfamily of protein kinases and Dbf4p has no sequence homology to the cyclins. The expression levels of Cdc7p are constant throughout the cell cycle, but Cdc7p kinase activity increases at the G₁–S boundary. This increase is due, at least in part, to its association with the regulatory subunit Dbf4p, whose expression levels reach a maximum at G₁–S (Jackson *et al.*, 1993; Cheng *et al.*, 1999; Oshiro *et al.*, 1999). Recent studies indicate that Cdc7p is required not only for initiation of DNA replication, but also for origin firing during the S phase (Bousset and Diffley, 1998; Donaldson *et al.*, 1998). Cdc7p interacts with the MCM2 protein, and Dbf4p has been shown to interact with the origin of DNA replication, although it is not clear whether this is direct or indirect (Dowell *et al.*, 1994; Lei *et al.*, 1997). A loss-of-function *mcm5* mutant can bypass the requirement for Cdc7p–Dbf4p, and Cdc7p–Dbf4p kinase phosphorylates several MCM proteins *in vitro* (Hardy *et al.*, 1997; Lei *et al.*, 1997). Taken together, these findings suggest that the Cdc7p–Dbf4p protein kinase complex may directly target the origin-associated components of pre-RCs, such as MCM proteins, during the initiation of DNA replication.

Like the Cdks, Cdc7p is conserved in evolution and its homologs have been found in fission yeast and metazoans (Masai *et al.*, 1995; Jiang and Hunter, 1997; Sato *et al.*, 1997; Hess *et al.*, 1998). In a previous study, we identified

a human Cdc7 homolog, HsCdc7. We demonstrated that, like Cdc7p, HsCdc7 has a protein kinase activity. Also like Cdc7p, the expression levels of HsCdc7 remain constant throughout the cell cycle, but its kinase activity is regulated during the cell cycle. HsCdc7 kinase activity is low during G₁ and increases in the S phase. The increase in HsCdc7 kinase activity at specific cell-cycle stages therefore results from post-translational regulation. Since activation of Cdc7p requires its regulatory subunit Dbf4p, we hypothesized that HsCdc7 also requires a regulatory subunit (e.g. a Dbf4-like protein) to regulate its kinase activity during the cell cycle. Here, we report the identification of HsDbf4, a HsCdc7 regulatory subunit, from human cells. We show that HsDbf4 binds to HsCdc7 and activates its kinase activity. Like yeast Dbf4p, the expression levels of HsDbf4 protein are regulated and coincide with the HsCdc7 kinase activity during the cell cycle. Purified baculovirus-expressed HsCdc7–HsDbf4 selectively phosphorylates the MCM2 subunit of the chromatin-associated MCM protein complex isolated by immunoprecipitation with MCM7 antibodies *in vitro*. Two-dimensional tryptic phosphopeptide-mapping analysis of *in vivo* ³²P-labeled MCM2 from HeLa cells reveals that several major tryptic phosphopeptides of MCM2 comigrate with those of MCM2 phosphorylated by HsCdc7–HsDbf4 *in vitro*, suggesting that MCM2 is a physiological HsCdc7–HsDbf4 substrate. Immunoneutralization of HsCdc7–HsDbf4 activity by microinjection of anti-HsCdc7 antibodies into HeLa cells blocks the initiation of DNA replication. These results, together with the known functions of Cdc7p–Dbf4p in yeast, indicate that HsCdc7–HsDbf4 is directly involved in regulating the initiation of DNA replication by targeting the MCM2 protein in mammalian cells.

Results

Identification, cloning and molecular features of the HsCdc7 regulatory subunit, HsDbf4

To identify the potential regulatory subunit of HsCdc7, we carried out a yeast two-hybrid screen using full-length HsCdc7 protein as the bait (for details see Materials and methods). We obtained four different proteins that interacted specifically with HsCdc7. A DDBJ/EMBL/GenBank database search revealed that one of these proteins contains an 80 amino acid stretch that has significant sequence similarity (~58%) to the C-terminal region of budding yeast Dbf4p (Figure 1C). We isolated the corresponding full-length cDNA, designated HsDbf4, from a HeLa cDNA phage library using the cDNA obtained from the two-hybrid screen as a probe. Analysis of the full-length HsDbf4 cDNA reveals that it encodes a protein of 674 amino acids with a predicted molecular mass of 77 kDa (Figure 1A and B). However, the apparent molecular mass of HsDbf4, as determined from its electrophoretic mobility on denaturing polyacrylamide gels, is significantly greater (see below). Unexpectedly, sequence analysis revealed that HsDbf4 shares rather limited homology to yeast Dbf4p. Only the 80 amino acid stretch in the central region of HsDbf4 (amino acids 256–336) is closely related to the C-terminal region of Dbf4p (amino acids 622–702; Figure 1C). Beyond these regions, the two proteins do not share any significant sequence homology.

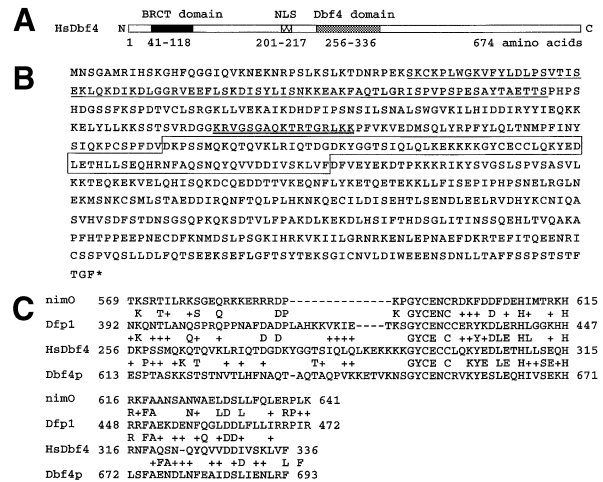


Fig. 1. Sequence and schematic structure of HsDbf4. (A) Schematic domain structure of HsDbf4. The boxes indicate the BRCT domain, NLS and Dbf4 domain. (B) Deduced amino acid sequence (single-letter amino acid code) of HsDbf4. The amino acids underlined represent the BRCT domain, the amino acids double underlined represent the putative NLS and the amino acids in the box represent the Dbf4 domain. (C) Alignment of amino acid sequence of Dbf4 domains from HsDbf4, Dbf4p, Dfp1 and nimO using the PSI-BLAST program on the National Center for Biotechnology Information database. Amino acids that are identical are indicated by a single letter between the sequence lines and amino acids that are similar are indicated by +.

The 80 amino acid stretch of HsDbf4 also shows significant sequence homology to Dfp1/Him1, the regulatory subunit of the *Schizosaccharomyces pombe* Cdc7p homolog Hsk1 (Brown and Kelly, 1998; Takeda *et al.*, 1999) and the *Emericella nidulans* G₁–S regulator, nimO (James *et al.*, 1999; Figure 1C). We designated this distinct 80 amino acid region as the Dbf4 domain (Figure 1A and B), although the exact biological functions of this domain are not clear (see Discussion). In addition, residues 201–217 form a putative bipartite nuclear localization sequence (NLS). Expression of GFP-tagged HsDbf4 fusion protein in human 293 cells and normal foreskin fibroblasts demonstrated that the tagged HsDbf4 protein was localized in the nucleus (W.Jiang and T.Hunter, unpublished data). HsDbf4 also has distinct hydrophobic clusters of amino acids in its N-terminal region (residues 41–118) with sequence similarity to BRCT domains. BRCT domains are found in the breast cancer susceptibility gene 1 product, BRCA1, and in several proteins involved in transcriptional regulation, DNA repair, recombination and cell-cycle control (Koonin *et al.*, 1996). It has been proposed that the BRCT domain is involved in protein–protein interaction. It is possible that HsCdc7–HsDbf4 might utilize this domain to specify its downstream targets. The sequence of HsDbf4 reported in this study is identical to several expressed sequence tag (EST) sequences derived from human 7q21 genomic clone RG135C18 (DDBJ/EMBL/GenBank accession No. AC005164), indicating that the *HsDbf4* gene is located on human chromosome 7q21.

HsDbf4 binds to HsCdc7 and activates HsCdc7 kinase activity *in vivo*

To determine whether HsDbf4 is a regulatory subunit of HsCdc7 kinase, we first examined whether HsDbf4 could

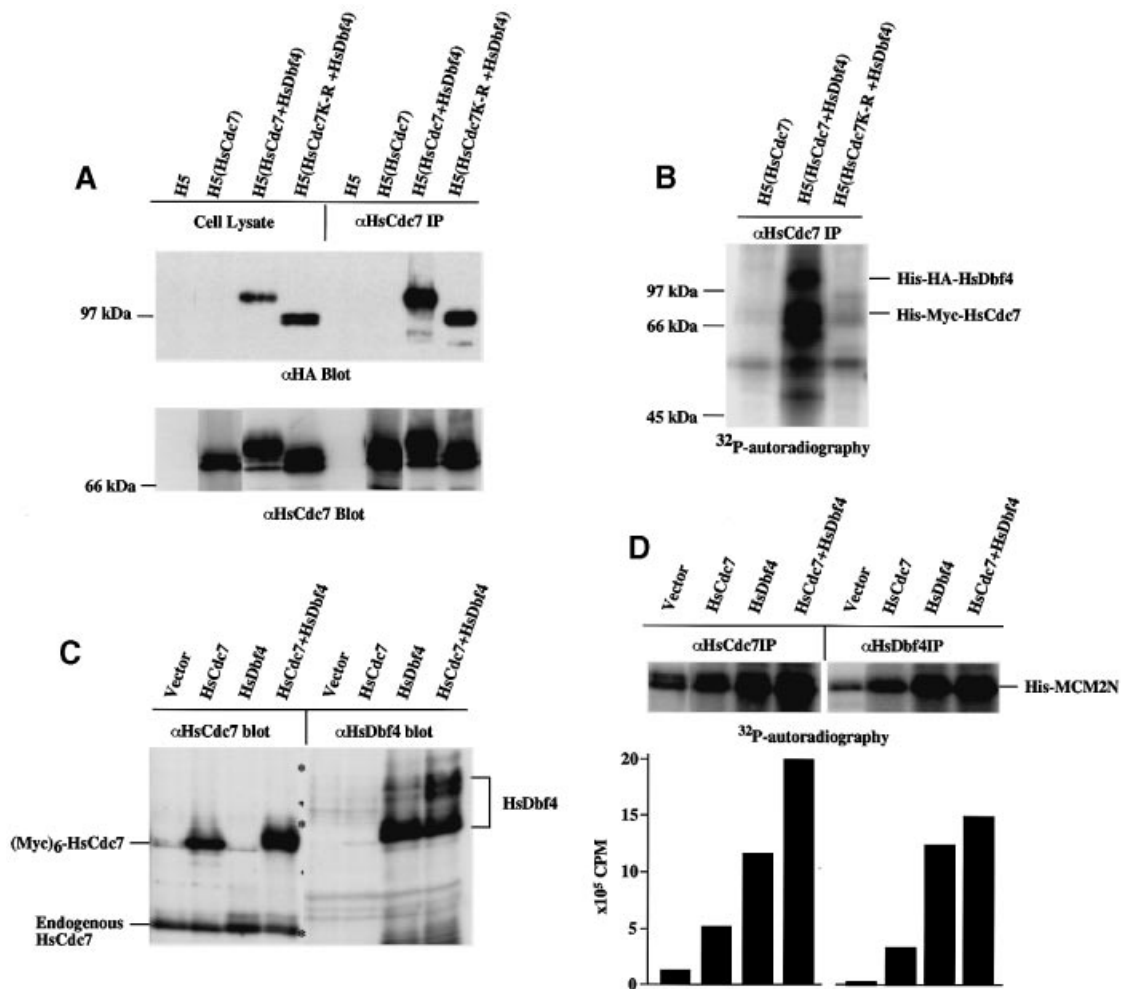


Fig. 2. Expression, association and kinase activity of HsCdc7-HsDbf4 complexes in insect Hi5 and human 293 cells. (A) Lysates and anti-HsCdc7 immunoprecipitates (α HsCdc7) from lysates of uninfected Hi5 cells or Hi5 cells infected or coinfecting with baculovirus expressing the indicated proteins were subjected to SDS-PAGE and then immunoblotting with anti-HA or anti-HsCdc7 antibodies. (B) Anti-HsCdc7 immunoprecipitates from lysates of Hi5 cells infected or coinfecting with baculovirus expressing indicated proteins were incubated with [γ -³²P]ATP. After incubation, the reaction products were separated on SDS-PAGE prior to autoradiography. (C) Lysates of 293 cells transiently transfected with the indicated plasmids were subjected to SDS-PAGE and then immunoblotting with affinity-purified anti-HsCdc7 and anti-HsDbf4 antibodies. *Molecular mass standards 116, 97 and 66 kDa (top to bottom). (D) Anti-HsCdc7 or anti-HsDbf4 immunoprecipitates from lysates of 293 cells described in (C) were incubated with His-MCM2N in the presence of [γ -³²P]ATP. After incubation, the reaction products were separated by SDS-PAGE prior to autoradiography (top) and then quantified using a PhosphorImager (bottom).

associate with HsCdc7 and activate its kinase activity in insect cells using a baculovirus-based expression system. Hi5 insect cells were infected with a baculovirus expressing His-Myc-tagged HsCdc7 alone or coinfecting with baculoviruses expressing His-Myc-tagged HsCdc7 and His-HA-tagged HsDbf4. As a control, Hi5 cells were also coinfecting with baculoviruses expressing His-HA-tagged HsDbf4 and His-Myc-tagged HsCdc7(K-R), in which the Lys in the kinase subdomain II (K90) required for ATP binding was mutated to Arg to inactivate catalytic activity (Jiang and Hunter, 1997). As shown in Figure 2A, high levels of ~70–75 kDa His-Myc-HsCdc7, His-Myc-HsCdc7(K-R) and ~95–105 kDa His-HA-HsDbf4 proteins were detected in baculovirus-infected cells by immunoblotting with anti-HsCdc7 and anti-HA antibodies, respectively. The electrophoretic mobilities of HsCdc7 and HsDbf4 proteins from Hi5 cells expressing His-Myc-HsCdc7 and His-HA-HsDbf4 were greater than those of HsCdc7 and HsDbf4 proteins from Hi5 cells expressing His-Myc-HsCdc7 alone or His-Myc-HsCdc7(K-R) and

His-HA-HsDbf4, implying that they might undergo post-translational modification, such as phosphorylation. To determine whether HsDbf4 could associate with HsCdc7, the lysates of Hi5 cells expressing His-Myc-HsCdc7, His-Myc-HsCdc7 together with His-HA-HsDbf4 or His-Myc-HsCdc7(K-R) together with His-HA-HsDbf4 were immunoprecipitated with anti-HsCdc7 antibodies. The immunoprecipitates were resolved by SDS-PAGE and then analyzed by immunoblotting with anti-HsCdc7 or anti-HA antibodies. His-HA-HsDbf4 was clearly detected in the anti-HsCdc7 immunoprecipitates from lysates of Hi5 cells coexpressing His-HA-HsDbf4 and His-Myc-HsCdc7 or His-HA-HsDbf4 and His-Myc-HsCdc7(K-R), but not in those of Hi5 cells expressing His-Myc-HsCdc7 alone or uninfected Hi5 cells, indicating that HsDbf4 associated with HsCdc7 in insect cells (Figure 2A).

Next, we determined the kinase activity of HsCdc7-HsDbf4 in immunoprecipitates. The cell lysates shown in Figure 2A were immunoprecipitated with anti-HsCdc7 antibodies and the protein kinase activities of these

immunoprecipitates were determined in the presence of [γ - 32 P]ATP. As shown in Figure 2B, autophosphorylation of His–Myc–HsCdc7 and His–HA–HsDbf4 was very weak in the anti-HsCdc7 immunoprecipitates from Hi5 cells expressing His–Myc–HsCdc7 alone or Hi5 cells expressing His–Myc–HsCdc7(K-R) and His–HA–HsDbf4. In contrast, autophosphorylation of His–Myc–HsCdc7 and His–HA–HsDbf4 was clearly detected in the anti-HsCdc7 immunoprecipitates from Hi5 cells expressing His–Myc–HsCdc7 and His–HA–HsDbf4. In addition, like the HsCdc7–HsDbf4 immunocomplex, the HsCdc7–HsDbf4 complex partially purified using nickel (NTA) agarose column chromatography displayed autophosphorylation kinase activity (data not shown and Figure 4A). These results demonstrated that HsDbf4 not only associated with HsCdc7, but also stimulated its kinase activity in insect cells. Since the HsCdc7–HsDbf4 complex has autophosphorylation activity, it seems possible that the electrophoretic mobility shift of HsCdc7 and HsDbf4 proteins in coinfecting Hi5 cells is due to phosphorylation.

To determine whether HsDbf4 could also activate HsCdc7 in mammalian cells, (Myc) $_6$ –HsCdc7 (Jiang and Hunter, 1997), full-length HsDbf4 or both were transfected and expressed in 293 cells using the cytomegalovirus (CMV)-based mammalian expression vectors. Lysates of transfected cells were resolved by SDS–PAGE and then immunoblotted with affinity-purified anti-HsCdc7 or anti-HsDbf4 antibodies (see Materials and methods). Consistent with our previously published results (Jiang and Hunter, 1997), anti-HsCdc7 antibodies specifically detected the endogenous 64 kDa HsCdc7 protein in all cells and recognized the 87 kDa (Myc) $_6$ –HsCdc7 protein in cells transfected with plasmid encoding (Myc) $_6$ –HsCdc7 (Figure 2C). Anti-HsDbf4 antibodies recognized a family of ~90–105 kDa proteins in cells transfected with the plasmid encoding HsDbf4 (Figure 2C). We were able to detect weak ~100–105 kDa bands in lysates from cells transfected with control vectors or plasmid encoding (Myc) $_6$ –HsCdc7 after long exposure of the film (data not shown and Figure 3A and C). These results indicated that anti-HsDbf4 antibodies recognized ectopically expressed and endogenous HsDbf4 proteins.

The cell lysates used in Figure 2C were subjected to immunoprecipitation with anti-HsCdc7 or anti-HsDbf4 antibodies and the protein kinase activities of these immunoprecipitates were determined using bacterially expressed purified His-tagged MCM2N (His–MCM2N, an N-terminal fragment of human MCM2 containing residues 1–285) as the substrate (Sato *et al.*, 1997). As shown in Figure 2D, phosphorylation of His–MCM2N was clearly increased in immunoprecipitates from cells overexpressing (Myc) $_6$ –HsCdc7 or HsDbf4 alone compared with that from control cells in which the observed activity was due to endogenous HsCdc7–HsDbf4 complexes. The increases in HsCdc7–HsDbf4 kinase activity isolated from these cells were 4- to 5-fold and 12- to 13-fold, respectively. The strongest phosphorylation of His–MCM2N was detected with immunoprecipitates from cells overexpressing both (Myc) $_6$ –HsCdc7 and HsDbf4 (14- to 20-fold). We also detected autophosphorylation of (Myc) $_6$ –HsCdc7 and HsDbf4 in immunoprecipitates from cells overexpressing both proteins (data not shown). These results indicated

that HsDbf4 could also activate HsCdc7 kinase in mammalian cells.

The expression of HsDbf4 protein is regulated and coincides with the HsCdc7 kinase activity during the cell cycle

To determine whether endogenous HsCdc7 and HsDbf4 form a complex in mammalian cells, 293, HeLa and U2OS cells were lysed and the lysates were immunoprecipitated with either anti-HsCdc7 or anti-HsDbf4 antibodies. These immunoprecipitates were subjected to SDS–PAGE and subsequently immunoblotted with anti-HsCdc7 or anti-HsDbf4 antibodies. As shown in Figure 3A, a family of ~100–105 kDa endogenous HsDbf4 proteins was detected in the immunoprecipitates made with anti-HsCdc7 and anti-HsDbf4 antibodies. Correspondingly, the ~64–65 kDa endogenous HsCdc7 protein was also detected in the immunoprecipitates made with anti-HsCdc7 and anti-HsDbf4 antibodies. In contrast, neither HsCdc7 nor HsDbf4 was detected in the immunoprecipitates with HsCdc7 or HsDbf4 antibodies that were prebound to their peptide antigen. These results demonstrated that, like ectopically expressed HsCdc7 and HsDbf4, endogenous HsCdc7 and HsDbf4 form a complex in mammalian cells.

Next, we examined the formation of the HsCdc7–HsDbf4 complex and its kinase activity during the cell cycle. 293 cells were synchronized at the G $_1$ –S to S phase using a thymidine block and then released into different stages of the cell cycle. The cell-cycle distributions of the synchronized cells were determined by flow cytometry (Figure 3B). The amounts of HsCdc7 and HsDbf4 proteins in cells at different stages of the cell cycle were analyzed by immunoblotting with anti-HsCdc7 and anti-HsDbf4 antibodies, respectively. Consistent with our previously published results (Jiang and Hunter, 1997), HsCdc7 protein levels remained constant during the cell cycle (Figure 3Ca). In contrast, immunoblotting analysis of HsDbf4 immunoprecipitates revealed that expression of HsDbf4 protein was clearly regulated during the cell cycle (Figure 3Cb). It was high during G $_1$ –S, S and G $_2$ –M (the decrease in HsDbf4 at 6 h is probably due to the increased content of G $_1$ cells, although HsDbf4 levels may begin to fall during mitosis), and was dramatically reduced when cells entered G $_1$. When cells entered the next cell cycle, HsDbf4 protein levels rose again in the subsequent S and G $_2$ –M phases. After the same blot was stripped and re-probed with anti-HsCdc7 antibodies, the levels of HsCdc7 protein detected in HsDbf4 immunoprecipitates fluctuated in the same way as that of HsDbf4 protein during the cell cycle (Figure 3Cc). These results indicated that, like yeast Dbf4p, the expression levels of HsDbf4 are regulated during the cell cycle, thereby regulating formation of HsCdc7–HsDbf4 complex during the cell cycle. Consistent with this, HsCdc7 immunoprecipitates from the same cell lysates exhibited cell-cycle regulated His–MCM2N kinase activity (Figure 3Cd). HsCdc7 kinase activity matched the expression levels of HsDbf4 protein and HsDbf4-bound HsCdc7 protein levels during the cell cycle. It was low when cells were in G $_1$, increased during G $_1$ –S and remained high during S to G $_2$ –M (Figure 3Cd). The increase in HsCdc7 kinase activity during G $_1$ –S to S was also observed in 293 cells that were fractionated at specific stages of the cell cycle using centrifugal elutriation (Jiang and

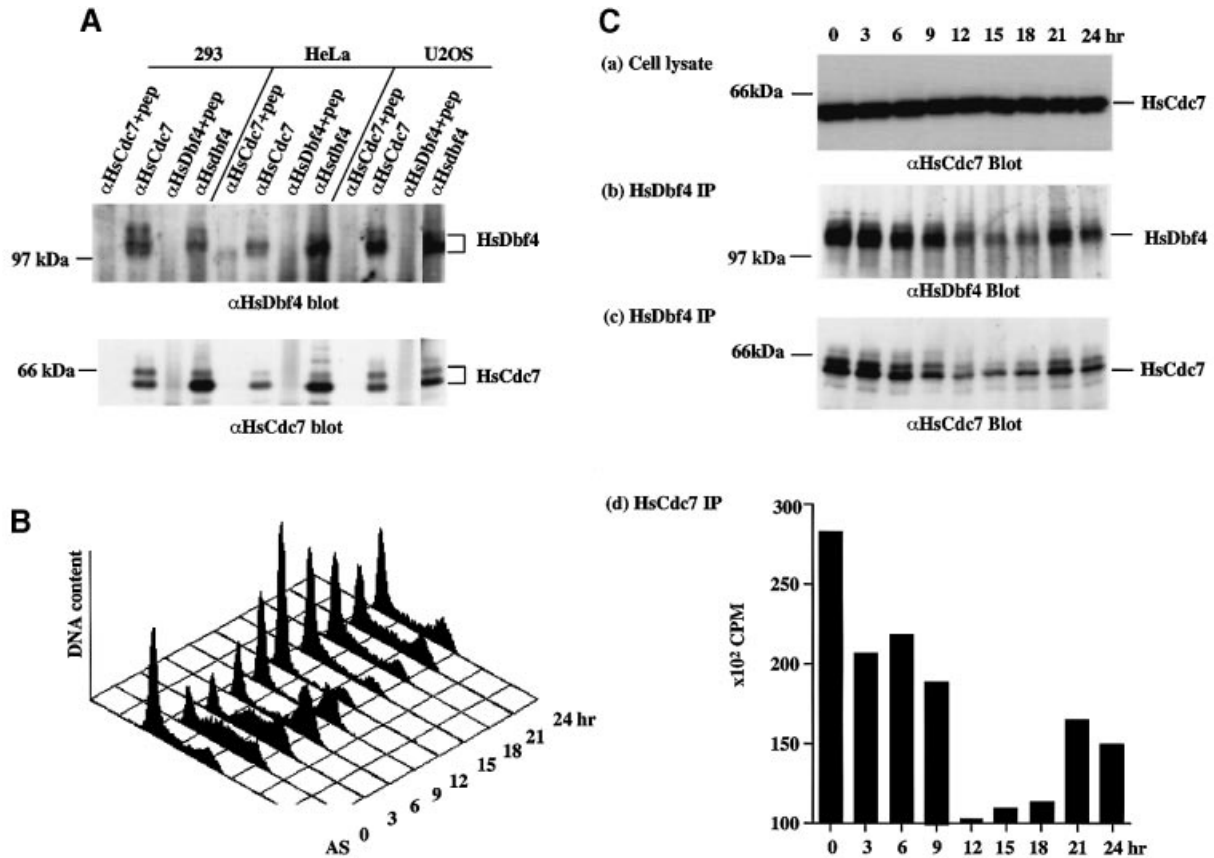


Fig. 3. The expression of HsCdc7 and HsDbf4 proteins, HsCdc7-HsDbf4 complex formation and its kinase activity during the cell cycle. (A) Cell lysates from the cells indicated were immunoprecipitated with anti-HsCdc7, anti-HsDbf4 or these antibodies prebound to their peptide antigens. The immunoprecipitates were then subjected to SDS-PAGE and immunoblotting with affinity-purified anti-HsCdc7 or anti-HsDbf4 antibodies. (B) 293 cells synchronized by a thymidine block and then released were collected at different time points and analyzed for DNA content by flow cytometry (As: asynchronous cells). (C) Cell lysates of synchronous 293 cells described in (B) were subjected to SDS-PAGE and immunoblotting with affinity-purified anti-HsCdc7 (a). Anti-HsDbf4 immunoprecipitates from lysates of synchronous 293 cells described in (B) were subjected to SDS-PAGE and immunoblotting with affinity-purified anti-HsDbf4 (b) or anti-HsCdc7 (c) antibodies. Anti-HsCdc7 immunoprecipitates from lysates of synchronous 293 cells described in (B) were incubated with His-MCM2N in the presence of [γ -³²P]ATP. After incubation, the reaction products were separated by SDS-PAGE prior to autoradiography and then quantified by a PhosphorImager (d).

Hunter, 1997). These results demonstrated that cell-cycle-regulated HsCdc7 kinase activity is dependent on the cell-cycle-regulated expression of its regulatory subunit, HsDbf4. Regulation of HsDbf4 expression appears to be partially exerted at the transcriptional level, since HsDbf4 RNA levels mirrored those of HsDbf4 protein (W.Jiang and T.Hunter, unpublished data).

HsCdc7-HsDbf4 selectively phosphorylates MCM2 subunit of chromatin-associated MCM complex *in vitro* and MCM2 is an *in vivo* HsCdc7-HsDbf4 substrate

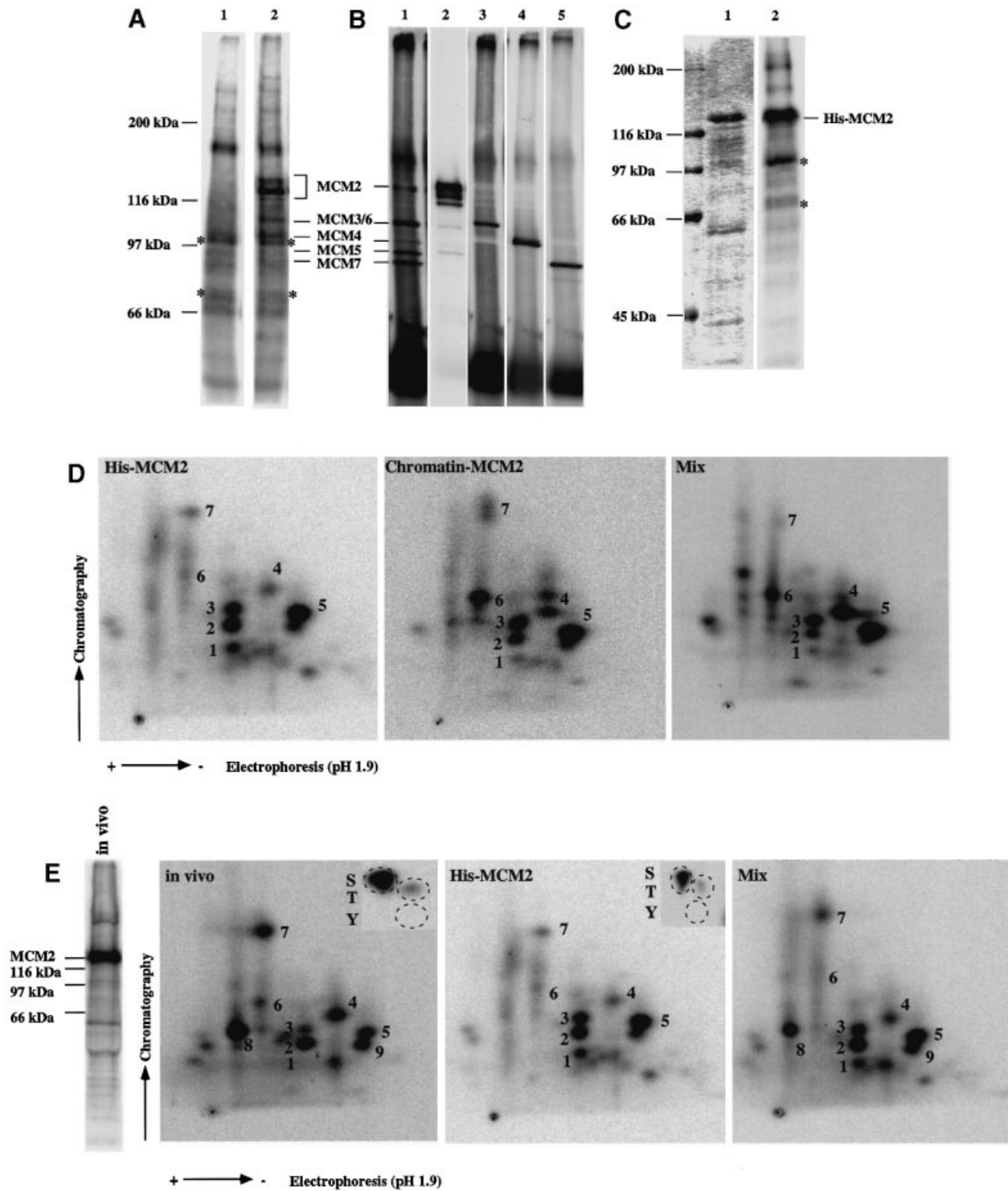
Recent genetic and biochemical studies suggest that MCM proteins may be the downstream targets of the Cdc7-Dbf4 kinase complex (Hardy *et al.*, 1997; Lei *et al.*, 1997; Sato *et al.*, 1997; Brown and Kelly, 1998). Several GST-MCM fusion proteins expressed in *Escherichia coli* have been shown to be phosphorylated by Cdc7-Dbf4 *in vitro* (Lei *et al.*, 1997; Sato *et al.*, 1997). Since MCM proteins form multimeric complexes that associate with chromatin *in vivo*, we wanted to determine whether MCM proteins in the chromatin-bound multimeric complexes were also phosphorylated by HsCdc7-HsDbf4. To this end, we performed subcellular fractionation and immunoprecipit-

ated chromatin-bound multimeric MCM complexes with anti-MCM7 antibodies from HeLa cells (see Materials and methods). The immunoprecipitates were subjected to SDS-PAGE and then analyzed by immunoblotting with antibodies against MCM proteins. As shown in Figure 4B, all six MCM proteins (MCM2-7) were detected in the MCM7 immunoprecipitates using anti-MCM-DEFD antibodies, which recognize all six MCM proteins (Hu *et al.*, 1993). The result was confirmed using anti-MCM2, -3, -4 and -7-specific antibodies.

In a parallel experiment, the MCM7 immunoprecipitates were incubated with partially purified baculovirus-expressed HsCdc7-HsDbf4 in the presence of [γ -³²P]ATP. As a control, protein A-agarose beads alone were also incubated with the purified HsCdc7-HsDbf4 in the presence of [γ -³²P]ATP. As shown in Figure 4A, the major protein phosphorylated specifically by HsCdc7-HsDbf4 migrated at ~120 kDa. Phosphorylation of the ~120 kDa protein was dependent upon the presence of both HsCdc7-HsDbf4 and the MCM complex. The phosphorylated ~120 kDa protein comigrated precisely with the MCM2 protein as determined by immunoblotting (compare Figure 4A with B). To determine whether the phosphorylated ~120 kDa protein is MCM2, we compared the two-

dimensional tryptic phosphopeptide map of this protein with that of bacterially expressed purified His-tagged MCM2 (His-MCM2) phosphorylated by HsCdc7-HsDbf4 *in vitro* (Figure 4C). As shown in Figure 4D, the two-dimensional tryptic phosphopeptide map of the ~120 kDa protein was virtually identical to that of His-MCM2. Seven major phosphopeptides (spots 1-7) of these two proteins comigrated when a mixture was analyzed. Therefore, we concluded that the ~120 kDa protein phosphorylated by HsCdc7-HsDbf4 in the MCM complex was MCM2.

In addition to the MCM2 protein, several minor phosphoproteins phosphorylated by HsCdc7-HsDbf4 were also detected (Figure 4A). These bands comigrated with the MCM3/6 and MCM4 proteins as determined by immunoblotting (compare Figure 4A with B). However, we have been unable to confirm that they are indeed MCM3/6 and MCM4 proteins by re-immunoprecipitation with specific anti-MCM3 and anti-MCM4 antibodies, possibly because the level of phosphorylation of these proteins by HsCdc7-HsDbf4 was very low. In addition, phosphorylation of MCM5 and MCM7 was not detected in



the reaction (Figure 4A). However, the MCM7 antibodies present in the MCM complex might affect the ability of HsCdc7-HsDbf4 to target MCM3-7 proteins. Future work is required to determine whether HsCdc7-HsDbf4 can phosphorylate one or more of the MCM3-7 proteins in a chromatin-associated MCM complex purified directly from cells without immunoprecipitation with anti-MCM antibodies. Nevertheless, under our conditions it is clear that the HsCdc7-HsDbf4 kinase selectively phosphorylated the MCM2 protein in the chromatin-associated MCM multimeric complex immunoprecipitated by MCM7 antibodies.

To determine whether MCM2 is a physiological HsCdc7-HsDbf4 substrate, HeLa cells were metabolically labeled with ^{32}P -orthophosphate and MCM2 protein was immunoprecipitated with affinity-purified anti-MCM2 antibodies. As shown in Figure 4E, the endogenous 120 kDa MCM2 was immunoprecipitated from *in vivo* ^{32}P -labeled cells. Phosphoamino acid (PAA) and two-dimensional tryptic phosphopeptide-mapping analyses of *in vivo* ^{32}P -labeled MCM2 were performed and then compared with those of His-MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro*. Like His-MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro*, the endogenous MCM2 was phosphorylated mainly on Ser and to a lesser extent on Thr. We observed nine (spots 1-9) phosphopeptides in the two-dimensional map of *in vivo* ^{32}P -labeled MCM2. When a mixture of *in vivo* ^{32}P -labeled MCM2 and His-tagged MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro* was analyzed, seven (spots 1-7) phosphopeptides from *in vivo* ^{32}P -labeled MCM2 comigrated with the phosphopeptides (spots 1-7) of His-tagged MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro*. These results, together with the findings that MCM2 is phosphorylated in G₁-S, S to G₂-M *in vivo* (Todorov *et al.*, 1995; Fujita *et al.*, 1998) coincident with the activation of HsCdc7-HsDbf4 during the cell cycle (Figure 3Cd), strongly suggest that MCM2 is an *in vivo* HsCdc7-HsDbf4 substrate. We are currently identifying the HsCdc7-HsDbf4 phosphorylation sites in MCM2.

HsCdc7-HsDbf4 is essential for the initiation of DNA replication

To determine whether HsCdc7-HsDbf4 plays a functional role in the initiation of DNA replication, we immuno-

neutralized HsCdc7-HsDbf4 activity by microinjection of affinity-purified anti-HsCdc7 antibodies into HeLa cells. Mitotic HeLa cells were microinjected with either affinity-purified anti-HsCdc7 antibodies or, as a control, affinity-purified anti-HsCdc7 antibodies that had been prebound to its peptide antigen. After injection, cells were cultured in medium in the presence of 5'-bromodeoxyuridine (BrdU) for 16 h to monitor the ability of daughter cells to initiate DNA replication in the next round of the cell cycle. As shown in Figure 5, like uninjected cells, injected mitotic cells were able to complete mitosis and cytokinesis, and microinjected antibodies could be detected in both daughter cells using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibodies. Of the control injected cells, >90% (34/36) replicated their DNA as determined by anti-BrdU antibody staining, a value that was not significantly different from uninjected cells. In contrast, the majority (36/42, 86%) of cells injected with anti-HsCdc7 antibodies failed to initiate DNA replication. Only a small proportion (6/42, 14%) of cells injected with anti-HsCdc7 antibodies exhibited very weak BrdU incorporation, suggesting that the quantity of anti-HsCdc7 antibodies in these cells may have been insufficient to titrate out all endogenous HsCdc7-HsDbf4 activity. These results, together with the established functional roles of Cdc7-Dbf4 in yeast, demonstrated that HsCdc7-HsDbf4 activity is required for initiation of DNA replication in mammalian cells.

Discussion

In the present study, we report the identification and characterization of HsDbf4. Although HsDbf4 only has one relatively short region of homology with budding yeast Dbf4p, several lines of evidence presented here demonstrate that HsDbf4 is a regulatory subunit of HsCdc7. First, HsDbf4 contains an 80 amino acid stretch in its central region (Dbf4 domain) that displays significant sequence homology with the C-terminal region of Dbf4p. This Dbf4 domain is also found in other Dbf4-related proteins, *S.pombe* Dfp1 and *E.nidulans* nimO (Figure 1C). Secondly, we found that HsCdc7 expressed in insect cells did not show any intrinsic kinase activity. However, when we coexpressed HsCdc7 and HsDbf4 in insect and

Fig. 4. Phosphorylation of MCM2 by HsCdc7-HsDbf4 kinase. (A) Proteins from chromatin-associated fraction of HeLa cells bound to protein A beads (1) or anti-MCM7-protein A beads (2) were incubated with partially purified baculovirus-expressed HsCdc7-HsDbf4 in the presence of [γ - ^{32}P]ATP. After incubation, the reaction products were separated by SDS-PAGE prior to autoradiography. *Autophosphorylation of HsDbf4 and HsCdc7. (B) Anti-MCM7 immunoprecipitates from chromatin-associated fraction of HeLa cells were subjected to SDS-PAGE and immunoblotting with anti-MCM-DEFD (1), anti-MCM2 (2), anti-MCM3 (3), anti-MCM4 (4) or anti-MCM7 (5) antibodies. The positions of various MCM proteins are indicated. (C) One microgram His-MCM2 was incubated with partially purified baculovirus-expressed HsCdc7-HsDbf4 in the presence of [γ - ^{32}P]ATP. After incubation, the reaction products were separated by SDS-PAGE and visualized by autoradiography (2) or Coomassie Brilliant Blue staining (1). *Autophosphorylation of HsDbf4 and HsCdc7. (D) ^{32}P -labeled chromatin-bound MCM2 from MCM7 immunoprecipitates in (A) (chromatin-MCM2) and His-tagged MCM2 phosphorylated by HsCdc7-HsDbf4 in (C) (His-MCM2) were eluted from SDS-PAGE gels and then digested with trypsin. The resulting phosphopeptides were separated by electrophoresis (pH 1.9 buffer) in the horizontal dimension (anode on the left) and chromatography (phospho-chromatography buffer) in the vertical dimension. Shown are two-dimensional tryptic phosphopeptide maps of: (chromatin-MCM2) ^{32}P -labeled chromatin-bound MCM2 from MCM7 immunoprecipitates phosphorylated by HsCdc7-HsDbf4 *in vitro* (2000 c.p.m.); (His-MCM2), His-tagged MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro* (2000 c.p.m.); (mix), mix of chromatin-MCM2 (1000 c.p.m.) and His-MCM2 (1000 c.p.m.). The major phosphopeptides, 1-7, are labeled. (E) HeLa cells were *in vivo* labeled with ^{32}P -orthophosphate for 4 h. MCM2 was immunoprecipitated using a monoclonal anti-MCM2 antibody. After washing, the immunoprecipitates were subjected to SDS-PAGE and visualized by autoradiography (*in vivo*). ^{32}P -labeled immunoprecipitated MCM2 protein from cells (*in vivo*) and His-tagged MCM2 protein phosphorylated by HsCdc7-HsDbf4 described in (C) (His-MCM2) were eluted from SDS-PAGE gels and then digested with trypsin. The resulting phosphopeptides were separated by electrophoresis and chromatography as described in (D). Shown are two-dimensional tryptic phosphopeptide maps of: (*in vivo*) MCM2 from *in vivo* ^{32}P -labeled cells (2000 c.p.m.); (His-MCM2) His-tagged MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro* (2000 c.p.m.); (mix) mix of *in vivo* (1000 c.p.m.) and His-MCM2 (1000 c.p.m.). The major phosphopeptides on the maps are labeled. The phosphoamino acid compositions of the *in vivo* and *in vitro* labeled MCM2 samples are shown as insets at the top right (S = P.Ser; T = P.Thr; Y = P.Tyr).

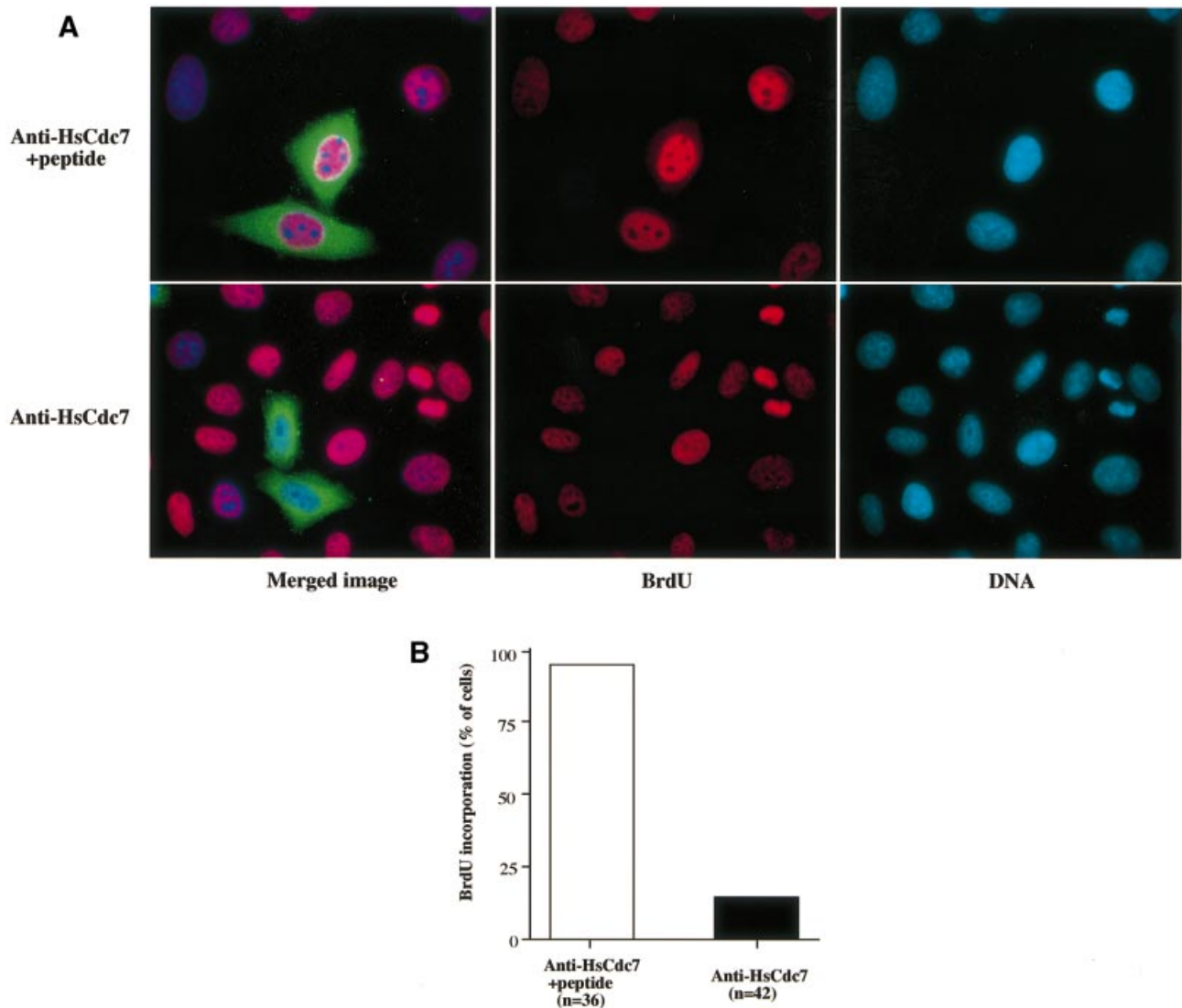


Fig. 5. Inhibition of DNA replication by microinjection of affinity-purified anti-HsCdc7 antibodies in HeLa cells. Mitotic cells grown on coverslips were microinjected with anti-HsCdc7 antibodies (3 mg/ml) and anti-HsCdc7 antibodies (3 mg/ml) that had been prebound to peptide antigen and then incubated with medium containing 10 μ M BrdU for 16 h. (A) Cells were fixed and stained with rat anti-BrdU monoclonal antibody followed by FITC-conjugated goat anti-rabbit, rhodamine-conjugated donkey anti-rat secondary antibodies and Hoechst 33258 as indicated. The immunofluorescence staining was analyzed using a fluorescence microscope. (B) Quantitation of BrdU incorporation in cells microinjected with the indicated antibodies. The number of injected cells scored is indicated.

mammalian cells, HsCdc7 bound to HsDbf4 and the HsCdc7–HsDbf4 complex displayed autophosphorylation and MCM2 kinase activity. Thirdly, like Dbf4p and Cdc7p, HsDbf4 protein levels are clearly regulated during the cell cycle, whereas HsCdc7 protein levels remain constant. HsDbf4 protein levels are low in G₁, increase during G₁–S and remain high in S and G₂–M. Coimmunoprecipitation experiments indicated that HsDbf4-associated HsCdc7 protein levels fluctuate in the same way as HsDbf4 levels. HsCdc7 kinase activity oscillates during the cell cycle with a pattern that matches the levels of HsCdc7–HsDbf4 complexes. These results clearly demonstrate that the cell-cycle-regulated HsCdc7 kinase activity is the result of cell-cycle-regulated expression of HsDbf4 protein. However, it is possible that additional events are required for activation of HsCdc7 in addition to binding HsDbf4. Taken together, our results indicate that HsDbf4 identified in this study is a bona fide regulatory subunit of HsCdc7. While this

paper was under review, Masai and colleagues reported identification of an HsCdc7 regulatory subunit, ASK (Johnston *et al.*, 1999; Kumagai *et al.*, 1999). ASK is identical to HsDbf4.

The regulation of HsCdc7 kinase activity by HsDbf4 is reminiscent of activation of Cdks by cyclins. Like Cdks, whose activities require their regulatory subunit cyclins, HsCdc7 kinase activity requires HsDbf4. During the cell cycle, expression levels of Cdks and HsCdc7 are constant, whereas the levels of the cyclins and HsDbf4 oscillate and coincide with the kinase activities of Cdk–cyclin and HsCdc7–HsDbf4, respectively. Nonetheless, HsCdc7 is not a member of Cdk family and HsDbf4 shows no sequence homology with the cyclins. All members of cyclin family contain a cyclin-box domain, which is required for binding and activating Cdks (Nigg, 1995). Thus far, the Dbf4 domain has been found only in Dbf4-like proteins. However, unlike the cyclin-box, the Dbf4

domains of Dbf4p are not essential for their association with their kinase subunits (Dowell *et al.*, 1994). This implies that the Dbf4 domain is not the primary Cdc7 kinase-binding motif in Dbf4 proteins, although recent evidence indicates that this domain may be important in the interaction of ASK/HsDbf4 with HsCdc7 (Kumagai *et al.*, 1999). In this regard, it is interesting to note that the Dbf4 box is located at the C-terminus of Dbf4 and Dfp1, but in the middle of HsDbf4. The fact that over most of its length the HsDbf4 protein is unrelated to Dbf4, and the fact that the large inserts in the HsCdc7 catalytic domain are unrelated to those in Cdc7 imply that even though human HsCdc7-HsDbf4 and budding yeast Cdc7-Dbf4 appear to have analogous cell-cycle roles, their exact functions and modes of regulation may differ in some regards.

The expression of HsDbf4 is regulated during the cell cycle, being induced at the G₁-S boundary and then decreasing again as cells enter the next G₁. The induction at G₁-S may occur in part at the transcriptional level, since HsDbf4 RNA levels increase at this time. However, the decrease as cells pass through the M phase into G₁ suggests that HsDbf4 may undergo regulated degradation during the M phase or early G₁, although the precise timing of the decrease in HsDbf4 levels remains to be determined. In budding yeast, the level of Dbf4 is regulated in the cell cycle, with low levels in G₁, an increase at G₁-S and then maintenance of a high level for the rest of the cycle. Dbf4 mRNA levels peak at G₁-S, but the mRNA is present throughout the cell cycle, and the major mechanism regulating Dbf4 protein levels appears to be changes in Dbf4 protein stability. Dbf4 is unstable in G₁ accounting for the low levels in G₁, and turnover of Dbf4 in G₁ requires anaphase-promoting complex (APC) activity (Cheng *et al.*, 1999; Oshiro *et al.*, 1999). The level of Dfp1 in fission yeast is also regulated in a similar manner in the cell cycle, with the low level in G₁ being due to increased turnover (Brown and Kelly, 1999; Takeda *et al.*, 1999). It will be important to determine whether HsDbf4 is degraded in an APC-dependent manner, and if so, how this is mediated.

Identification of the downstream targets of Cdc7 is a critical step toward unveiling the mechanisms by which initiation of DNA replication is controlled and executed. One possibility is that the Cdc7-Dbf4 kinase may directly target protein components of the pre-RC for DNA replication. We have found that purified baculovirus-expressed HsCdc7-HsDbf4 selectively phosphorylated the MCM2 protein in the chromatin-associated MCM multimeric complex *in vitro*. Two-dimensional tryptic phosphopeptide-mapping analysis of *in vivo* ³²P-labeled MCM2 from HeLa cells revealed that several major tryptic phosphopeptides of MCM2 comigrated with those of MCM2 phosphorylated by HsCdc7-HsDbf4 *in vitro*, suggesting that MCM2 is a physiological HsCdc7-HsDbf4 substrate. Consistent with our results, it has recently been reported that several MCM proteins (MCM2, -3, -4 and -6) expressed as GST-fusion proteins can be phosphorylated by Cdc7p as well as HsCdc7, and MCM2 protein in MCM multimeric complexes purified from *S.pombe* can be phosphorylated by Hsk1-Dfp1 *in vitro* (Lei *et al.*, 1997; Sato *et al.*, 1997; Brown and Kelly, 1998). Several reports showed that the six MCM proteins interact with each

other to form multimeric complexes *in vivo* (Chong *et al.*, 1995; Kubota *et al.*, 1995; Lei *et al.*, 1996) and that their phosphorylation state changes during the cell cycle. MCM proteins are hypophosphorylated in G₁ and become hyperphosphorylated during the G₁-S transition, which coincides with the gradual dissociation of MCM proteins from chromatin and the initiation of DNA replication (Todorov *et al.*, 1995; Fujita *et al.*, 1998). The heterotrimeric MCM complex containing MCM4, -6 and -7 has DNA helicase activity, and such activity can be inhibited by the presence of MCM2 (Ishimi, 1997; Ishimi *et al.*, 1998). Since HsCdc7-HsDbf4 kinase activity increases dramatically during G₁-S, phosphorylation of MCM2 by HsCdc7-HsDbf4 kinases *in vivo* could regulate MCM multimeric complex helicase activity at replication origins during replication, thus accounting for the essential role of HsCdc7-HsDbf4 in initiation of DNA replication. It is noteworthy that the molecular structure of MCM proteins is highly conserved during evolution (Kearsey and Labib, 1998). Given that the Dbf4 domain exists in Dbf4 proteins from various organisms and is not necessary for binding to Cdc7, it will be of interest to determine whether it might be involved in targeting Cdc7-Dbf4 kinase complex to its downstream substrate MCM2.

In summary, we have identified a HsCdc7 regulatory subunit HsDbf4 and determined the expression of the HsDbf4 protein, formation of the HsCdc7-HsDbf4 complex and its kinase activity during the cell cycle. We present evidence that HsCdc7-HsDbf4 kinase phosphorylates the MCM2 protein *in vitro* and *in vivo*, and that HsCdc7-HsDbf4 kinase activity is required for the initiation of DNA replication in mammalian cells. Future work will focus on defining how HsDbf4 activates and interacts with HsCdc7, identifying the HsCdc7-HsDbf4 phosphorylation sites in MCM2 and determining the role of phosphorylation of MCM2 in the initiation of replication, which should provide important insights into the mechanisms that control the initiation of DNA replication in mammalian cells.

Materials and methods

Identification and cloning of HsDbf4

A yeast two-hybrid screen, carried out as described previously (Hannon *et al.*, 1993), was used to isolate cDNA encoding proteins that interact with HsCdc7. Briefly, a *SalmI-SallI* cDNA fragment encoding full-length HsCdc7 was subcloned into plasmid pAS2 in-frame with the yeast GAL4 DNA-binding domain to generate the pAS2-HsCdc7 bait plasmid. *Saccharomyces cerevisiae* Y190 cells were transformed with the pAS2-HsCdc7 bait plasmid to produce a stable strain. The Y190-pAS2-HsCdc7 strain was then transformed with a pGADGH HeLa cDNA library which expresses various proteins fused with the GAL4 transcription activation domain. Of 30 000 colonies screened, 19 showed specific interaction with HsCdc7; by sequence analysis these clones were found to correspond to four distinct genes. One of them encoded a protein that revealed a limited sequence homology to the budding yeast Dbf4p, designated HsDbf4. To obtain full-length HsDbf4 cDNA, a HeLa cDNA library constructed in λGEX5 was screened using the cDNA fragment from the two-hybrid screen as a probe. A screen of 10⁶ phage plaques yielded 20 positive clones. DNA sequences of these clones were determined using the dideoxy-sequencing method. Two clones contained the entire open reading frame (ORF), and conceptual translation of full-length HsDbf4 cDNA reveals a 674 deduced amino acid ORF. The original two-hybrid clone also proved to contain the entire HsDbf4-coding region.

Antibodies

Polyclonal antibodies were developed in rabbits against a synthetic peptide, CADNLLTAFFSSPSTSTFTGF, corresponding to the C-terminal

residues 655–674 of HsDbf4 coupled to keyhole limpet hemocyanin via the added N-terminal Cys. Polyclonal antibodies against HsDbf4 were purified from the serum of rabbits #6097 and #6098 using a Sepharose 4B affinity column coupled to the HsDbf4 synthetic peptide (Jiang and Hunter, 1997). Because affinity-purified anti-HsDbf4 antibodies had a low titer, they were not useful for immunoneutralization experiments. Rabbit polyclonal antibodies against human HsCdc7 and the anti-hemagglutinin (HA) 12CA5 monoclonal antibody were used as described (Jiang and Hunter, 1997). Rabbit polyclonal antibodies against MCM-DEFD peptide, *Xenopus* MCM3, -4 and -7, which cross-react with their human homologs and mouse monoclonal antibody against human MCM2 were kindly provided by Drs R.Knippers (Konstanz, Germany), L.Sun and J.Newport (UCSD, San Diego, USA), and K.Stoeber and R.Laskey (University of Cambridge, UK), respectively. Mouse monoclonal anti-human MCM2 and anti-BrdU antibodies were purchased from Transduction Laboratories and Harlan Sera-Laboratories, respectively.

Expression and purification of recombinant HsCdc7 and HsDbf4 in insect cells

The *NcoI*–*XhoI* cDNA fragments containing either the Myc-tagged HsCdc7 or HsCdc7(K-R) mutant (Jiang and Hunter, 1997) and the *XhoI*–*XhoI* cDNA fragment containing the HA-tagged HsDbf4 coding sequence were subcloned into the pBlueBacHis-2 baculovirus vector. Production of recombinant HsCdc7, HsCdc7(K-R) and HsDbf4 baculoviruses in Sf9 cells and purification of the recombinant proteins in Hi5 insect cells were performed as described previously (Jiang *et al.*, 1998).

Cell culture, expression plasmids, transfection and cell-cycle analysis

The 293, HeLa and U2OS (obtained from ATCC) human cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). To generate His-tagged MCM2 proteins, fragments encoding full-length MCM2 and residues 1–285 were amplified from an *NdeI*–*BamHI* fragment derived from a human MCM2 cDNA by PCR using Pfu polymerase, and subcloned into the pET28a bacterial expression vector. To express HsCdc7 and HsDbf4 in mammalian cells, the *SmaI*–*SmaI* full-length HsDbf4 cDNA was subcloned into the pREVF mammalian expression vector. All PCR products were verified by DNA sequencing. The pCS3HsCdc7 plasmid has been described previously (Jiang and Hunter, 1997). Cell transfections were carried out using the calcium phosphate precipitation method as described by Jiang and Hunter (1997). Cell-cycle synchronization and flow-cytometric analysis were performed as described previously (Jiang and Hunter, 1997).

Immunoblotting, immunoprecipitation and protein kinase assays

Cells were lysed in NP-40 lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM DTT, 10 U/ml aprotinin, 20 µg/ml leupeptin and 10% glycerol. Standard protocols for protein extraction, immunoprecipitation, immunoblotting and kinase assay were used as described previously (Jiang and Hunter, 1997). Where indicated affinity-purified antibodies were blocked by preincubation with peptide immunogen (1 µg of peptide per µg antibody). The nuclear and chromatin fractionation was performed as described previously (Fujita *et al.*, 1997). In brief, HeLa cells were lysed with mCSK buffer (Fujita *et al.*, 1997), fractionated and the insoluble nuclear and chromatin fraction was then solubilized with DNase I (1000 U/ml) in the presence of 1 mM ATP, prior to immunoprecipitation of the MCM complex with anti-MCM7 antibodies.

In vivo metabolic labeling, phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping

For *in vivo* metabolic labeling, asynchronous HeLa were washed once with phosphate-free medium and then incubated with fresh phosphate-free medium supplemented with 10% dialyzed calf serum and 2 mCi/ml ³²P-orthophosphate for 4 h. PAA analysis and two-dimensional analysis of tryptic phosphopeptides were performed as described (Boyle *et al.*, 1991).

Antibody microinjection and immunofluorescence staining

HeLa cells were grown on glass coverslips for 1–2 days and mitotic cells were injected with affinity-purified anti-HsCdc7 antibodies (3 mg/ml) or anti-HsCdc7 antibodies (3 mg/ml) that had been prebound to the peptide antigen (2 mg/ml) using a semiautomatic micromanipulator/injector (Eppendorf micromanipulator 5172 and microinjector 5242). After injection, coverslips were placed into fresh medium containing 10 µM BrdU

for 16 h before fixation. Immunofluorescence staining was performed as described by Jiang and Hunter (1997).

Accession number

The HsDbf4 sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank database under accession No. AF160249.

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