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CDC7 KINASE COMPLEX AS A MOLECULAR SWITCH FOR DNA REPLICATION

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1. ABTRACT

Cdc7 kinase and its activator Dbf4 protein, originally identified in budding yeast *Saccharomyces cerevisiae*, are widely conserved in eukaryotes including fission yeast and human. Dbf4-related activators bind and stimulate kinase activity of Cdc7-like catalytic subunit. Its kinase activity is cell cycle-regulated, mainly through availability of the activation subunit whose level increases at G1/S boundary and is maintained at a high level throughout S phase. MCM2 protein is among physiologically important substrates. Genetic studies in fission yeast indicate that Cdc7-related kinase complex also functions in meiosis, uninduced mutagenesis, DNA replication checkpoint signaling and maintenance of chromatin structures during S phase.

2. INTRODUCTION

It is well established mainly from the extensive studies of bacterial DNA replication that the major step of regulation of DNA replication occurs at the initiation (1). Once replication is initiated, DNA chains are elongated at a relatively constant rate until the entire genome is replicated. Therefore, rate of genome replication is regulated by frequency of origin firing which alters in response to nutritional conditions and other environmental factors. In the normal course of replication of a bacterial genome, DnaA protein is the initiator which binds to the chromosomal origin (*oriC*) and activates its firing (2). The level of DnaA protein stays at a relatively constant level during the cell cycle, and its activity is regulated by association with nucleotide cofactors; ATP-bound or ADP-bound form as an active or inactive form, respectively (3). It is predicted that the ligand state of DnaA protein is regulated by sensing nutritional conditions to adjust frequency of origin firing in coordination with cell growth (4). To date, however, the nature of the activator(s) which triggers initiation of DNA replication by activating DnaA protein at each cell cycle remains unclear.

In eukaryotes, DNA replication is strictly regulated during cell cycle, occurring once and only once during S phase (5). A number of factors conserved in wide varieties of eukaryotes are known to play crucial roles in initiation and elongation stages of DNA replication. These include ORC (Origin Recognition Complex), MCM (Minichromosome Maintenance), Cdc6, Cdc45, RPA (Single-stranded DNA binding proteins), and DNA polymerases. Although assembly of a prereplicative complex (preRC) at an origin, which involves actions of ORC, Cdc6 and MCM proteins, is prerequisite for initiation of DNA replication (6,7), it is not sufficient for triggering of DNA replication. Genetic evidence from budding yeast *Saccharomyces cerevisiae* has indicated that initiation of DNA replication requires actions of at least two distinct serine/threonine kinases, namely Cdc28-Clb5, 6 and Cdc7-Dbf4 kinases (8,9). Since protein synthesis is no longer required for initiation of S phase after the function of Cdc7 is executed, it was predicted that Cdc7 acts at the stage immediately before or at least very close to the initiation of S phase (10). Furthermore, more recently, Cdc7 was shown to be required for activation of each replication origin on the chromosome (11,12). These pieces of evidence strongly point to the critical function of Cdc7 as a molecular switch for initiation of DNA replication.

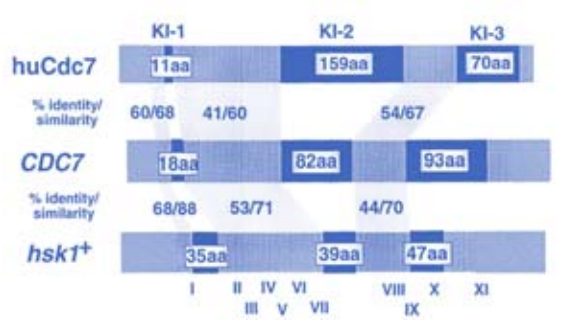

Figure 1. Schematic drawing of structures of Cdc7-related kinase catalytic subunits from human, budding yeast and fission yeast. Black and gray regions represent kinase inserts (KI-1, KI-2 and KI-3) and kinase conserved domains, respectively. Figures between the bars indicate % identity and similarity of each conserved region between the two proteins. Roman figures indicate the kinase domains as defined by Hanks et al. (51).

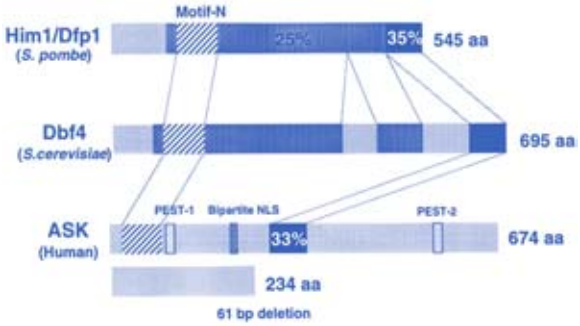

Figure 2. Schematic drawing of structures of Cdc7-related kinase regulatory subunits from fission yeast, budding yeast and human Motif-C's of Him1/Dfp1 and ASK share 35 % and 33% identity, respectively, with that of Dbf4 protein. An alternative spliced form of ASK, a 234 amino acid-long C-terminally truncated polypeptide due to 61 bp insertion, is also shown (19).

In spite of strong genetic evidence for essential role of Cdc7-Dbf4 kinase in initiation of S phase in budding yeast (13,14), it has not been clear whether DNA replication in higher eukaryotes is regulated by related kinases. We and others have demonstrated the presence of kinase complexes related to Cdc7-Dbf4 in other eukaryotes including fission yeast, *Xenopus* and mammals (15-20). Available genetic and biochemical evidence is consistent with the notion that Cdc7 phosphorylates essential components of replication complexes to trigger firing of replication origins, and regulates replication checkpoint control as well as chromatin structures during S phase.

3. IDENTIFICATION OF Cdc7-Dbf4-RELATED KINASE COMPLEXES IN EUKARYOTES OTHER THAN BUDDING YEAST

A kinase related to Cdc7 was first identified in fission yeast *Schizosaccharomyces pombe* on the basis of structural similarity (15). The isolated gene *hsk1*$^+$ was shown to be essential for initiation of S phase, thus strongly suggesting that *hsk1*$^+$ is the functional homologue of Cdc7 in fission yeast. We and others further isolated cDNA's encoding Xenopus, mouse and human Cdc7-related kinases, suggesting conservation of regulatory mechanisms of DNA replication by this family of kinases (16-18). In order to isolate putative homologues of Dbf4, two-hybrid screening was conducted and him1+ (Hsk1 interacting molecule 1) and ASK (Activator of S phase Kinase) was identified, and were subsequently shown to be regulatory subunits for Hsk1 and huCdc7, respectively (19,20). him1+ turned out to be identical to dfp1+ described by Brown and Kelly as a subunit for Hsk1 protein (22). HsDbf4 was more recently described and was shown to be identical with ASK (23). Furthermore, nim0 of Aspergillus, originally identified as a mutant defective in DNA replication, was shown to encode a Dbf4-like protein (24). Thus, Cdc7-Dbf4-related kinase complexes are likely to be conserved in all the eukaryotes (table 1).

3.1. Conserved motifs in Cdc7 regulatory subunits

The primary sequences of the catalytic subunit of Cdc7 kinase complexes are conserved, and identity between mammalian and fission yeast Hsk1 is about 45 % in the kinase conserved domain (16,17). Cdc7 is unique in that it contains two or three stretches of amino acids inserted within the conserved kinase domains (figure 1). These "kinase insert" sequences are more diverged and appear to be involved in species-specific interaction with cognate regulatory subunits. Although Dbf4 and its functional homologues in other eukaryotes are functionally similar to cyclins in terms of kinase activation of the catalytic subunit and periodic appearance during cell cycle, their primary structures bear no similarity to known cyclin molecules. The degree of conservation of amino acid sequences between different species is much lower in the regulatory subunits. Overall identity between budding yeast Dbf4 and fission yeast Him1/Dfp1 is 25 % and almost no similarity was detected between Dbf4 and ASK except for two small stretches of amino acids, motif-N and motif-C, which are conserved in all the Cdc7 regulatory subunits identified so far (figure 2; 19,20). Analysis of deletion derivatives of Him1/Dfp1 showed that motif-C is essential for viability as well as for full kinase activation, whereas motif-N is dispensable for mitotic functions. This is consistent with earlier report that a C-terminal region of budding yeast Dbf4 containing motif-C is essential and can interact with Cdc7 in the two-hybrid assay (14,21). We found that deletion of motif-N results in loss of checkpoint functions as well as in sensitivity to DNA damages (20; Ogino *et al.,* unpublished results; see below). It is intriguing that motif-N, which share some conserved residues of BRCT (BRCA C-terminal domain) motif (25), is present also in fission yeast Cut5/Rad4 protein essential for both initiation of DNA replication and S/M and DNA damage checkpoint responses (26,27).

3.2. Conservation of physiological functions of Cdc7-related kinases

The original *cdc7* and *dbf4* temperature-sensitive mutants arrest with dumbbell forms containing 1C DNA content at a non-permissive temperature, suggesting their essential roles in G1/S transition (8,28). Fission yeast *hsk1*$^+$ and *him1*$^+$/*dfp1*$^+$ are essential for viability and null mutants arrest with 1C DNA content. Characterization of a newly isolated *hsk1* temperature-sensitive mutant also indicates

Table 1. Cdc7-related kinase complexes identified in various eukaryotes

Species	Catalytic subunit	Regulatory subunit	*In vitro* substrates
S. cerevisiae	Cdc7(507 aa)	DBDF4(695 aa)	MCM2, 3, 4, 6
S. pombe	hsk1$^+$(507 aa)	him1$^+$ Idfp1$^+$I rad35$^+$(545 aa)	DNA polα p180 SpMCM2ÅiCdC19Åj
Xenopus	XeCdc7 (483 aa)	unidentified	
Mammals (human and mouse)	huCdc7 (574 aa) muCdc7(564 aa)	huASK (674 aa) muASK(663 aa)	huMCM2, MCM4 and MCM6 ORC4, 5 Geminine SV40Tag BPVE1

that Hsk1 is essential for initiation of S phase (Takeda *et al.*, submitted for publication). Antibodies against *Xenopus* Cdc7 blocked DNA replication *in vivo* and *in vitro* (29). In order to dissect roles played by mammalian Cdc7-related kinase, two approaches were taken. First, specific antibodies against ASK were microinjected into fibroblast cells, and its effect on cell cycle progression was examined. Two independent antibodies inhibited DNA synthesis when injected into human primary fibroblast KD cells in G1 state. The DNA replication was restored when the antigen was coinjected with antibody. The results strongly indicate that ASK functions are essential for initiation of S phase in mammalian cells (19). Secondly, in our attempt to generate mutant mice which lack both alleles of the muCdc7 genes to assess the functions of mammalian Cdc7 catalytic subunit on cellular and animal levels, we discovered that homozygous null mutant mice were not generated, while we were able to obtain heterozygous mice lacking one allele of muCdc7. Examination of embryos indicated that homozygous null embryos died between day 3.5 and 6.5, indicating that muCdc7 functions are essential for early embryonic development or for cell proliferation per se or for both. However, inability to generate ES cells with homozygous null genotype in the absence of a trans-gene expressing the wild-type muCdc7 suggests that muCdc7 is essential for cell proliferation itself (Kim *et al.*, unpublished results). A recent report that microinjection of anti-Cdc7 antibody inhibits DNA synthesis in human cells also supports our conclusion (23). Taken together, mammalian Cdc7-related kinase complexes play pivotal roles in cell cycle progression, most likely in S phase initiation, as was discovered in yeasts.

4. REGULATION OF Cdc7 KINASE

4.1. Expression during cell cycle and in tissues

Expression of Cdc7 catalytic and regulatory subunits was examined in fission yeast and mammalian cells. In both organisms, expression of regulatory subunits (him1+/dfp1+ and ASK) oscillates during cell cycle both at mRNA and protein levels. The protein level of the regulatory subunit increase at late G1/S boundary and is kept at a high level all through S phase, whereas that of catalytic subunits is relatively constant during cell cycle (figure 3; 19,20). Expression of both catalytic and regulatory subunits for mammalian Cdc7 decreases after arrest by serum depletion, and increases in response to growth stimulation by addition of serum or growth factors (17,19). Cdc7-dependent kinase activity also fluctuates during cell cycle as a result of oscillation of the level of the regulatory subunit. In fission yeast, the level of Him1/ Dfp1 protein is very low in cells arrested at START in cdc10 mutant, and increases in cells arrested at S phase in cdc22 or in cdc19 mutants. Since the level of him1+/dfp1+ mRNA at START is relatively high, Him1 protein may be actively degraded during M/ G1 phase (19,30). Him1/Dfp1 protein expressed ectopically from constitutively active promoter is also unstable during M/ G1 phase, supporting post-transcriptional control of the protein abundance (30). Furthermore, APC-dependent degradation of budding yeast Dbf4 protein was recently reported (31,32).

Analysis of the promoter region for muCdc7 and human ASK revealed the presence of multiple putative E2F and Sp1 binding sites and indicated that the 230 or 290 bp promoter-proximal region, respectively, is sufficient to confer serum responsiveness in each promoter (17; Yamada *et al.*, unpublished data). The promoter region of *him1*$^+$ does not contain perfect matches to *Mlu*I box sequences, which are recognized by Cdc10/Res1-Res2 transcription factor and are known to play crucial roles in cell cycle-dependent expression in yeasts (33,34). Consistent with it, the transcription of *him1*$^+$ is not dependent on Cdc10 transcription factor.

huCdc7 mRNA is expressed more or less ubiquitously in various tissues including brain (16). ASK mRNA is expressed in most tissues examined except for brain and kidney. Both huCdc7 and ASK are expressed at a high level in testis. ASK is particularly abundant in testis and mRNA of different sizes are also detected (19). Genetic characterization of budding yeast Cdc7 and fission yeast Hsk1 suggested critical roles of Cdc7 kinase in the process of meiosis (35,36;Takeda *et al.*, unpublished data). It remains to be characterized how Cdc7 regulates meiosis. ASK is generally expressed at a high level in various cancer cell lines (19). Overexpression of huCdc7 mRNA in some thyroid tumors was also reported (37). Further investigation is needed to draw any conclusions on possible involvement of Cdc7/ASK in carcinogenesis.

4.2. Cell cycle regulation of Cdc7 kinase activity

We have shown that huCdc7 kinase activity is high during S phase, when ASK expression is high, and decreases in G2 and G1 as ASK protein level diminishes

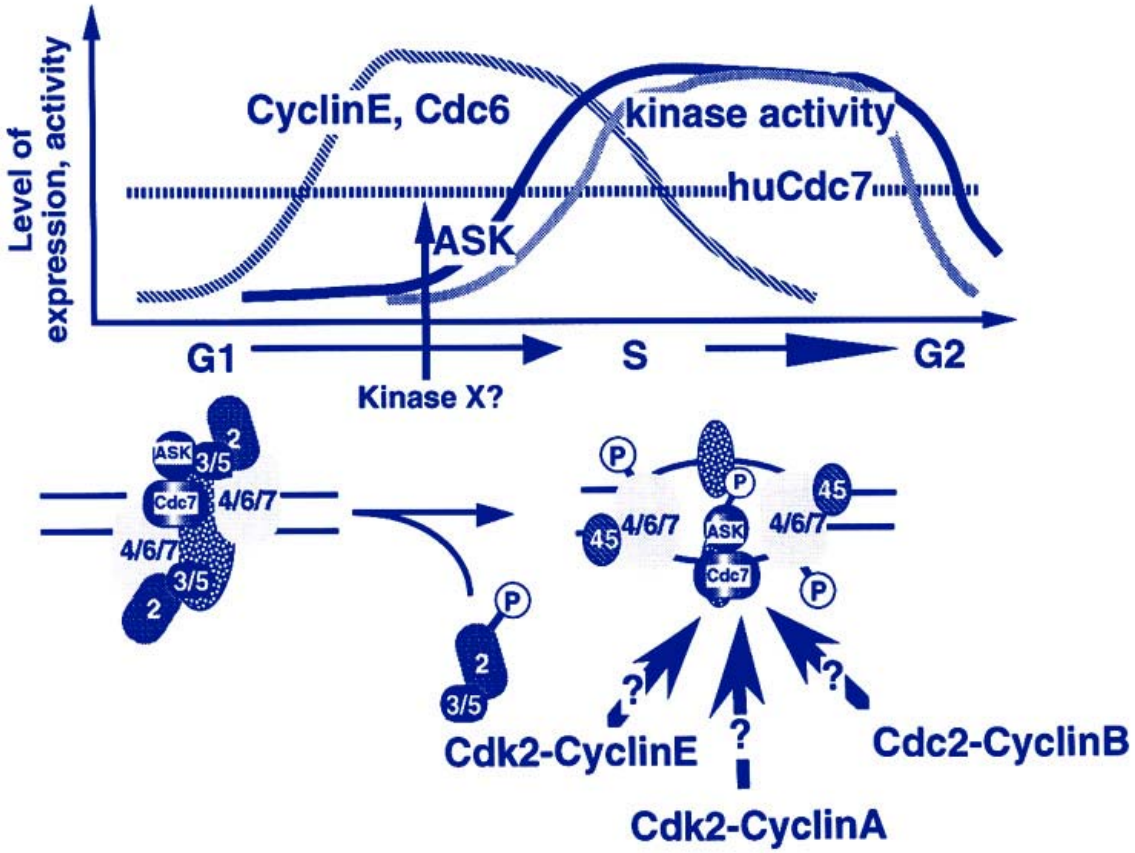

Figure 3. Cell cycle regulation of huCdc7 and ASK protein levels as well as huCdc7 kinase activity and possible mode of origin activation by Cdc7. Kinase X represents an unknown kinase which may phosphorylate threonine 376 of huCdc7 for possible activation of the kinase activity. The lower figure indicates that huCdc7-ASK present in the prereplication complex may trigger origin activation (duplex unwinding) by activating MCM complex. A possibility that inhibitory MCM2 and 3/5 subunits may be removed upon phosphorylation of MCM2 is described, although this is at present hypothetical.

(19). Although the kinase activity of Cdc7 closely parallels with the level of regulatory subunit, additional regulation by modification may exist. In *Saccharomyces cerevisiae*, substitution of the conserved threonine at position 281, corresponding to the activation threonine of CDK, with alanine but not with serine results in impaired function (36,38). A similar mutant of huCdc7 shows significantly reduced kinase activity in vitro, indicating that Cdc7 activity may be regulated by phosphorylation of this conserved threonine (Masai *et al*., unpublished data).

Cdc7 kinase complexes autophosphorylate both subunits *in vitro*, and phosphorylation of the regulatory subunits is detected specifically during S phase which is probably caused by autophosphorylation due to increased kinase activity during S phase (20). Both catalytic and regulatory subunits are localized in chromatin-enriched fractions, although they are not readily extractable by nuclease treatment, suggesting association of the Cdc7 kinases with nuclear structures (20). Association of Cdc7-Dbf4 proteins with chromatin during S phase was recently reported in budding yeast (39,40).

5. TARGETS OF Cdc7 KINASE

One-hybrid interaction assays indicated that *Saccharomyces cerevisiae* Dbf4 protein associates with the chromosomal origins (41). Evidence for genetic interaction of *DBF4* with components of replication complexes at the origins as well as biochemical evidence for its association with chromatin suggests that the targets of Cdc7 kinases are present within the replication complexes on the chromosomes. *In vitro* phosphorylation experiments first indicated that components of MCM protein complexes may be important substrates of Cdc7 (16). Genetic and biochemical evidence in budding yeast showed that MCM2 is the physiologically important substrate of Cdc7-Dbf4 kinase complex (42). In fission yeast, purified Hsk1 kinase complex phosphorylated specifically MCM2 protein in the purified MCM complex (22). Hyperphosphorylated forms of MCM2 are detected specifically during S phase in yeasts as well as in mammalian cells (42; Takeda et al., Cho et al., unpublished results) and this phosphorylation appears to be mediated by Cdc7 in vivo. A bypass suppressor of S. cerevisiae cdc7 mutations was mapped on MCM5 protein, lending genetic

support of Cdc7-MCM interaction (42). The purified huCdc7-ASK complex phosphorylated MCM2 protein in vitro to a significant level in the purified MCM2/4/6/7 complex. Multiple sites on MCM2 are phosphorylated by huCdc7. MCM4 and 6 were also phosphorylated by huCdc7 albeit to much less extent (Masai et al., manuscript in preparation). In vitro phosphorylation assays showed that other components of putative replication complexes, including Orc4, Orc5, and geminine, as well as viral initiators SV40 T antigen and bovine papilloma virus E1 protein can also be phosphorylated by purified huCdc7 kinase complex, although physiological significance of these phosphorylation events is unclear (our unpublished results).

How does Cdc7 activate prereplicative complexes by phosphorylating their components? This is still an open question. Although the MCM complex is an essential component for preRC, its precise functions in initiation of DNA replication are not clear. Association of MCM components with moving replication forks (43) as well as discovery of helicase activity in the MCM4/6/7 subassembly (44) suggests a possibility that MCM may play critical roles at the replication fork as a part of DNA helicase. It is tempting to speculate that huCdc7 regulates essential functions of the MCM through phosphorylation of its components. MCM2 was reported to inhibit the helicase activity of MCM4/6/7 complex *in vitro* (45), and phosphorylation of MCM2 and other subunits may potentiate the helicase activity of MCM by facilitating reorganization of MCM assemblies at the origins. We have found that huCdc7-mediated phosphorylation of MCM2 *in vivo* resulted in its relocation to chromatin-unbound fractions (Sato *et al*., unpublished data). It is also possible that interactions with other replication proteins are affected by phosphorylation of MCM proteins, which may lead to formation of replication complexes capable of inducing DNA unwinding at the origins.

6. GENETIC ANALYSES OF Cdc7 KINASES IN YEASTS

6.1. Roles in meiosis and induced mutagenesis

Characterization of budding yeast *cdc7*(ts) mutants indicated its roles in meiosis and induced mutagenesis. *cdc7*(ts)/*cdc7*(ts) diploid cells fail to form ascospores at a semipermissive temperature, and this defect is caused by failure to commit to gentic recombination and formation of synaptonamal complexes (35,36). *cdc7*(ts) mutants exhibit varying degrees of mutability in response to DNA damaging agents (46). Cdc7 kinase activity may be required for these cellular processes as well, as judged from the analyses of various mutant Cdc7 proteins. However, the downstream events, e.g phosphorylation of substrates, may be quite different in these processes compared to those in the mitotic pathways.

6.2. Roles in DNA replication checkpoint control

Genetic analyses of fission yeast Hsk1 kinase revealed novel functions associated with Cdc7 kinases. A temperature-sensitive mutant of *hsk1*, *hsk1-89*, was isolated and its phenotype was analyzed in detail (Takeda *et al*., manuscript submitted). *hsk1-89* showed temperature-sensitive phenotype, being unable to generate colonies at temperatures above 30°C, due to three amino acid substitutions in the C-terminal kinase conserved domains which resulted in highly attenuated kinase activity. *hsk1-89* exhibited mainly initiation defect at 30°C, generating 1C DNA cells in nearly half the population at 2 hour. Further incubation at this temperature lead to appearance of cut cells with <1C DNA content, indicative of loss of replication checkpoint control. Theses defects are extremely enhanced when *hsk1-89* is combined with *cdc19-P1*, a mutant of fission yeast homologue of MCM2 (47). S phase specific phosphorylation of Cdc19 was abrogated in *hsk1-89* mutant, strongly suggesting that Hsk1-mediated phosphorylation of Cdc19 is essential for initiation of S phase. The checkpoint defect was extremely enhanced in combination with *rad3* mutation. The cells viability was significantly reduced in *hsk1-89 rad3* double mutant, with significant increase of the population of small cut cells. The impairment of Rad3-dependent checkpoint responses in *hsk1-89* was indicated by significant reduction of Cds1 kinase activation in response to HU in the mutant. We conclude that Hsk1 kinase may be involved in DNA replication checkpoint responses. Similar checkpoint regulation of Dbf4 and Genetic and two-hybrid interactions between CDC7-DBF4 and RAD3, the *cds1*$^+$ homologue of budding yeast, were also recently reported (40, 48).

6.3. Roles in maintenance of chromatin structures during S phase

Unexpectedly, *hsk1-89* accumulated near 2C cells at 37°C and exhibited aberrant nuclear structures including fragmented or unequally segregated chromosomes. This phenotype was specifically enhanced when *hsk1-89* was combined with *rad21-K1*, a temperature-sensitive mutant of the fission yeast homologue of cohesin essential for sister chromatid cohesion during S phase (49). Similar phenotype was observed in *rad21-K1* mutant at a non-permissive temperature. *rad21-K1* is sensitive to thiabendazole (TBZ), since defect of sister chromatid cohesion affect microtubule functions. Growth of *hsk1-89* is sensitive to TBZ to the extent similar to that of *rad21* mutant, in agreement with loss of cohesion functions in *hsk1-89*. We speculate that infrequent origin firing in *hsk1-89* results in insufficient establishment or maintenance of sister chromatid cohesion during S phase, which eventually leads to defective mitosis and apparent defect of S/M transition. Dependence of sister chromatid cohesion on DNA replication has recently been suggested in *S. cerevisiae* (50). We propose a possibility that sister chromatid cohesion may be coupled with firing of replication origins by Hsk1 kinase activity

7. CONCLUSIONS

Cdc7-related kinase complexes are conserved from yeasts to human, and they play conserved essential functions for initiation of DNA replication. The kinase activity of Cdc7 depends on Dbf4-like regulatory subunits and increases at G1/S boundary through S phase, when the level of the regulatory subunit increases. MCM2 protein within the MCM complex is likely to be a physiologically

important target of Cdc7. Genetic analyses in yeasts indicated roles of Cdc7 kinase in meiosis and induced mutagenesis as well as in DNA replication checkpoint control and maintenance of proper chromatin.

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