A Novel Intermediate in Initiation Complex Assembly for Fission Yeast DNA Replication

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Assembly of initiation factors on individual replication origins at onset of S phase is crucial for regulation of replication timing and repression of initiation by S-phase checkpoint control. We dissected the process of preinitiation complex formation using a point mutation in fission yeast *nda4-108/mcm5* that shows tight genetic interactions with *sna41⁺/cdc45⁺*. The mutation does not affect loading of MCM complex onto origins, but impairs Cdc45-loading, presumably because of a defect in interaction of MCM with Cdc45. In the *mcm5* mutant, however, Sld3, which is required for Cdc45-loading, proficiently associates with origins. Origin-association of Sld3 without Cdc45 is also observed in the *sna41/cdc45* mutant. These results suggest that Sld3-loading is independent of Cdc45-loading, which is different from those observed in budding yeast. Interestingly, returning the arrested *mcm5* cells to the permissive temperature results in immediate loading of Cdc45 to the origin and resumption of DNA replication. These results suggest that the complex containing MCM and Sld3 is an intermediate for initiation of DNA replication in fission yeast.

INTRODUCTION

Initiation of DNA replication in eukaryotic cells is regulated through an ordered assembly of replication complexes at replication origins (reviewed in Kelly and Brown, 2000; Bell and Dutta, 2002; Kearsey and Cotterill, 2003). Replication origins are recognized and bound by the origin recognition complex (ORC). In the G1 phase of the cell cycle, MCM (minichromosome maintenance) complex composed of Mcm2-7 associates with replication origins depending on ORC, Cdc6/Cdc18, and Cdt1, forming a prereplicative complex (pre-RC). At the onset of S phase, several factors, including Cdc45 and Sld3, associate with pre-RC to form a preinitiation complex (pre-IC), which is capable of origin-unwinding, and promoting assembly of replication forks at replication origin.

The MCM complex is considered to be involved in unwinding of replication origins because a heterohexameric complex composed of Mcm4, 6, and 7 has a DNA helicase activity in vitro (Ishimi, 1997; Lee and Hurwitz, 2001). However, the helicase activity has not been detected with the Mcm2-7 heterohexamer, which seems to be the intrinsic MCM complex in cells. Thus, modification of the MCM complex and/or its association with other factors might be required for activation.

Cdc45 plays a crucial role in activation of replication origins. In *Xenopus* egg extracts, Cdc45 is required for unwinding of template DNA (Walter and Newport, 2000). S phase cyclin-dependent kinase (S-CDK) and Cdc7-Dbf4 kinase (Dbf4-dependent kinase [DDK]) are necessary for assembly of Cdc45 with pre-RC (Mimura *et al.,* 2000; Zou and Stillman, 2000). Because some MCM subunits are efficient substrates for CDK or DDK in vitro (Sato *et al.,* 1997; Fujita

et al., 1998), their phosphorylation might have roles in assembly of Cdc45 with pre-RC. Furthermore, several factors such as Sld3, Dpb11, Mcm10, and the GINS complex are shown to be required for chromatin loading of Cdc45 (Masumoto *et al.,* 2000; Kamimura *et al.,* 2001; Van Hatten *et al.,* 2002; Wohlschlegel *et al.,* 2002; Gregan *et al.,* 2003; Kubota *et al.,* 2003; Takayama *et al.,* 2003). *SLD3* was initially isolated as a synthetic lethal gene with *dpb11-1* in budding yeast and it exhibits a tight genetic interaction with *CDC45* (Kamimura *et al.,* 1998). Coimmunoprecipitation of Sld3 with Cdc45 depending on cross-linking was observed throughout the cell cycle and association of Sld3 and Cdc45 with origins depended on each other (Kamimura *et al.,* 2001). In fission yeast, however, Cdc45 was coimmunoprecipitated with Sld3 without cross-linking selectively in S phase (Nakajima and Masukata, 2002). The mechanism of Cdc45-loading in either organism has yet to be elucidated.

In budding yeast, the *CDC46/MCM5* gene has been identified as an extragenic suppressor for *cdc45-1* (Hennessy *et al.,* 1991). In fission yeast, in turn, the defects of the *nda4-108/mcm5* mutation are suppressed by a mutation in the *sna41/cdc45* gene (Miyake and Yamashita, 1998). The genetic interactions conserved among these species suggest that Mcm5 plays an important role in the interaction of MCM with Cdc45. Furthermore, the *bob1-1/mcm5* mutation in budding yeast bypasses requirement of DDK for DNA replication (Hardy *et al.,* 1997). The *bob1-1/mcm5* mutation allows loading of Cdc45 onto origin in the absence of DDK and structural changes of origin DNA in the α-factor arrested G1 cells (Geraghty *et al.,* 2000; Sclafani *et al.,* 2002). These findings are consistent with involvement of Mcm5 in the interactions between MCM and Cdc45.

Replication origins are likely to contain information required to promote DNA replication, because replication initiates from fixed regions in almost all eukaryotes. Structures of replication origins are well understood in budding yeast. Budding yeast origins are composed of modular elements within a 100–200-base pair region. A consensus sequence, designated as the ARS consensus sequence (ACS), is essen-

tial for origin function (Marahrens and Stillman, 1992). The ACS is the binding site for ORC complex (Bell and Stillman, 1992) and the pre-RC is formed in the vicinity of ACS (Diffley *et al.*, 1994). On the other hand, fission yeast, similar to higher eukaryotes, requires a long chromosome region as a replication origin. Short essential consensus sequences like the budding yeast ACS have not been found. Instead, stretches of asymmetrically placed adenine/thymine residues are essential for the origin activity on both the ARS plasmid (Clyne and Kelly, 1995; Dubey *et al.*, 1996; Okuno *et al.*, 1999) and the chromosome (Takahashi *et al.*, 2003). Adenine/thymine stretches are the binding sites for ORC both in vitro (Kong and DePamphilis, 2001; Lee *et al.*, 2001; Takahashi and Masukata, 2001; Kong and DePamphilis, 2002) and in vivo (Kong and DePamphilis, 2002; Takahashi *et al.*, 2003). The *ars2004*, an efficient replication origin (Okuno *et al.*, 1997), contains three essential regions I, II, and III in about a 1-kb segment. The regions I and III, consisting of adenine stretches, are collectively required for the ORC binding, whereas the nonadenine region II stimulates loading of MCM. Interestingly, MCM in pre-RC is localized preferentially to a region near the initiation site, which is distant from ORC-binding sites (Takahashi *et al.*, 2003). Multiple ORC binding sites and a preferential MCM binding near the initiation site seem to be general organization of fission yeast replication origins, although MCM binds to the proximal region of one of the ORC binding sites in the *ars3001* (Kong and DePamphilis, 2002). Hence, the organization of fission yeast origins resembles those in multicellular organisms, such as the chorion amplification origin in *Drosophila*, which consists of multiple DmORC binding regions and a preferential initiation site (Austin *et al.*, 1999). These results suggest that fission yeast replication origins are good model system for understanding the complex replication origins in higher eukaryotes. Thus, it should be important to analyze assembly of replication components such as those in pre-IC on fission yeast origins.

In the present study, we examined molecular assembly of replication factors onto the replication origins in a fission yeast *nda4-108/mcm5* mutant. We show here that the *mcm5* mutation does not affect pre-RC formation but greatly reduces loading of Cdc45 onto origins. In contrast, Sld3 associates with origins in the *mcm5* mutant, suggesting independence from Cdc45-loading. Sld3 colocalizes with Mcm6 within the origin during the *mcm5*-arrest. Immediately after return to the permissive temperature from the *mcm5*-arrest, Cdc45 associates with origins and DNA replication initiates. Thus, the complex containing MCM and Sld3 but not Cdc45 appears to be a physiological intermediate for initiation of DNA replication.

MATERIALS AND METHODS

Strains and Media

The *Schizosaccharomyces pombe* haploid strains used are listed in Table 1. *h⁻ nda4-108*, *h⁻ nda1-376*, and *h⁻ nda3-KM311* were gifts from Dr. M. Yanagida (Kyoto University, Japan). Fission yeast strains were cultured in complete YE medium (0.5% yeast extract, 3% glucose) and minimal EMM medium (Moreno *et al.*, 1991). Media containing 2% agar were used for plating.

Cell Cycle Synchronization

To arrest the cell cycle in G1 phase, cells were incubated at 30°C for 32 h in EMM medium lacking nitrogen source but supplemented with 250 µg/ml leucine, and then the cell cycle was resumed by addition of a nitrogen source. For synchronization from G2/M boundary, *cdc25-22* and *cdc25-22 mcm5* cells were grown at 28°C to log phase and then incubated at 36°C for 3 h. The cell cycle was restarted at 20°C, the restrictive temperature for *mcm5*. The *nda3-KM311* and *sna41-928/cdc45 nda3-KM311* cells grown at 28°C were incubated

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Source
HM144	*h⁻ nda1-376*	Our stock
HM5	*h⁻ leu1-32 nda1-376*	M. Yanagida
YYY72	*h⁻ nda1-376 Δcig2::ura4⁺*	This work
YYY51	*h⁻ nda4-108*	Our stock
HM9	*h⁻ leu1-32 nda4-108*	M. Yanagida
YYY84	*h⁻ nda4-108 Δcig2::ura4⁺*	This work
YYY36	*h⁻ cdc19-P1*	S. L. Forsburg
YYY74	*h⁻ cdc19-P1 Δcig2::ura4⁺*	This work
HM130	*h⁻ cdc21-M68*	S. E. Kearsey
YYY75	*h⁻ cdc21-M68 Δcig2::ura4⁺*	This work
OGM1	*h⁻ mis5-268*	M. Yanagida
YYY86	*h⁺ mis5-268 Δcig2::ura4⁺*	This work
YYY164	*h⁺ orp1-4*	P. Nurse
YYY163	*h⁺ orp1-4 Δcig2::ura4⁺*	This work
OGC17	*h⁻ cdc18-K46*	H. Nishitani
YYY159	*h⁻ cdc18-K46 Δcig2::ura4⁺*	This work
YYY63	*h⁻ sna41-928*	Our stock
YYY67	*h⁻ nda4-108 sna41-928*	Our stock
HM65	*h⁻ leu1-32 sna41-928*	Our stock
HM59	*h⁻ cdc25-22*	Our stock
YYY32	*h⁻ nda4-108 cdc25-22*	This work
YYY97	*h⁻ FLAG-cdc45*	T. Uchida
YYY100	*h⁻ cdc25-22 flag-cdc45*	This work
YYY104	*h⁻ nda4-108 cdc25-22 flag-cdc45*	This work
YYY136	*h⁺ ura4-D18 cdc25-22 5flag-sld3(ura4⁺)*	R. Nakajima
YYY137	*h⁻ ura4-D18 nda4-108 cdc25-22 5flag-sld3(ura4⁺)*	This work
HM128	*h⁻ leu1-32 nda3-KM311*	M. Yanagida
YYY173	*h⁻ ura4-D18 nda3-KM311 5flag-sld3(ura4⁺)*	This work
YYY156	*h⁻ ura4-D18 sna41-928 nda3-KM311 5flag-sld3(ura4⁺)*	This work

The following are names of genes relevant to this study in *S. pombe* and in *Saccharomyces cerevisiae*: *S. pombe*: *nda1⁺/cdc19⁺*, *cdc21⁺*, *nda4⁺*, *mis5⁺*, *sna41⁺*; *S. cerevisiae*: *MCM2*, *MCM4*, *MCM5*, *MCM6*, *CDC45*.

at 20°C for 4 h to block the cell cycle at M phase and then released at 37.5°C, the restrictive temperature for *sna41-928/cdc45*.

Epitope Tagging of cdc45⁺

To express FLAG-Cdc45 protein, the recombinant *cdc45* gene carrying a FLAG epitope sequence between the first and second codons was made as described below. The N-terminal region of *cdc45⁺* was amplified by PCR from pSNA41 containing a 3072-base pair *Sau*3AI genomic fragment of the *cdc45⁺* (Nakajima and Masukata, 2002) using primers 5′-AACATATGGACTACAAGGAC-GACGATGACAAGTTCATCAAGAGATCCG-3′, containing an *Nde*I site and a FLAG epitope sequence, and 5′-AAGGATCCTCATTACTGGAATCGG-3′, and the product digested with *Nde*I and *Bam*HI was cloned into pUCTL1. The upstream region of the *cdc45⁺* gene was PCR-amplified from pSNA41 using primers 5′-GGAATTCCATATGTAATAAATAAACGTTACT-3′ and the M13 reverse primer. The *Pst*I-*Nde*I fragment containing the upstream of *cdc45⁺* and *Nde*I-*Sph*I fragment containing the FLAG-tagged N-terminal region of *cdc45* were cloned into *Pst*I-*Sph*I site of pBS-SNA41, resulting in an integration plasmid pFL-cdc45. A diploid strain *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 cdc45⁺/cdc45::ura4⁺* was transformed with the *Bst*BI-*Xba*I fragment of pFL-cdc45 and 5-fluoroorotic acid (5-FOA)-resistant transformants were selected. Integration of the *flag-cdc45* gene at the *cdc45⁺* locus was confirmed by PCR and immunoblotting. A haploid *cdc45⁺::flag-cdc45* strain was obtained by tetrad analysis. YYY97 *h⁻ cdc45⁺::flag-cdc45* without auxotrophy was used to construct YYY100 *h⁻ cdc25-22 flag-cdc45* and YYY104 *h⁻ nda4-108 cdc25-22 flag-cdc45* by mating with appropriate strains.

Immunoprecipitation and Immunoblotting

For immunoprecipitation of Mcm6, *S. pombe* haploid cells (5 × 10⁸ cells) were sequentially washed with cold water and HNG buffer (10 mM HEPES-NaOH [pH 7.5], 50 mM sodium acetate, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, 2 µg/ml leupeptin, 50 µg/ml TLCK, 2 µg/ml aprotinin) and then
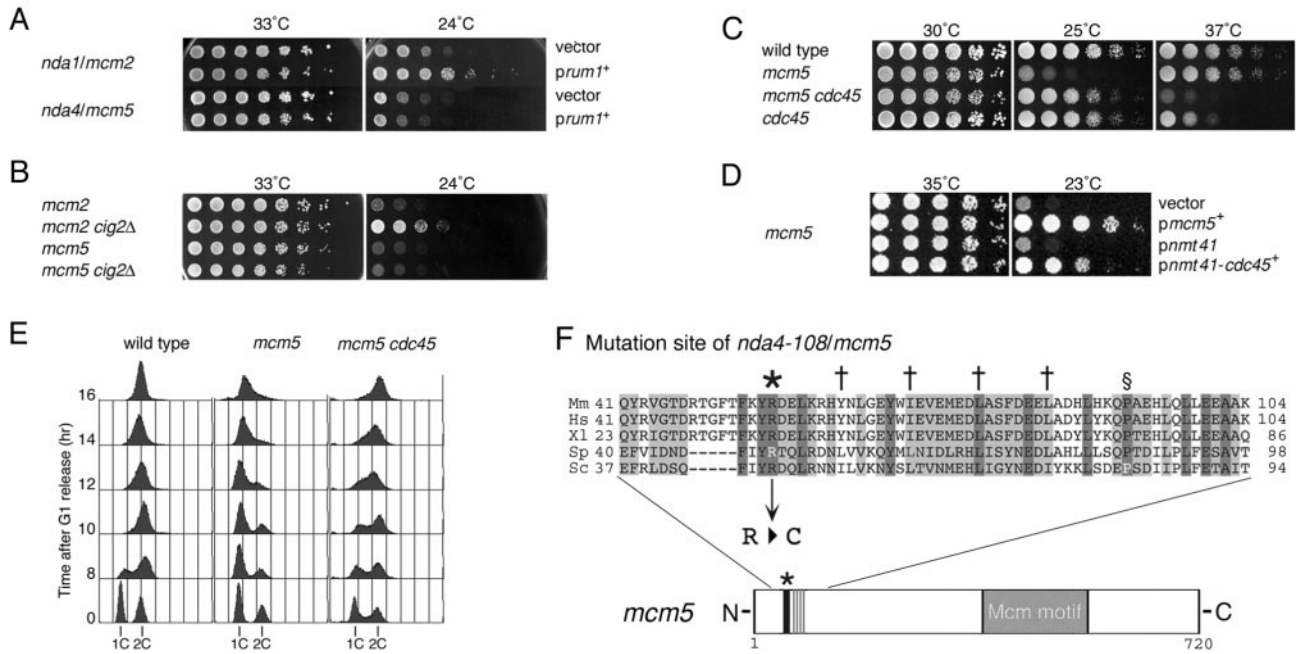
Figure 1. Genetic interactions between *mcm5*⁺ and *cdc45*⁺. (A) Suppression of *mcm2* and *mcm5* by multicopy *rum1*⁺. Five-fold serial dilutions of *nda1-376/mcm2 leu1* and *nda4-108/mcm5 leu1* cells carrying a vector or *rum1*⁺ plasmid were spotted onto EMM plates and incubated at permissive (33°C) and restrictive (24°C) temperatures. (B) Effects of *cig2*Δ on the *mcm2* and the *mcm5* mutations. Serial dilutions of *cig2*⁺ and *cig2*Δ derivatives of *mcm2* and *mcm5* cells were spotted onto YE plates as in A and their growth was examined. (C) Suppression of *mcm5* by the *cdc45* mutation. Growth of wild-type (972), *mcm5, sna41-928/cdc45*, and *mcm5 cdc45* cells was examined as in B. (D) Effects of overexpression of Cdc45 on *mcm5*. Growth of *mcm5 leu1* derivatives carrying vector (*ars2004-LEU2*), p*mcm5*⁺, p*nmt41*-vector and p*nmt41-cdc45*⁺ plasmids was examined as in A. (E) Effects of the *cdc45* mutation on DNA replication in the *mcm5* mutant. Wild-type (972), *mcm5*, and *mcm5 cdc45* cells arrested in G1 phase by culturing at 30°C in EMM medium lacking a nitrogen source were released at 20°C by adding a nitrogen source, and their DNA contents were analyzed by flow cytometry. Positions of 1C and 2C DNA are indicated. Minor peaks at 2C DNA content at 0 h correspond to cells arrested in G2 phase under conditions used. (F) Site of the *nda4-108/mcm5* mutation. Amino acid sequences of N-terminal regions of Mcm5 of mouse (Mm), human (Hs), *Xenopus laevis* (Xl), *S. pombe* (Sp), and *Saccharomyces cerevisiae* (Sc), respectively, are aligned for maximum homology. Identical amino acids are shaded and similar amino acids are boxed in gray. The asterisk indicates the position of the amino acid altered by the *nda4-108* mutation. Leucine residues in a putative leucine-zipper motif and the mutation site for the *S. cerevisiae bob1-1/mcm5* are indicated by † and §, respectively (Hardy *et al.*, 1997).

suspended in 0.4 ml of HNG buffer. Cells were disrupted with acid-washed glass beads by a bead beater (45 s, 6 times with 2-min intervals). Total cell extracts were obtained by centrifugation at 15,000 rpm for 10 min. For immunoprecipitation, the extracts were incubated at 4°C for 1.5 h with 20 μl of 10% (wt/vol) protein A-Sepharose (Sigma-Aldrich, St. Louis, MO) preincubated with rabbit anti-Mcm6 antibody. The Sepharose beads were washed five times with cold HNG buffer and then suspended in an equal volume of SDS-sample buffer (120 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol).

For immunoprecipitation of Cdc45, haploid cells (3 × 10⁸ cells) were washed with cold water and EB buffer (25 mM HEPES-NaOH [pH 7.5], 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 0.25% Triton X-100, 1 mM PMSF, 2 μg/ml leupeptin, 50 μg/ml TLCK, 2 μg/ml aprotinin) and then suspended in 0.5 ml of EB buffer. After cells were disrupted with glass beads, the extracts were incubated with 70 U of DNaseI (TAKARA) at 4°C for 30 min and then centrifuged at 15,000 rpm for 30 min to obtain the total extracts. A portion of the extracts was incubated at 4°C for 1.5 h with 50 μl of anti-rabbit IgG-conjugated magnetic beads (Dynal Biotech.) preincubated with rabbit anti-Cdc45 antibody. The magnetic beads were washed five times with cold EB buffer and suspended in SDS-sample buffer. Immunoblotting was performed as described previously (Takahashi and Masukata, 2001), with rabbit polyclonal antibodies against Mcm2, Mcm3 (Sherman and Forsburg, 1998), Mcm4 (Sherman *et al.*, 1998), Mcm5, Mcm6, Mcm7 or Cdc45 at 1:200, 1:3000, 1:2000, 1:200, 1:2000, 1:500 or 1:1000 dilutions, respectively. Rabbit anti-Mcm3 and Mcm4 antibodies were gifts from Dr. S. Forsburg.

Chromatin Immunoprecipitation (ChIP) Assay and ChIP Scanning

ChIP assays were performed as described previously (Ogawa *et al.*, 1999). Three sets of primers were used to amplify a segment in the *ars2004* (239 base pairs) and adjacent nonorigin regions non-ARSa (295 base pairs) and non-

ARSb (195 base pairs) on the chromosome II. Another three sets of primers to amplify *ars3002* segment (246 base pairs) and nonorigin regions non-ARSc (195 base pairs) and non-ARSd (289 base pairs) on the chromosome III. The primers that amplify *ars3002*, non-ARSa and non-ARSb, are identical to those in the previous work (Ogawa *et al.*, 1999). The nucleotide sequences of the remaining primers are ars2004F, 5′-CTTTTGGGTAGTTTTCGGATCC-3′; ars2004R, 5′-ATGAGTACTTGTCACGAATTC-3′; non-ARScF, 5′-GAATCT-TCAGACCTTGCAGC-3′; non-ARScR, 5′-ACGGACTCCAACAACCATTTG-3′; non-ARSdF, 5′-TTGCTGGCAGGGGTGAGTTG-3′; and non-ARSdR, 5′-TATGCTCTTTTCCCTCCACC-3′. ChIP scanning analyses were carried out as described previously (Takahashi *et al.*, 2003). The nucleotide sequences of the primers for amplification of segments 8A and non-ARS were ars2004−8AF, 5′-GAGTTAAGAGTGAAATAAGATTCATGGC-3′; ars2004−8AR, 5′-CCT-TACCCTTTCCTACCTGCC-3′; non-ARS-F, 5′-TCGAAGATCCTACCGCT-TTC-3′; and non-ARS-R, 5′-CTTGCGCTGAAGCTTTAGTAAAAG-3′.

RESULTS

CDK Modulation Suppresses Mutations in pre-RC Components except for nda4/mcm5

To identify factors that have functional interaction with MCM complex, we screened for fission yeast genes on a multicopy plasmid, which suppressed the cold sensitivity of the *nda1-376/mcm2*. We thereby obtained the *rum1*⁺ gene, encoding an inhibitor of cyclin-dependent kinase (CDK; Figure 1A). Because Rum1 is required for maintenance of the G1 phase by inhibiting the CDK kinase activity (Benito *et al.*, 1998), we speculate that the suppression was due to transient inhibition of CDK. Consistent with this idea, deletion

of the *cig2⁺* gene (*cig2Δ*), encoding a B-type cyclin that controls entry into S phase (Mondesert *et al.*, 1996), suppressed the growth defect of the *mcm2* mutant at restrictive temperatures (Figure 1B). It has been shown that deletion of *cig2* suppresses the growth defect of *cdc19-P1/mcm2*, allelic to *nda1-376/mcm2*, *cdc18-K46* and *cdc21-M68/mcm4* (Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998; Grallert *et al.*, 2000). In addition, we here found that *mis5-268/mcm6* and *orp1-4/orc1* were suppressed by *cig2Δ* or by the multicopy *rum1⁺* (unpublished data). A possible explanation for such wide-spectrum suppression is that extended G1 phase by transient inhibition of CDK provides time to form pre-RC in the mutants that have defects in pre-RC formation. Indeed, the *nda1-376/mcm2* and *orp1-4/orc1* mutants are defective in chromatin loading of Mcm6 (T. Asahara and H. Masukata, unpublished results). An exception that was not suppressed by the multicopy *rum1⁺* or *cig2Δ* was the *nda4-108/mcm5* mutation (Figure 1, A and B). Therefore, we hypothesized that the *mcm5* mutation may have a defect rather in a later step than the pre-RC formation.

DNA Replication in the mcm5 Mutant Is Rescued by a Mutation in the cdc45

It has been shown that the defects of *nda4/mcm5* mutation are suppressed by an extragenic mutation in the *sna41/cdc45* gene, and the second mutation itself endows the temperature-sensitive growth (Miyake and Yamashita, 1998). In an independent screening, we obtained another allele of *sna41/cdc45*, identical to the *sna41-928/cdc45* mutation (S. Miyake, personal communication). The *sna41-928/cdc45* mutation suppressed the growth defect of *mcm5* mutant at 25°C and, in turn, the temperature sensitivity of the *sna41-928/cdc45* mutation was enhanced by the *mcm5* mutation (Figure 1C). In addition, overexpression of *cdc45⁺* from the inducible promoter (*nmt41*) partially suppressed the cold sensitivity of the *mcm5* mutant (Figure 1D). These genetic observations suggest that the *mcm5* mutant has a defect in a step where Cdc45 functions.

To test whether the *cdc45* mutation rescued DNA replication of the *mcm5* mutant, DNA contents of *mcm5* and *mcm5 cdc45* cells were examined after synchronization of the cell cycle from G1 phase by depleting nitrogen source (see MATERIALS AND METHODS). Wild-type, *mcm5*, and *mcm5 cdc45* cells arrested in G1 phase were released at the restrictive temperature for the *mcm5* by adding a nitrogen source. The results of flow cytometry analysis showed that wild-type cells with 1C DNA content shifted to 2C DNA cells by 10 h after release, whereas proportion of *mcm5* cells with 1C DNA did not decrease significantly even at 16 h (Figure 1E), showing a defect in an early step of DNA replication as described previously (Miyake *et al.*, 1993). In contrast to the results of *mcm5* cells, 1C DNA peak of *mcm5 cdc45* cells gradually shifted to 2C at 16 h, although the increment of DNA content was slower than that in wild-type cells. These results show that DNA replication in the *mcm5* mutant is rescued in some extent by the *cdc45* mutation.

Determination of the nucleotide sequence of the *nda4-108/mcm5* mutant gene revealed that substitution of cysteine for a conserved arginine residue at position 50 from the N-terminus of Mcm5 (R50C; Figure 1F). The mutation is located proximal to the putative leucine zipper motif. Because leucine-zippers are considered to be involved in protein-protein interactions, the *mcm5* mutation may affect possible interactions between the N-terminus of Mcm5 and other factors for initiation of DNA replication.
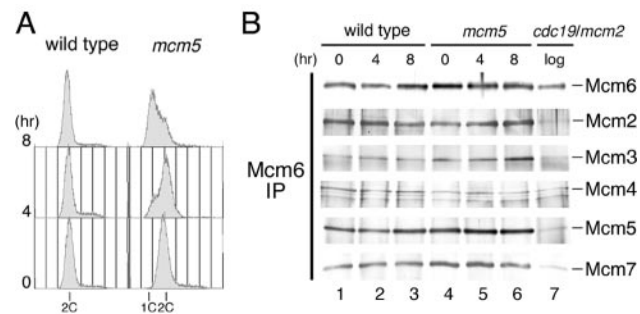

Figure 2. Stable MCM complexes are maintained in the *mcm5* mutant. (A) Wild-type (*972*) and *mcm5* cells grown at 33°C were incubated at 20°C, and DNA contents were analyzed by flow cytometry. (B) Immunoprecipitates with anti-Mcm6 antibody from the extracts of wild-type and *mcm5* cells cultured as in A were analyzed by Western blotting with antibodies against Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7. An extract prepared from exponentially growing *cdc19-P1/mcm2* cells was also analyzed in lane 7.

The mcm5 Mutation Does not Affect MCM Complex Formation

To determine the step affected by the *mcm5* mutation at a molecular level, we first examined MCM complex formation. It has been reported that MCM complex is unstable in the *cdc19-P1/mcm2* mutant (Sherman *et al.*, 1998). Exponentially growing wild-type and *mcm5* cells were cultured at 20°C for 4 and 8 h. The results of flow cytometry analysis showed that *mcm5* cells with 1C DNA content accumulated at 8 h at 20°C, showing that cells were arrested with unreplicated DNA (Figure 2A). To examine whether MCM complexes were maintained, Mcm6 was immunoprecipitated with polyclonal antibody and analyzed by Western blotting with antibodies against various MCM subunits. The results in Figure 2B showed that all other components of MCM complex, Mcm2, 3, 4, 5, and 7, were coprecipitated with Mcm6 from the *mcm5* extracts at similar efficiencies with those from wild-type extracts. As reported previously, amounts of Mcm2, Mcm3, 5, and 7 coprecipitated with Mcm6 from the *cdc19-P1/mcm2* extracts were greatly reduced, even when cultured at a permissive temperature (Sherman *et al.*, 1998; Figure 2B, lane 7). These results show that MCM complexes containing the mutant Mcm5 are stably maintained under restrictive conditions.

Cell Cycle–specific Interaction between Cdc45 and MCM Is Impaired by the mcm5 Mutation

On the basis of tight genetic interactions between *mcm5⁺* and *cdc45⁺*, we tested the possibility that physical interaction between Mcm5 and Cdc45 was affected by the *mcm5* mutation. Because MCM-Cdc45 interaction was observed only in G1 and S phases in fission yeast (Uchiyama *et al.*, 2001b), the cell cycle was synchronized using the *cdc25-22* mutation, which causes cell cycle arrest at the G2/M boundary (Russell and Nurse, 1986). The *cdc25* (wild-type for *mcm5*) and *mcm5 cdc25* cells grown at 28°C, a permissive temperature for both mutations, were first incubated at 36°C for 3 h for arrest and then released into 20°C. Progression of the cell cycle was monitored by assessing the proportion of cells with divided nuclei (Figure 3B). In fission yeast, nuclear division is not immediately followed by cytokinesis, which occurs at a similar time with S phase of the next cell cycle. Thus, binucleate cells correspond to those in G1-S phases. For wild-type cells, the proportion of binucleate cells in-
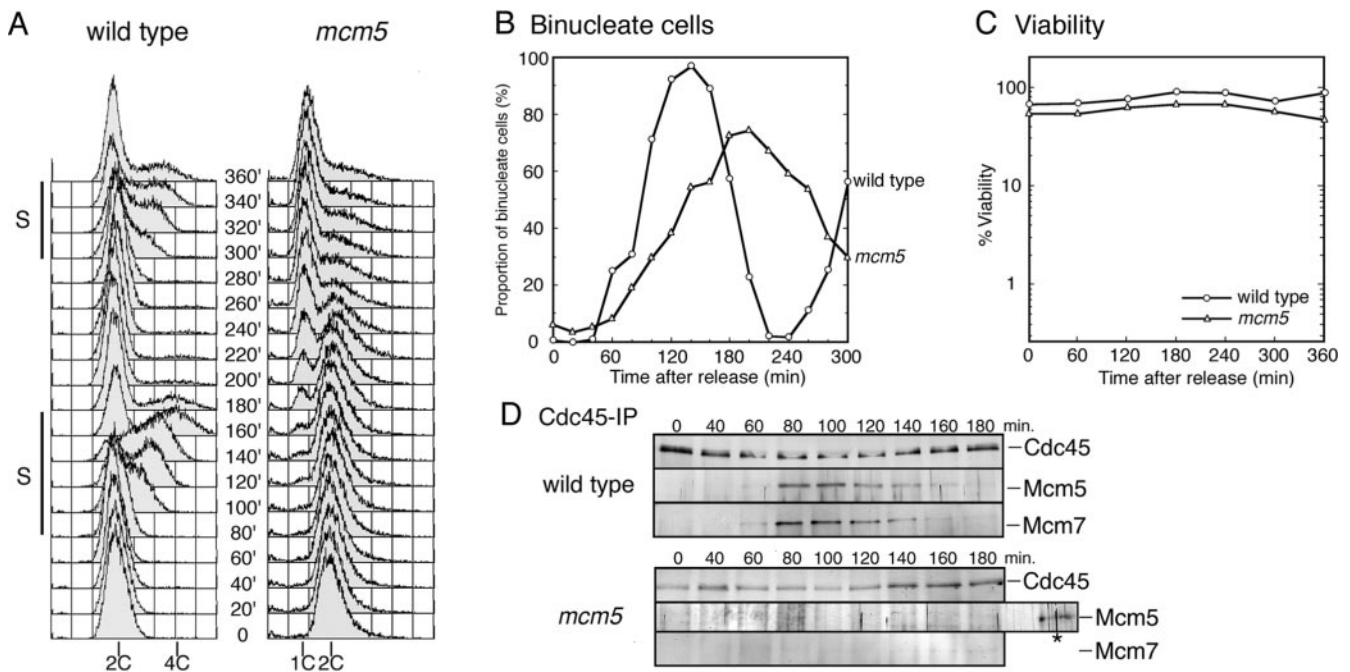
Figure 3. Coimmunoprecipitation of MCM subunits with Cdc45 is reduced in the *mcm5* mutant. Wild-type (*cdc25-22*) and *mcm5* (*mcm5 cdc25-22*) cells grown in EMM medium at 28°C were incubated at 36°C for 3 h to arrest at the G2/M boundary. Cells were then shifted to 20°C, which is the restrictive temperature for *mcm5*. (A) DNA contents analyzed by flow cytometry are shown. Approximate periods of the first and the second S phases are indicated along with positions of 1C, 2C, and 4C DNA. (B) Percentages of binucleate wild-type and *mcm5* cells are shown by circles and triangles, respectively. (C) Viability of wild-type (○) and *mcm5* (△) cells was determined as percentage of number of colonies formed at the permissive temperature among cell numbers measured by Sysmex F-520. (D) The immunoprecipitates with anti-Cdc45 antibody were analyzed by Western blotting with antibodies against Cdc45, Mcm5, and Mcm7. The immunoprecipitates from wild-type cells at 80 min was included as a positive control in a lane marked by an asterisk.

creased from 60 min with a peak at 140 min, and the second increase was observed from 260 min (Figure 3B). In the *mcm5* mutant, the proportion of binucleate cells increased from 80 min and peaked at 200 min with a slight delay for unknown reasons (Figure 3B). Flow cytometry analysis of wild-type showed that cells 2C-4C DNA content appeared during 100–160 min and 300–360 min, indicative of S phases (Figure 3A, left). In contrast, for the *mcm5* mutant 1C DNA cells increased from 180 min and remained even after 360 min (Figure 3A, right). Increase of 1C DNA cells well correlates with decrease in proportion of binucleate cells (Figure 3, A and B). These results show that the *mcm5* cells arrest without significant DNA synthesis. It should be noted that *mcm5* cells maintained their viability at the restrictive temperature even at 360 min (Figure 3C), suggesting that the mutation does not cause an irreversible defect.

Extracts prepared at indicated time points were treated with DNaseI to eliminate interactions mediated by chromosome DNA, and then Cdc45 was immunoprecipitated with anti-Cdc45 polyclonal antibody. The results of Western blotting showed that Mcm5 and Mcm7 subunits were coimmunoprecipitated with Cdc45 in wild-type extracts specifically at 80–140 min (Figure 3D), consistent with previous observations (Uchiyama *et al.*, 2001b). In contrast, neither Mcm5 nor Mcm7 was pulled down with Cdc45 from the *mcm5* extracts at any time point (Figure 3D). These results show that interaction between MCM complex and Cdc45 was greatly reduced in the *mcm5* mutant.

Cdc45-loading onto Replication Origins Is Diminished in the mcm5 Mutant

To examine the effect of the *mcm5* mutation on origin-association of MCM and Cdc45, we used a chromatin-immunoprecipitation (ChIP) assay. The cell cycle was synchronized as described above using the *cdc25* mutation. DNA immunoprecipitated with Mcm6 or FLAG-Cdc45 was used for PCR to determine the enrichment of the *ars2004* or *ars3002*, efficient replication origins on chromosomes II and III, respectively (Zhu *et al.*, 1994; Okuno *et al.*, 1997). We used three sets of primers to amplify *ars2004* or *ars3002*, and two adjacent nonorigin regions as controls (Figure 4A). PCR amplification with these primers from the total cellular DNA prepared without immunoprecipitation yielded similar amounts of products (Figure 4, B and C, lanes WCE). On the other hand, the *ars2004* and the *ars3002* fragments were preferentially amplified from Mcm6-IP DNA of wild-type during 60–140 min and then diminished (Figure 4Bi), whereas surrounding bands were not significantly amplified at any time point, showing that Mcm6 associated preferentially with the origins during G1-S phase, as described previously (Ogawa *et al.*, 1999). In contrast, when Mcm6-IP DNA from *mcm5* cells was used as a template, the *ars2004* and the *ars3002* fragments were amplified at 80 min and did not decrease even at 360 min (Figure 4Ci). These results indicate that Mcm6 persistently associates with the origins in *mcm5* cells. DNA immunoprecipitated with the anti-Mcm2 antibody yielded similar results as shown for Mcm6-IP (unpublished data). Taken together, the MCM
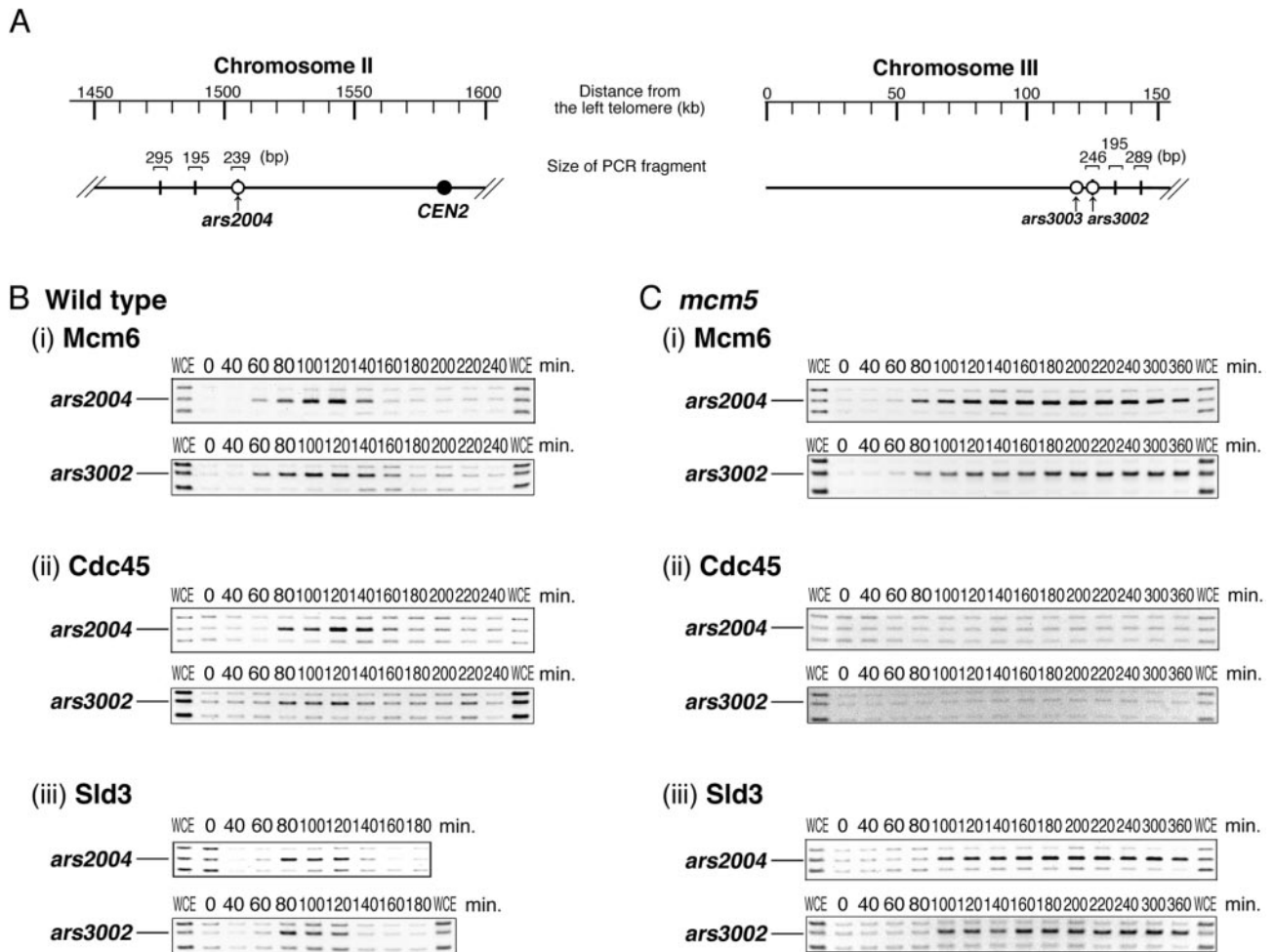
Figure 4. Sld3 efficiently associates with origins, whereas Cdc45 does not in the *mcm5* mutant. Wild-type and *mcm5* cells were arrested at G2/M boundary and released at 20°C as described for Figure 3. (A) Positions of segments examined by ChIP analysis are shown along with the distance from the left telomere. PCR reactions were carried out using three sets of primers that amplify *ars2004* or *ars3002* origin and adjacent nonorigin regions on same chromosome. Synchronized wild-type (B) and *mcm5* (C) cells taken at indicated time points were cross-linked and DNA fragments immunoprecipitated with antibodies were analyzed by PCR with three sets of primers. PCR products amplified from total cellular DNA without immunoprecipitation are shown in lane WCE. Positions of *ars2004* (239 base pairs) and *ars3002* (246 base pairs) bands are indicated. DNA was immunoprecipitated with anti-Mcm6 antibody (i) and with anti-FLAG antibody (ii) from cell extracts of YYY100 (*cdc25 flag-cdc45*) and YYY104 (*mcm5 cdc25 flag-cdc45*). Sld3-associated DNA (iii) was immunoprecipitated with anti-FLAG antibody from YYY136 (*cdc25 5flag-sld3*) and YYY137 (*mcm5 cdc25 5flag-sld3*).

complex persistently associates with replication origins in the *mcm5* mutant. The findings demonstrate that the *mcm5* mutant does not affect pre-RC formation but has a defect in a later step.

To examine association of Cdc45 with origins in *mcm5* cells, we used strains in which the *cdc45*⁺ gene was replaced with FLAG-tagged *cdc45*. As shown in Figure 4Bii, the *ars2004* and the *ars3002* fragments were selectively amplified from the FLAG-Cdc45-IP DNA in wild-type cells (*cdc25 flag-cdc45*) at 80–140 min. In contrast, in *mcm5* cells, the *ars2004* and the *ars3002* fragments were not amplified significantly from FLAG-Cdc45-IP DNA at any time point (Figure 4Cii). These results show that cell cycle–specific association of Cdc45 with the origins is greatly reduced in *mcm5* cells.

### Sld3 Binds to Replication Origins without Cdc45 in the mcm5 Mutant

Sld3 is a factor that has tight genetic interactions with Cdc45 and is required for origin-loading of Cdc45 in both fission

yeast and budding yeast (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002). In budding yeast loading of Sld3 onto the origin depends on Cdc45. Thus, we tested whether loading of Sld3 onto origins was also impaired in the *mcm5* mutant, using strains with FLAG-tagged Sld3. FLAG-Sld3-ChIP analyses were carried out after synchronization as described above. In wild-type cells, the *ars2004* and the *ars3002* fragments were preferentially amplified at 80–120 min, showing Sld3-association with the origin during S phase (Figure 4Biii). In contrast, Sld3 in *mcm5* cells started to associate with the origins at 100 min and remained even at 360 min (Figure 4Ciii). These results show that Sld3 efficiently binds to the origins without origin-association of Cdc45.

Loading of Cdc45 and DNA Replication upon Return to Permissive Condition

Because the *mcm5* mutant retains the viability at the restrictive temperature, the process of initiation of DNA replica-
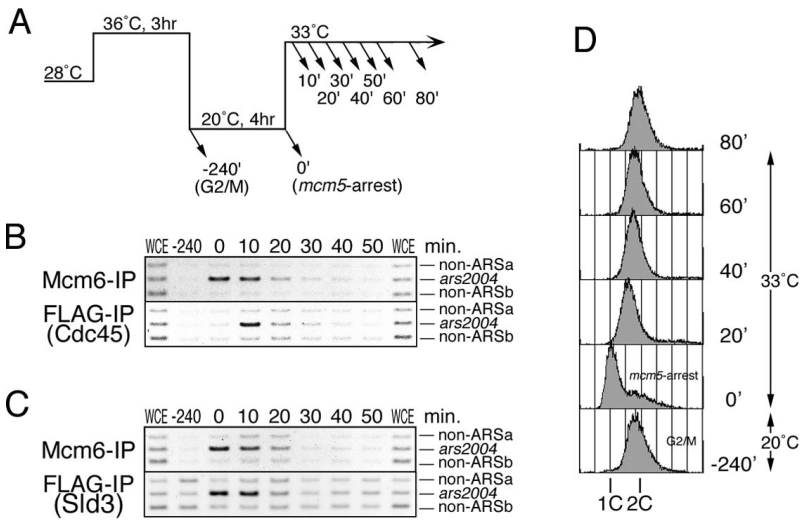
Figure 5. Quick restoration of initiation reaction from *mcm5*-arrest. (A) Experimental scheme. *mcm5* (*mcm5 cdc25*) cells were first arrested at G2/M boundary by incubation at 36°C for 3 h and then incubated at 20°C for 4 h to arrest at Mcm5-execution point. Cells were then released from the *mcm5*-arrest at 33°C and samples were collected at indicated time points. (B) Synchronized *mcm5* (*mcm5 cdc25 flag-cdc45*) cells as in A were subjected to immunoprecipitation with anti-Mcm6 and anti-FLAG antibodies. (C) Samples were same as in B except that FLAG-Sld3 was immunoprecipitated with anti-FLAG antibody. (D) DNA contents were analyzed by flow cytometry for samples collected in B.

tion might be arrested with a physiological intermediate in the *mcm5* mutant. To assess this possibility, we examined loading of Cdc45 and subsequent dissociation of Mcm6 and Sld3 from the origin, when the arrested *mcm5* cells were returned to the permissive temperature (Experimental scheme in Figure 5A). The ChIP analysis with anti-Mcm6 antibody and anti-FLAG(Sld3) antibody showed that the *ars2004* fragment, which was amplified efficiently at the arrested condition, clearly diminished at 20 min, showing efficient translocation or dissociation of Mcm6 and Sld3 from the origin (Figure 5, B, top panel, and C). In contrast, by FLAG-Cdc45-IP, the *ars2004* fragment, which was not amplified in *mcm5* arrested cells (0 min), was preferentially amplified at 10 min after return to the permissive temperature, and then diminished (Figure 5B, bottom panel). These results indicate that Cdc45 binds to the origin immediately after Mcm5 function is restored. Consistent with these results, the DNA content of the *mcm5* cells increased from 1C to 2C within 40 min after return to the permissive temperature (Figure 5D), suggesting that replication of chromosome DNA is resumed in a short period. Taken together, it is likely that the complex formed in the *mcm5* mutant can readily resume the normal initiation.

Proficient Origin Association of Sld3 without Cdc45 in the *cdc45* Mutant

If the complex containing MCM and Sld3 without Cdc45 observed in the *mcm5* mutant is a physiological one, it could

be observed in the other conditions. To test this possibility, we examined association of initiation factors in the temperature-sensitive *cdc45* mutant, which also maintains the viability at the restrictive temperature (R. Nakajima and H. Masukata, unpublished results). For synchronization, we used a cold-sensitive *nda3* mutation, which is defective in polymerization of β-tubulin (Hiraoka *et al.*, 1984). The *sna41-928/cdc45 nda3 flag-sld3* and *nda3 flag-sld3* (wild-type for *cdc45*) cells grown at 28°C were incubated at 20°C for 4 h for M phase arrest, and then released at 37.5°C, which is the restrictive temperature for the *cdc45*. The *nda3 flag-sld3* (as a control) cells were released in the presence of hydroxyurea (HU), which depletes the nucleotide pool and perturbs replication fork progression in the early S phase. Flow cytometry analysis showed that 1C DNA cells accumulated in both HU-arrested wild-type and *cdc45* cells (Figure 6A). At the indicated time points, DNA recovered by immunoprecipitation with anti-Mcm6, anti-FLAG and anti-Cdc45 antibodies, respectively, were analyzed by PCR. At 0 min, both wild-type and *cdc45* cells being arrested at metaphase, no significant amplification of *ars2004* fragment was observed from Mcm6-IP or FLAG-Sld3-IP or Cdc45-IP (Figure 6, B and C). In HU-arrested wild-type cells, *ars2004* fragment was preferentially amplified at 60–90 min from Mcm6-IP, FLAG-Sld3-IP and Cdc45-IP, showing association of these proteins with the replication origin at S phase (Figure 6B). In contrast, the *ars2004* fragment was not preferentially recovered at any time point by Cdc45-IP from the *cdc45* mutant, whereas the
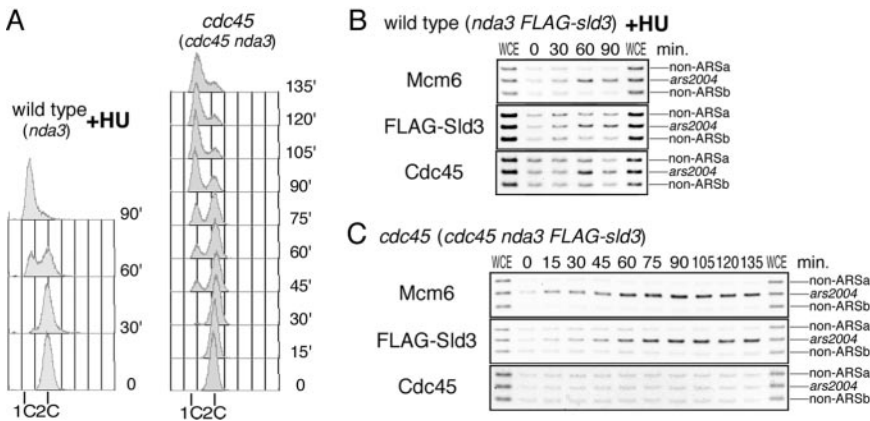
Figure 6. Sld3 associates with the origin in the *cdc45* mutant. Wild-type (*nda3 5flag-sld3*) cells grown at 28°C were incubated at 20°C for 4 h to arrest in M phase and incubated at 37.5°C in the presence of 12 mM HU, whereas *cdc45* (*cdc45 nda3 5flag-sld3*) cells synchronized similarly were incubated at 37.5°C without HU. (A) DNA contents were analyzed by flow cytometry. Cell extracts of wild-type (B) and *cdc45* (C) were divided into three and subjected to immunoprecipitation with anti-Mcm6, anti-FLAG (Sld3), and anti-Cdc45 antibodies, respectively. DNA recovered by immunoprecipitation was analyzed by PCR as described for Figure 4.
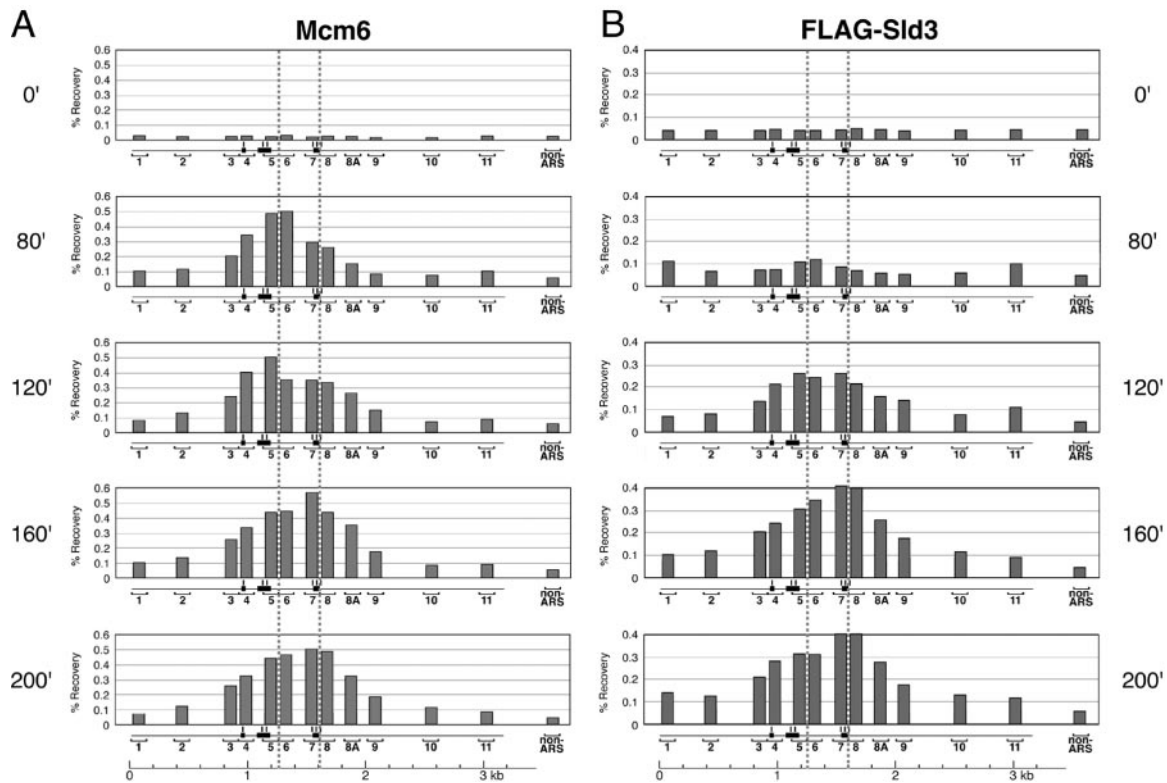
Figure 7. Colocalization of Mcm6 and Sld3 within the *ars2004*. The *mcm5 cdc25 5flag-sld3* cells arrested at G2/M boundary as described for Figure 3 were released at 20°C. Total cellular DNA and DNA immunoprecipitated with anti-Mcm6 and anti-FLAG (Sld3) were prepared at the indicated time points, serially diluted and used as the templates for PCR amplification with 12 primer sets within the *ars2004* and a primer set in the non-ARS region. PCR products were separated by 2% agarose gel electrophoresis and the corresponding DNA bands stained with ethidium bromide were quantitated with LAS1000-plus Image analyzer (Fuji Film). Recoveries of segments by immunoprecipitation with Mcm6 (A) and with FLAG-Sld3 (B) determined as the ratio of immunoprecipitated DNA against total DNA are presented by histograms above the corresponding regions. Locations of three essential regions for ARS activity and 12 fragments amplified by PCR are indicated below the panels. Peak positions of Mcm6-localization in G1 and *mcm5*-arrested cells are shown by vertical broken lines between fragments 5–6 and 7–8, respectively. A representative result from three independent experiments is shown.

recovery of the fragment by Mcm6-IP or Sld3-IP increased during 15–60 min and remained stable (Figure 6C). These results indicate that the *cdc45* mutant has a defect in Cdc45-loading but allows proficient association of Sld3 with the origin, confirming independent loading of Sld3 from Cdc45.

Colocalization of Sld3 with Mcm6 in the Origin during *mcm5* Arrest

We have previously shown that MCM complex is located in a narrow region distant from ORC-binding sites within *ars2004* in G1-arrested cells (Takahashi *et al.*, 2003). Here, we addressed whether Sld3 colocalizes with MCM in *mcm5*-arrested cells, which allow us to map the location of Sld3. We adopted the ChIP-scanning method using 12-primer sets amplifying within *ars2004* as well as non-ARS control primer sets (Figure 7) as described previously (Takahashi *et al.*, 2003). Recoveries of DNA fragments by Mcm6-IP and Sld3-IP from synchronized *mcm5 cdc25 flag-sld3* cells were determined by quantitative PCR analyses. When cells were arrested at the G2/M boundary (0 min), no region was significantly recovered by Mcm6-IP or Sld3-IP (Figure 7, A and B, top panels). At 80 min after release, origin region was recovered by Mcm6-IP several times more efficiently than non-ARS control fragment (Figure 7A). An apparent peak of the recovery resides at segments 5 and 6, showing binding of Mcm6 at a narrow region. These results are very similar to

those described previously for pre-RC in G1 arrested cells (Takahashi *et al.*, 2003). At this time point, Sld3-IP did not enrich any specific fragment (Figure 7B), showing the absence of Sld3 in pre-RC. At 120 min, however, recovery of regions of *ars2004* by Sld3-IP increased, as distribution of Mcm6 became slightly broader than that at 80 min. At 160 and 200 min, recoveries of segments 7–9 by Mcm6-IP and Sld3-IP became higher than other regions and the profiles of Sld3 and Mcm6 localization were almost identical (Figure 7, A and B). These results show that Sld3 colocalizes with Mcm6 within the origin in *mcm5*-arrested cells. It should be noted that the peak of Mcm6 localization shifted by ~300 base pairs from segments 5–6 to 7–8 (indicated by two broken lines in Figure 7, A and B). This may indicate that a significant fraction of Mcm6 changes its localization within the origin as Sld3 associates.

DISCUSSION

At the onset of S phase, the pre-RC (prereplicative complex) formed on replication origin is converted into pre-IC (preinitiation complex), which is capable of origin DNA unwinding and of assembly of DNA polymerases to initiate DNA replication. DNA helicase activity of Mcm2-7 complex should be activated during pre-IC formation. Several factors such as Cdc45, Sld3, Dpb11, Mcm10, and GINS complex as well as

DDK and S-CDK kinases are involved in this step. However, because origin-loading of these factors appears to be mutually dependent, little is known about the precise process of pre-IC formation and MCM activation. In this report, taking advantage of a point mutation that caused a defect in a specific function of the gene product, we were able to identify a novel step in pre-IC assembly in fission yeast. In the *nda4/mcm5* mutant, which does not affect MCM complex stability or pre-RC formation, loading of Cdc45 is impaired, but Sld3 can proficiently associate with the origin. Furthermore, the *sna41/cdc45* mutation reduces loading of Cdc45 onto the origin without affecting Sld3-loading. These results strongly suggest that origin-association of Sld3 is a step independent from Cdc45-loading. ChIP-scanning experiments revealed that Sld3 colocalizes with Mcm6 in the origin in the *mcm5* mutant. The complex containing MCM and Sld3 but not Cdc45 might be a physiological intermediate for the initiation of DNA replication, because Cdc45-loading and DNA replication are resumed immediately after return to permissive conditions.

Involvement of the N-terminal Region of Mcm5 in Interactions with Cdc45

It has been previously shown that defects of the *nda4-108/mcm5* mutation are suppressed by a mutation in the *sna41⁺/cdc45⁺* gene (Miyake and Yamashita, 1998). The results of suppression of *mcm5* mutation by overexpression of Cdc45 in addition to synthetic enhancement of temperature sensitivity of *cdc45* by *mcm5* presented in this work provide strong evidence for a tight genetic interaction between *mcm5⁺* and *cdc45⁺* (Figure 1, C and D). This genetic interaction suggests that physical interaction between Mcm5 and Cdc45 is affected by the *mcm5* or *cdc45* mutation. Indeed, we demonstrated that coprecipitation of Mcm5 and Mcm7 with Cdc45 was greatly reduced in the *mcm5* mutant (Figure 3D). This could be the cause for failure in origin-loading of Cdc45 (Figure 4C). Consistent with the results of genetics, the *cdc45* mutation that rescues the replication defect of *mcm5*, in turn, causes a defect in Cdc45-loading at its restrictive temperature (Figure 6C). These results suggest that the interaction between Mcm5 and Cdc45 is affected by these mutations.

Based on the results of previous studies and those presented here, conditional lethal *mcm* mutations are classified into three groups due to the defects in MCM functions. First group includes mutations, such as *cdc19-P1/mcm2* (Sherman *et al.*, 1998), that affect stability of the MCM complex. The mutations in the second group, such as *nda1-376/mcm2*, have a defect in loading of MCM complex, which is stable, onto the origin (Y. Yamada, unpublished results). The third group affects neither MCM complex stability nor MCM-loading, but is defective in a later stage. The *nda4-108/mcm5* belongs to this group. In budding yeast, all the mutations reported, including *cdc46-1* that has genetic interaction with *CDC45*, appear to belong to the first group (Hennessy *et al.*, 1990). Thus, the effects of *nda4-108/mcm5* mutation on the initiation process might be allele specific, although no other *mcm5* mutation is available in fission yeast to test this possibility.

The *mcm5* mutation alters a conserved amino acid residue in the N-terminal region of Mcm5 (Figure 1F). A leucine zipper motif, which may be involved in protein-protein and DNA-protein interactions, is predicted near the *mcm5* mutation. Furthermore, the budding yeast *mcm5-bob1* mutation has been mapped proximal downstream of the leucine zipper motif (Hardy *et al.*, 1997; Figure 1F). The *mcm5-bob1* bypasses the requirement of DDK for initiation of DNA replication and stimulates association of Cdc45 with origins

in G1 phase (Sclafani *et al.*, 2002). Studies of *Archaea* MCM structure suggest that the *mcm5-bob1* mutation appears to increase structural flexibility in the N-terminus of Mcm5 (Fletcher *et al.*, 2003). Taken these results together with those obtained with the *nda4/mcm5* mutation, possible structural change caused either by DDK-phosphorylation of MCM subunits or by the *mcm5-bob1* mutation may allow the N-terminal region of Mcm5 to interact with Cdc45.

Sequential Assembly of pre-IC Components

We have previously shown that binding of Cdc45 to the chromatin depends on the functional Sld3 (Nakajima and Masukata, 2002). On the other hand, we show here that Sld3 can efficiently bind to replication origins without detectable association of Cdc45 in the *mcm5* and *cdc45* mutants (Figures 4 and 6). Furthermore, temperature-sensitive mutations of *psf3*, a component of fission yeast GINS complex, inhibit loading of Cdc45 but not Sld3 onto the origin (H. Yabuuchi and H. Masukata, unpublished results). Although the results obtained by mutations are attributed to specific alleles, these results strongly suggest that Sld3-loading is independent of Cdc45-loading in fission yeast.

One cannot exclude the possibility that a step detected in a certain mutant could be an aberrant dead-end product. However, the following observations suggest that this is not the case. The *mcm5* mutant retains the viability at the restrictive temperature (Figure 3C). Furthermore, Cdc45 is loaded onto the origin immediately after return to the permissive temperature, and replication of the bulk chromosome is efficiently resumed (Figure 5, B and D). These results suggest that the complex containing MCM and Sld3 but not Cdc45 is a functional intermediate in initiation of DNA replication.

Loading of Cdc45 and Sld3 is interdependent in budding yeast (Kamimura *et al.*, 2001; Takayama *et al.*, 2003). Origin association of Sld3 is decreased in *cdc45-1* mutant. The finding in this study that loading of Sld3 does not depend on Cdc45 in fission yeast is somewhat different from the observations in budding yeast. Although the possibility that the difference could be due to allele specificity is not excluded, there are two other possible explanations, not exclusive each other, for this difference. Interactions of Sld3 with other factors may be more stable in fission yeast than those in budding yeast. For example, fission yeast Sld3 is coprecipitated with Mcm6 and Cdc45 without cross-linking, whereas coprecipitation of Sld3 with Cdc45 depends on cross-linking in budding yeast (Kamimura *et al.*, 2001; Nakajima and Masukata, 2002). For the second explanation, the process of pre-IC assembly could be diverse in some extents among eukaryotes. In *Xenopus* egg extracts, Cut5, a possible counterpart of Dpb11, associates with the chromatin without GINS (Kubota *et al.*, 2003), whereas loading of these components are mutually dependent in budding yeast (Kamimura *et al.*, 2001; Takayama *et al.*, 2003). More careful investigation in various replication systems should be required to elucidate the principle scheme of pre-IC formation.

Distinct Roles of Sld3 and Cdc45 in Initiation of Replication

What are the roles of Cdc45 and Sld3 in pre-IC assembly and MCM helicase activation? It has been shown, by yeast two-hybrid analyses, that budding yeast Sld3 interacts with Psf1, a component of GINS complex, and with Dpb11 (Takayama *et al.*, 2003). Interaction of Dpb11 with Sld2 depends on phosphorylation of Sld2 by S-CDK and is required for loading of DNA Pol ϵ (Masumoto *et al.*, 2002; Noguchi *et al.*, 2002). We detected a reduced but significant level of origin association of Dpb2, the second largest subunit of DNA Pol

ε, by ChIP assay in the *mcm5*-arrested condition (unpublished data). Hence, Sld3 may play an important role in assembly of initiation factors including DNA Pol ε onto replication origins. It has been suggested that Cdc45 is involved in chromatin-loading of DNA Pol α (Mimura and Takisawa, 1998; Aparicio *et al.*, 1999), and genetic interaction between Cdc45 and DNA Pol α has been suggested (Uchiyama *et al.*, 2001a). Thus, Cdc45 may function in recruiting Pol α onto origins. These results suggest that Cdc45 and Sld3 are involved in assembly of distinct factors.

In the *mcm5* mutant arrested at the restrictive temperature, we could not detect single-stranded DNA in the origin either by ChIP assay with anti-Ssb2 (RPA) antibody or by genomic foot-printing with potassium permanganate modification (unpublished data). These results suggest that MCM helicase is not activated. It has been reported that complexes containing MCM and Cdc45 from *Xenopus* egg extracts exhibit DNA helicase activity (Masuda *et al.*, 2003). Considering genetic and physical interactions of Cdc45 and MCM, Cdc45 may play a direct role in activation of MCM helicase. We show that localization of Sld3 and Mcm6 is changed from initial MCM binding site in *ars2004* (Figure 7). This result could be interpreted by the possibility that Sld3-associated MCM may move along the DNA without unwinding. The interaction between MCM and DNA might be altered by association of Sld3. A possibility that additional association of MCM and subsequent Sld3-loading occur at the secondary preferred site in later periods is not excluded.

Initiation of replication is regulated by two independent kinase pathways, DDK and CDK. Chromatin loading of Cdc45 requires activation of both DDK and CDK (Bousset and Diffley, 1998; Donaldson *et al.*, 1998; Zou and Stillman, 2000). However, the apparent dependency of Cdc45-loading on DDK should be, at least in part, mediated by Sld3-loading in fission yeast, because DDK-dependent origin-loading of Sld3 does not depend on Cdc45 (Nakajima and Masukata, 2002). Thus, Sld3-loading is a step critical for temporal regulation of origin firing and for repression of late origin firing by replication checkpoint. On the other hand, one of the steps regulated by CDK is demonstrated to be complex formation between Dpb11/Cut5 and Sld2/Drc1 (Masumoto *et al.*, 2002; Noguchi *et al.*, 2002). Taken these results together, DDK and CDK have distinct execution points, namely Sld3 loading and assembly of Dpb11/Cut5-Sld2/Drc1 complex, respectively, although some other step could be regulated by these kinases. Hence, loading of Sld3 before Cdc45 might be important for step-wise activation of initiation complex to ensure that origin is unwound only after assembly of replication factors.

Although many replication factors are conserved from yeast to human, a possible homolog of Sld3 has not been identified in higher eukaryotes. Considering essential role of Sld3 in assembly of pre-IC, its functional homolog should be present in higher eukaryotes, although its amino acid sequence might be diversified. Future biochemical studies are necessary to understand the roles of replication factors in initiation of replication.

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