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CDC45, a Novel Yeast Gene That Functions with the Origin Recognition Complex and Mcm Proteins in Initiation of DNA Replication

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The *CDC45* gene of *Saccharomyces cerevisiae* was isolated by complementation of the cold-sensitive *cdc45-1* mutant and shown to be essential for cell viability. Although *CDC45* genetically interacts with a group of *MCM* genes (*CDC46*, *CDC47*, and *CDC54*), the predicted sequence of its protein product reveals no significant sequence similarity to any known Mcm family member. Further genetic characterization of the *cdc45-1* mutant demonstrated that it is synthetically lethal with *orc2-1*, *mcm2-1*, and *mcm3-1*. These results not only reveal a functional connection between the origin recognition complex (ORC) and Cdc45p but also extend the *CDC45-MCM* genetic interaction to all known *MCM* family members that were shown to be involved in replication initiation. Initiation of DNA replication in *cdc45-1* cells was defective, causing a delayed entry into S phase at the nonpermissive temperature, as well as a high plasmid loss rate which could be suppressed by tandem copies of replication origins. Furthermore, two-dimensional gels directly showed that chromosomal origins fired less frequently in *cdc45-1* cells at the nonpermissive temperature. These findings suggest that Cdc45p, ORC, and Mcm proteins act in concert for replication initiation throughout the genome.

Eukaryotic DNA replication is tightly regulated during the cell cycle. Each chromosomal replication origin normally fires once and only once per S phase, suggesting that the cell cycle control of DNA replication is primarily achieved at the level of initiation. To understand how this is accomplished, we seek to identify the basal initiation machinery operating at replication origins and determine how it interacts with cell cycle regulatory components.

Initiation of DNA replication requires the participation of replicators, which are the *cis*-acting DNA elements of the origins, as well as initiator proteins that recognize replicators (59). In *Saccharomyces cerevisiae*, replicators have been identified by virtue of their ability to support chromosome-independent replication on plasmids and are thus termed autonomously replicating sequences (ARSs) (reviewed in references 9, 19, and 60). Linker-scanning analysis revealed that these ARSs typically consist of one essential A element containing the 11-bp ARS consensus sequence and two to three B elements that are also important for origin function (31, 45, 53, 61). Furthermore, the origin recognition complex (ORC), a six-subunit initiator protein, has been purified and shown to bind to both the A and B1 elements in an ATP-dependent manner (2, 3, 54, 56). Genetic and two-dimensional (2D) gel analyses of mutant strains that affect ORC subunits have demonstrated that ORC is essential for replication initiation in vivo (3, 4, 21, 22, 26, 30, 37, 40, 41). However, constant ORC-ARS binding throughout the cell cycle was suggested by in vivo footprinting experiments (17), thereby implying that ORC alone is not sufficient to render replication initiation cell cycle dependent. In support of this idea, an extended ORC footprint was found in G_1 phase but not after replication was initiated (17). This observation leads to the hypothesis that some unknown initiation regulators associate with ORCs at origins during G_1 phase, and together they assemble into competent prereplication complexes (pre-RCs). To fire in S phase, these pre-RCs need to be triggered, probably in part by the action of cell cycle-regulated protein kinases. Once replication is launched, the competent complexes are irreversibly disassembled, so that no reinitiation can occur until the next S phase (reviewed in references 18, 49, and 64).

Recent work has suggested that Cdc6p is one of the candidates for such initiation regulators. Cdc6p has been shown to be an unstable protein whose de novo synthesis in G_1 phase is required to promote replication initiation (51, 52). As elucidated by several recent studies, Cdc6p apparently plays a critical role at replication origins. First, 2D gels demonstrated that only a subset of chromosomal origins fired in *cdc6* mutant cells even at the permissive temperature (40). Second, the *cdc6-1* mutant is synthetically lethal with both *orc2-1* and *orc5-1* mutants, and overexpression of *CDC6* can suppress the defect of *orc5-1* (40). Third, Cdc6p has been shown to be required for both formation and maintenance of the in vivo footprint of pre-RCs (13, 15, 52), and baculovirus-expressed Cdc6p coimmunoprecipitated with purified ORC (40).

Like Cdc6p, proteins of the Mcm2-3-5 family are strongly implicated in the regulation of replication initiation (reviewed in references 12, 34, and 63). To date, five members of the family have been identified in *S. cerevisiae* (16, 24, 27, 65, 66) (there is predicted to be a sixth Mcm protein encoded by a newly identified open reading frame [ORF], YGL201c). All of these proteins, including Mcm2, Mcm3, Mcm5/Cdc46, Cdc47, and Cdc54, have substantial sequence homology and interact with each other genetically (16, 27, 65, 66). The role of Mcm proteins in replication has been indicated by the high plasmid loss rate and incomplete chromosomal duplication in *mcm* mutants at their nonpermissive temperatures (28, 44). More interestingly, the nuclear localization of Mcm proteins was suggested to be cell cycle regulated by immunostaining and subcellular fractionation experiments (16, 27, 67). The protein are present in the cytoplasm for most of the cell cycle but only

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
W303-1A	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	R. Rothstein (62)
W303-1B	*MAT*α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	R. Rothstein (62)
W303-1A/B	*MAT***a**/α diploid cross of W303-1A and W303-1B	R. Rothstein (62)
DBY2027	*MAT***a** *ade2-1 lys2-801 leu2-3,112 ura3-52 cdc45-1*	D. Botstein (48)
YB0302	*MAT*α *leu2-3,112 his3-11,15ura3-52 mcm2-1*	B.-K. Tye (44)
YB0303	*MAT*α *leu2-3,112 his4Δ34 ura3-52 mcm3-1*	B.-K. Tye (44)
JRY4125	W303-1B; *orc2-1*	J. Rine (21)
YB0059	W303-1A; *orc2-1*	This laboratory (40)
YB0044	W303-1A; *cdc6-1*	This laboratory (40)
YB0298	W303-1A; *cdc45-1*	This study
YB0299	W303-1B; *cdc45-1*	This study
YB0300	W303-1A/B; *cdc45*Δ::*HIS3*	This study

tightly bound to the nucleus during the G_1 phase. The association of the Mcm proteins in the nucleus and with chromatin from late M to early S phase coincides with the competency of the pre-RCs at origins. In support of the hypothesis that Mcm proteins play a critical role in establishing the competent initiation complex, immunodepletion of an Mcm3-containing complex from *Xenopus* egg extract abolished its ability to support G_2 nuclei replication (11, 36, 42).

cdc45-1 was originally isolated as a cold-sensitive (*cs*) cell division cycle (*cdc*) mutant (48). Intriguingly, two temperature-sensitive (*ts*) mutants of *MCM* genes, *cdc46-1* and *cdc47-1*, were isolated as extragenic suppressors of *cdc45-1* (48) and therefore implicated Cdc45p in DNA replication. The suppression by *cdc46* was later shown to be allele specific (28). In the study presented here, the gene encoding Cdc45p is cloned and shown to be essential for cell viability. More detailed characterization of the *cdc45-1* mutant establishes a functional connection between Cdc45p and ORC and also extends the known *CDC45-MCM* genetic interaction to all characterized *MCM* genes involved in replication initiation. Furthermore, 2D gels were used to examine the frequency of firing of individual origins in mutant cells. The results clearly show that Cdc45p functions at replication origins and affects the frequency of initiation.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this study are listed in Table 1. Strains YB0298 and YB0299 were obtained by backcrossing strain DBY2027 four times to W303-1. Strain YB0300 was constructed by one-step gene replacement of *CDC45* in the W303-1 diploid (see below for details). Yeast cells were grown in YPD, synthetic complete medium (SCM), or dropout medium (33). SCM plates with 1 mg of 5-fluoroorotic acid (5-FOA) per ml were used to select against Ura^+ yeast cells.

DNA manipulations and genetic techniques. All standard techniques of molecular cloning were performed essentially as described previously (57). Yeast transformations were done by the lithium acetate method (58). Other yeast genetic manipulations were conducted as described previously (25, 33).

Cloning of *CDC45*. To clone *CDC45*, strain DBY2027 (*cdc45-1*^{cs}) was transformed with a yeast genomic library in YCp50 (a kind gift of K. Arndt, Cold Spring Harbor Laboratory) and screened for growth at 15°C. Plasmids were recovered from the yeast transformants and transformed into *Escherichia coli* DH5α, and the *cdc45-1*-complementing activity was confirmed by retransformation into the *cdc45-1* mutant. Three plasmids [pEM45.1(9-1), pEM45.1(9-2) and pEM45.1(10-1)] with different inserts were isolated and subsequently shown to contain overlapping genomic fragments.

To narrow down the region responsible for the complementing activity, a 3.7-kb *Sau*3A-*Sal*I fragment containing the only substantial ORF was subcloned from pEM45.1(10-1) into the yeast vector pRS416. The resultant plasmid (pEM45.3) was transformed into DBY2027 and shown to retain the *cs*-complementing activity. To confirm that the ORF within the fragment was the sole element responsible for complementation, its coding sequence was cut into two halves at either the *Eco*RI or the *Sac*I site. The fragments containing partial ORF sequences (Fig. 1B) were then subcloned into pRS416 (plasmids pEM45.5-8) and tested for the ability to complement the cold sensitivity of *cdc45-1*. All these plasmids failed to rescue the mutant at 15°C.

Gene disruption of *CDC45*. For the purposes of gene disruption and protein tagging, a *Not*I restriction site was created right before the stop codon of the *CDC45* ORF by a standard PCR-based method. The genomic sequence between *Spe*I and *Not*I sites that includes the entire *CDC45* ORF was removed from the 3.7-kb insert in pEM45.3 and replaced with a *HIS3* selection marker (Fig. 1C). The *HIS3*-disrupted genomic fragment was subsequently released from the plasmid, gel purified, and transformed into the W303-1 diploid. Southern blot analysis was performed on the His^+ transformants to confirm that only one copy of the endogenous *CDC45* was successfully disrupted.

Synchronization of yeast cells and fluorescence-activated cell sorting (FACS) analysis. Cultures of yeast cells (*MAT***a**) were grown to log phase at 30°C, diluted to an optical density at 600 nm of 0.2, and then arrested with 10 μg of α-factor (Sigma) per ml at 30°C for 2 h. Thereafter, α-factor was removed by spinning the cells at low speed and washing them twice with fresh YPD medium. The washed cells were then resuspended in fresh YPD medium containing 100 μg of pronase E per ml at various test temperatures.

In hydroxyurea (HU; Sigma) arrest experiments, cells released from the α-factor block were arrested again with 0.1 M HU at 30°C for at least 1.5 h. Subsequently, arrested cells were spun by low-speed centrifugation, washed twice with fresh YPD, and finally released into fresh YPD at various test temperatures.

In time course FACS assays, approximately 5×10^6 cells were collected at each time point. The collected cells were first washed with 0.4 ml of deionized H_2O and then fixed in 1.4 ml of 70% ethanol at 4°C for more than 2 h. Fixed cells were subsequently resuspended in 0.5 ml of 50 mM sodium citrate (pH 7.4), sonicated briefly, and treated sequentially with 0.25 mg of RNase A per ml and 1 mg of proteinase K per ml at 50°C for 1 h each. The sample volume was then adjusted to 1 ml with 50 mM sodium citrate, and cells were stained with 10 μg of propidium iodide per ml at room temperature for at least 30 min.

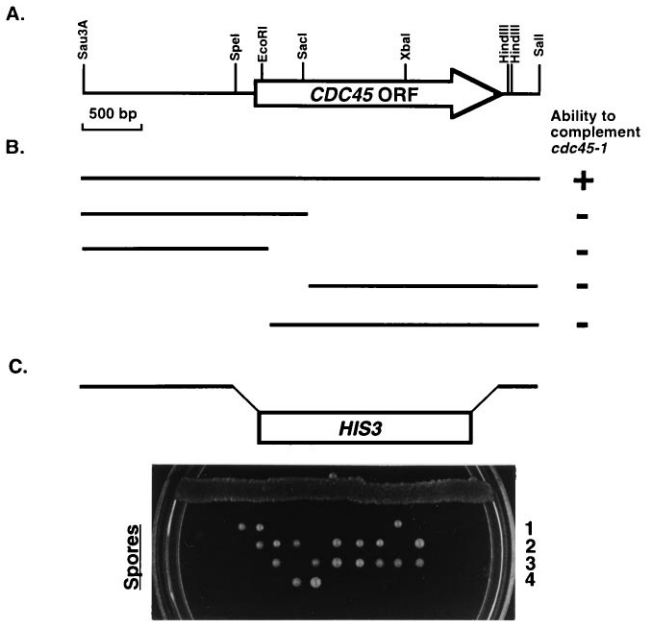

FIG. 1. Structure and genetic analyses of the 3.7-kb genomic fragment containing the *CDC45* gene. (A) Physical map of the 3.7-kb *Sau*3A-*Sal*I genomic fragment. The extent and orientation of the *CDC45* ORF are shown by the arrow. (B) Complementation analysis. Thick lines represent a series of plasmid inserts derived from the *Sau*3A-*Sal*I fragment. The *CDC45* ORF was disrupted at either the *Eco*RI or the *Sal*I site. All genomic fragments shown were subcloned into the yeast vector pRS416. Complementation was scored as the ability to complement the cold sensitivity of *cdc45-1* at 15°C. (C) Disruption of the genomic *CDC45* gene. A *Not*I restriction site was created right before the stop codon of *CDC45* ORF by a PCR-based method. The fragment between *Spe*I and *Not*I sites was excised and replaced with a *HIS3* gene. The disrupted *Sau*3A-*Sal*I fragment was released from the plasmid and used in one-step *CDC45* replacement. Tetrads of the *cdc45*Δ::*HIS3*/*CDC45* diploid were dissected on YPD plates and grown at 23°C.

Plasmid stability assay. Transformants containing either plasmid pDK243 or plasmid pDK368-7 were grown to early stationary phase in SCM-Leu at 23°C. The initial fraction (F_i) of cells that contained the plasmid was determined by plating the same number of cells onto SCM and SCM-Leu plates and incubating them at 23°C. The SCM-Leu cultures were then diluted, and the cells were inoculated into YPD medium and grown at either 18 or 23°C. After 15 doublings, the final fraction (F_f) of cells that retained the plasmid was determined in the same way as F_i. The loss rate was calculated as $1 - 10^{m}$, where $m = [\log(F_f) - \log(F_i)]$/number of cell doubling (41).

2D origin and fork migration analyses. Total DNA was prepared by CsCl gradient centrifugation (32). In the origin analyses, DNA was digested with *Nco*I, and replication intermediates were enriched by BND-cellulose chromatography (39). In the fork migration analyses, DNA was cut with *Nhe*I prior to the first-dimensional gel electrophoresis and then in-gel digested with *Nco*I before the second run (7). Approximately 50 μg of DNA was used for each 2D gel sample. Neutral/neutral (N/N) 2D gels were run, blotted, and probed as described elsewhere (6, 40). The 5-kb *Nco*I-*Nco*I genomic fragment containing *ARS1* and the 3-kb *Nco*I-*Nhe*I fragment adjacent to *ARS1* were used as the probes for origin and fork migration analyses, respectively. All 2D images were visualized by using a Fuji BAS1000 phosphorimager and photographed directly from the computer screen.

Nucleotide sequence accession number. The sequence of the *CDC45* ORF and its flanking region was deposited in GenBank under accession number U65790.

RESULTS

Cloning of the *CDC45* gene. *cdc45-1* is a recessive *cdc* mutant arrested at 15°C (48). Three different *cdc45-1*-complementing plasmids were isolated from a YCp50-based yeast genomic library. Restriction mapping revealed that these plasmids contained overlapping genomic inserts. The complementing activity was further narrowed down to a 3.7-kb fragment which was sequenced and shown to contain only one substantial ORF of 650 amino acids (Fig. 1A and 2). Disruption of this ORF completely abolished the ability of the genomic fragment to complement the cold sensitivity of *cdc45-1* (Fig. 1B).

To test whether this *cdc45-1*-complementing ORF is *CDC45*, we crossed a null mutant of the ORF (for details, see below) with the original *cdc45-1* strain (DBY2027). Since the ORF is essential for viability, the *orf*Δ haploid strain was maintained by wild-type ORF on a *URA3* plasmid (pEM45.3). Once an *orf*Δ/*cdc45-1* diploid was generated, the *URA3* plasmid-containing cells were selected against by 5-FOA. The *orf*Δ/*cdc45-1* diploid strain obtained from 5-FOA selection displayed the cold-sensitive phenotype as did the original *cdc45-1* haploid (data not shown), showing that the recessive *cdc45-1* mutant allele dominated the diploid phenotype in the absence of the ORF. This result indicates that *orf*Δ haploid does not carry wild-type *CDC45*, and therefore the wild-type ORF deleted has to be the *CDC45* gene. Furthermore, the cold sensitivity of the *orf*Δ/*cdc45-1* diploid could be rescued by a plasmid carrying a single copy of wild-type ORF but not vector alone, confirming that the absence of the ORF was responsible for the dominant phenotype of the otherwise recessive *cdc45-1* mutation. To prove that *orf*Δ is indeed a null allele of *CDC45*, 40 tetrads derived from the sporulated *orf*Δ/*cdd45-1* diploid were dissected and cold sensitivity of the surviving spores was tested. Among these tetrads, the majority (35 of 40) gave two viable spores and the rest gave fewer. More importantly, all of the spores that survived were cold sensitive, showing that no recombination occurred between the *cdc45-1* and *orf*Δ alleles. Based on these results, we conclude that the ORF that we have cloned is the bone fide *CDC45* gene.

During the course of this work, genomic sequences from *S. cerevisiae* chromosome XII were released to the genome database (23, 47). One ORF of unknown function (YLR103c/YSCL8004.11) was found to be either identical to our sequence (47) or different in only one base (23) (nucleotide 1727 is C instead of A in our clone; consequently, amino acid 576 is Tyr instead of Lys). Sequence analysis of the predicted *CDC45* gene product suggests that it is a 74.2-kDa acidic protein (pI = 4.37) with a putative bipartite nuclear targeting sequence (55). Although *CDC45* genetically interacts with a group of *MCM* genes (28), its gene product does not show significant sequence similarity to any Mcm protein. Tsd2, an *Ustilago maydis* protein involved in DNA synthesis (50), is highly related to the Cdc45 protein sequence by GenBank BLAST searching (1). The sequences of the two proteins have 35% identity and 54% similarity. Furthermore, two perfect *Mlu*I cell cycle boxes are found in the 5′ region proximal to the coding sequence, suggesting that *CDC45* may be periodically expressed in late G_1 phase as are many other genes involved in DNA replication (reviewed in reference 46).

***CDC45* is essential for cell viability.** The entire *CDC45* coding sequence and approximately 200 bp 5′ flanking region were deleted from the 3.7-kb genomic fragment (from plasmid pEM45.3) that complemented the cold sensitivity of *cdc45-1* and were replaced with a *HIS3* selection marker (Fig. 1C). One-step gene replacement was carried out by transforming a His^- wild-type diploid strain (W303-1) with the *HIS3*-disrupted genomic fragment. Subsequent Southern blot analysis of the His^+ transformants confirmed that one copy of the endogenous *CDC45* was replaced by *HIS3*, while the other copy remained intact (data not shown). The resultant *cdc45*Δ::*HIS3*/*CDC45* diploid was sporulated, and tetrads were dissected. Of the 30 tetrads analyzed, 26 showed 2 viable:2 nonviable spore segregation, and the remaining 4 yielded only one viable spore (Fig. 1C and data not shown). In addition, all viable spores were His^-, indicating that no *cdc45*Δ::*HIS3* haploid survived. Direct microscopic examination of the *cdc45*Δ::*HIS3* spores revealed that they had divided only once before death. To confirm that we did not incidentally inactivate any essential gene other than *CDC45*, the *cdc45*Δ::*HIS3*/*CDC45* diploid was transformed with a single copy of wild-type *CDC45* on a *URA3* plasmid before sporulation. Many tetrads generated from this diploid strain yielded more than two viable spores, and some of them were His^+. All His^+ survivors were also Ura^+, showing that the lethality of *cdc45*Δ::*HIS3* haploid was rescued by the wild-type *CDC45* on the *URA3* plasmid. Consistently, none of the His^+ spores could survive on 5-FOA plates, confirming that the viability of *cdc45*Δ::*HIS3* haploid completely relied on the presence of the rescuing plasmid. Thus, the lethality observed is exclusively due to the specific deletion of *CDC45.*

***cdc45-1* is synthetically lethal with *orc2-1*.** To generate *cdc45-1* strains in the same strain background as the existing *orc* mutants, the original *cdc45-1*^{cs} mutant (DBY2027) was backcrossed four times to W303-1 haploid. The resulting *cdc45-1* strains (YB0298 and YB0299) exhibited a cold-sensitive phenotype identical to that of the original mutant and grew more slowly than the isogenic wild-type strain at 37°C. Both defects of the newly generated strains were fully rescued by the wild-type *CDC45* on a plasmid. Furthermore, 4′,-diamidino-2-phenylindole (DAPI) staining of the backcrossed *cdc45-1* cells showed that their terminal morphology at 15°C was also identical to that of the original mutant: cells were arrested at medial nuclear division, with a single large bud and the nucleus in the neck between the mother and daughter (data not shown).

The backcrossed *cdc45-1*^{cs} strain was crossed to *orc2-1*^{ts}, which is also in the W303-1 strain background. Tetrads from the *ORC2 cdc45-1*/*orc2-1 CDC45* double heterozygote were dissected, and the segregants were grown at 23°C. Among the 23 tetrads analyzed, 28 spores were deduced to be *cdc45-1 orc2-1* double mutants based on the phenotype of their surviving siblings. The majority (22 of 28) of these spores formed

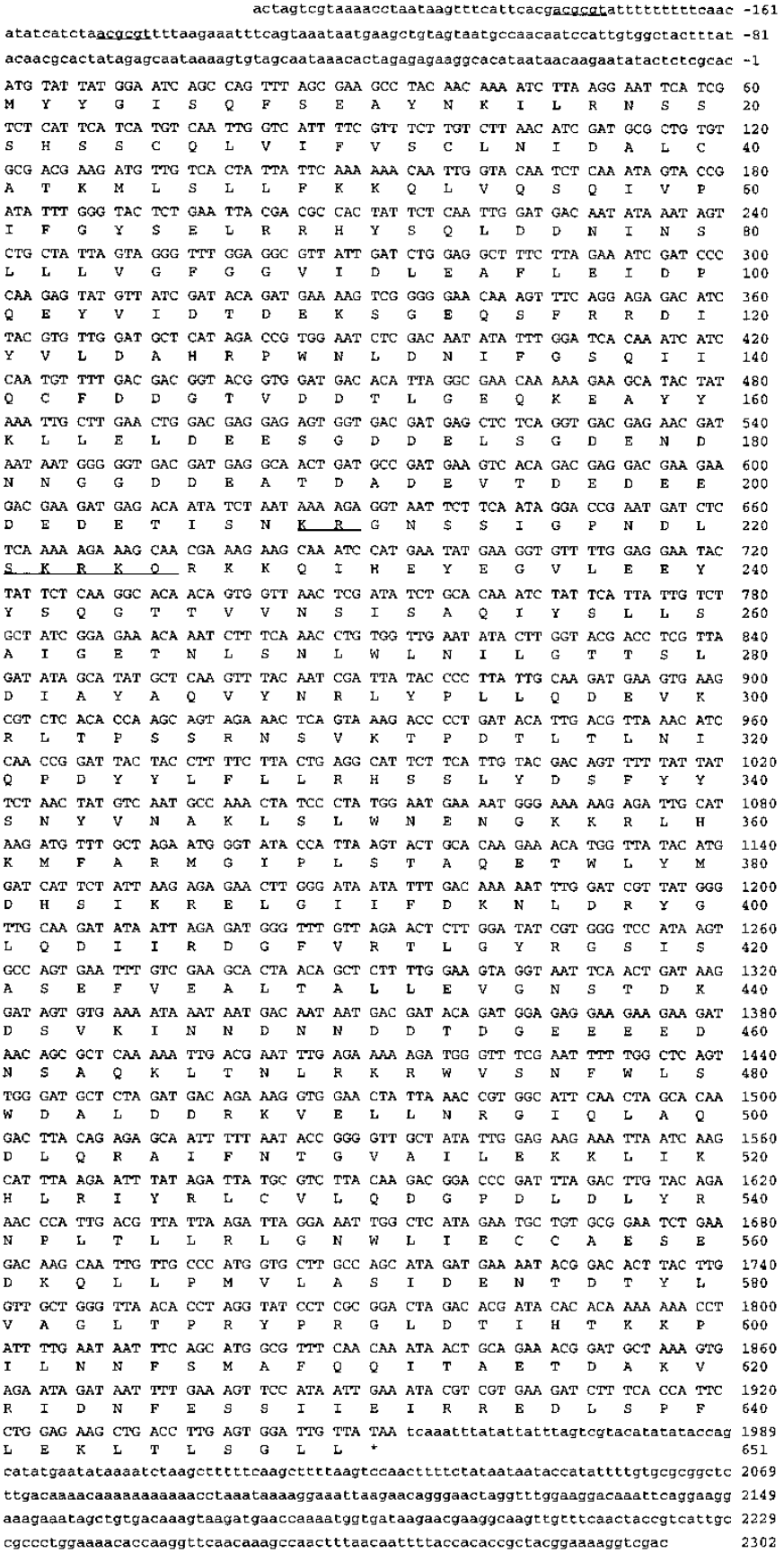

FIG. 2. DNA sequence of a 2.5-kb *Spe*I-*Sal*I fragment containing the *CDC45* ORF and deduced amino acid sequence of the *CDC45* gene product. The two *Mlu*I cell cycle boxes and the putative bipartite nuclear localization signal are underlined.

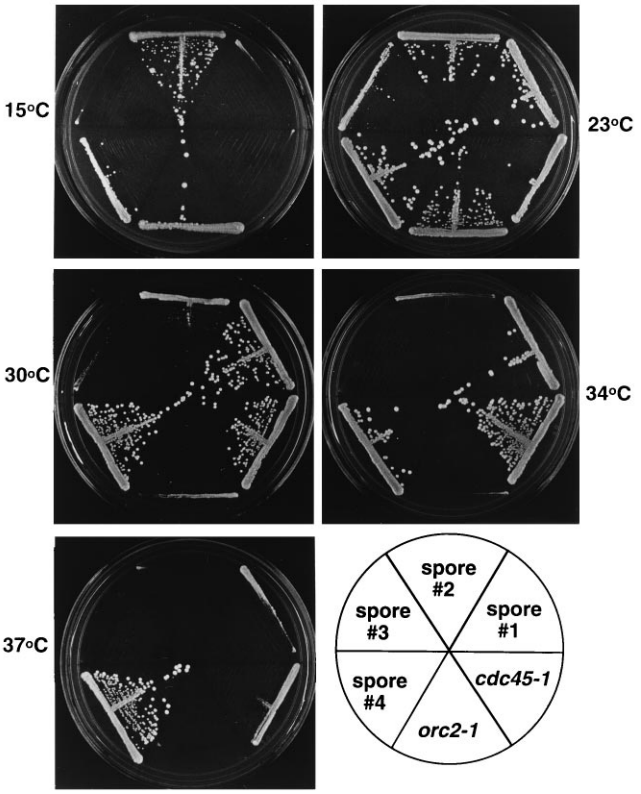

FIG. 3. *cdc45-1 orc2-1* double mutant is nonviable at 30°C. Spores 1 to 4 are siblings from the same tetrad generated by an *ORC2 cdc45-1*/*orc2-1 CDC45* double heterozygous diploid. *cdc45-1* and *orc2-1* single mutants serve as *ts* and *cs* controls at various temperatures. Spore 1 carries *cdc45-1*, spore 2 carries *orc2-1*, spore 3 carries both *cdc45-1* and *orc2-1*, and spore 4 carries neither of the mutant alleles.

microcolonies that were invisible to the naked eye and failed to be recovered. Nevertheless, six cold/heat-double-sensitive spores did form tiny but visible colonies, and they were recovered at 23°C and analyzed further. All six double-sensitive haploid strains grew much more slowly than the *cdc45-1* and *orc2-1* single mutants at 23°C, and none of them survived at 30°C, a permissive temperature for both *cdc45-1* and *orc2-1* single mutants. One such tetrad containing a single *cdc45-1 orc2-1* double mutant is shown in Fig. 3. To verify whether these double-sensitive spores were indeed *cdc45-1 orc2-1* double mutants, we transformed them with plasmids carrying either wild-type *CDC45* or wild-type *ORC2*. The cold sensitivity and temperature sensitivity were rescued by *CDC45* and *ORC2*, respectively, confirming the double-mutant identity of these spores. Taken together, these results show that *cdc45-1* and *orc2-1* are synthetically lethal.

Double mutants from a *cdc45-1* and *orc5-1* cross were constructed and analyzed in the same way. At 23°C, all cold/heat-double-sensitive spores formed colonies that were similar to those of *ts* and *cs* single mutants. However, the double-sensitive spores grew more slowly than both single mutants at 30°C and were lethal at 34°C, a temperature at which *cdc45-1* cells grow normally and *orc5-1* cells form small colonies (data not shown). The cold sensitivity and temperature sensitivity of these spores were also rescued by wild-type *CDC45* and *ORC5*, respectively.

***cdc45-1* is synthetically lethal with *mcm2-1* and *mcm3-1*.** The genetic interaction between *CDC45* and *MCM* genes was first revealed as the suppression of *cdc45-1* by *cdc46-1* and *cdc47-1* (48). *CDC54*, another *MCM* gene, is also implicated in the same genetically interacting group since *cdc54-1* can be specifically suppressed by *cdc46-5* (28). To test whether *CDC45* genetically interacts with the remaining members of the *MCM* family, we crossed *cdc45-1* with *mcm2-1* and *mcm3-1*. The *cdc45-1* strains used in these crosses (YB0298 and YB0299) had been backcrossed four times to W303-1, whereas the *mcm2-1* and *mcm3-1* mutants were in a different strain background. As controls, the wild-type W303-1 haploid and a *cdc6-1* mutant in the W303-1 strain background were also crossed to *mcm2-1* and *mcm3-1* (38). Diploid cells from each cross were sporulated, and at least 20 tetrads from each cross were dissected. Among all tetrads analyzed, neither *cdc45-1 mcm2-1* nor *cdc45-1 mcm3-1* double mutants could be recovered at 23°C, which is permissive for all the single mutants. In contrast, all spores derived from the control crosses were viable at 23°C, indicating that the synthetic lethality observed was not due to incompatible strain backgrounds. In another control experiment, the *cdc45-1* strain was transformed with *CDC45* on a *URA3* plasmid before it was crossed to the *mcm* mutants. *cdc45-1 mcm2-1* and *cdc45-1 mcm3-1* double mutants were recovered only in the presence of the *CDC45*-containing plasmid. Taken together, the data show that *cdc45-1* is synthetically lethal with *mcm2-1* and *mcm3-1*.

***cdc45-1* cells are defective for entry into S phase.** To further investigate the role of *CDC45* in yeast DNA replication, the DNA content of asynchronous *cdc45-1* (YB0298 and YB0299) and isogenic wild-type cells was measured by FACS at various time points after a shift from the permissive to the nonpermissive temperature. At 30°C, a permissive temperature, wild-type and *cdc45-1* cells showed similar FACS profiles except that the mutant cell population contained slightly more (10 to 15%) S-phase cells (Fig. 4A). Soon after the temperature was shifted down to 15°C, the number of *cdc45-1* cells in S phase gradually declined. Concomitantly, mutant cells with a 1C DNA content started to accumulate. These redistributions were specific to the mutant cell population and suggested that entry into S phase slowed down. Between 2 to 3 h, the 1C peak of *cdc45-1* cells started to shorten, and the shoulder between the 1C and 2C peaks began to rise. By 6 h, a significant fraction of mutant cells had entered S phase and accumulated partially replicated DNA. As the time course continued, the FACS peak of the replicating *cdc45-1* cells moved very slowly toward the 2C position, indicating that the mutant genome was being duplicated at an extremely slow rate. After 20 h of incubation at 15°C, most mutant cells were eventually arrested with an approximate 2C DNA content (Fig. 4A). When the same experiment was carried out at 11°C, a more restrictive temperature, most mutant cells were arrested, with the genome duplicated to a lesser extent. These observations suggested that at nonpermissive temperatures, *cdc45-1* cells could still replicate, albeit very slowly, most if not all of their genome.

The defect of entry into S phase was more pronounced in the *cdc45-1* cells that had been synchronized by α-factor. Exponentially growing *MAT***a** *cdc45-1* (YB0298) or W303-1A cells were synchronized with α-factor at 30°C and then released into fresh medium at 15°C. Small aliquots were withdrawn from the cultures at various time points. In addition to analyzing DNA content, we also determined the percentage of cells that remained viable at each time point, by plating out a certain number of cells onto YPD plates and growing them at the permissive temperature. Within 2 h after release, the majority of wild-type cells had entered into S phase, and by 3 h, DNA replication was virtually completed in almost all wild-type cells (Fig. 4B). In contrast, no entry into S phase was observed in

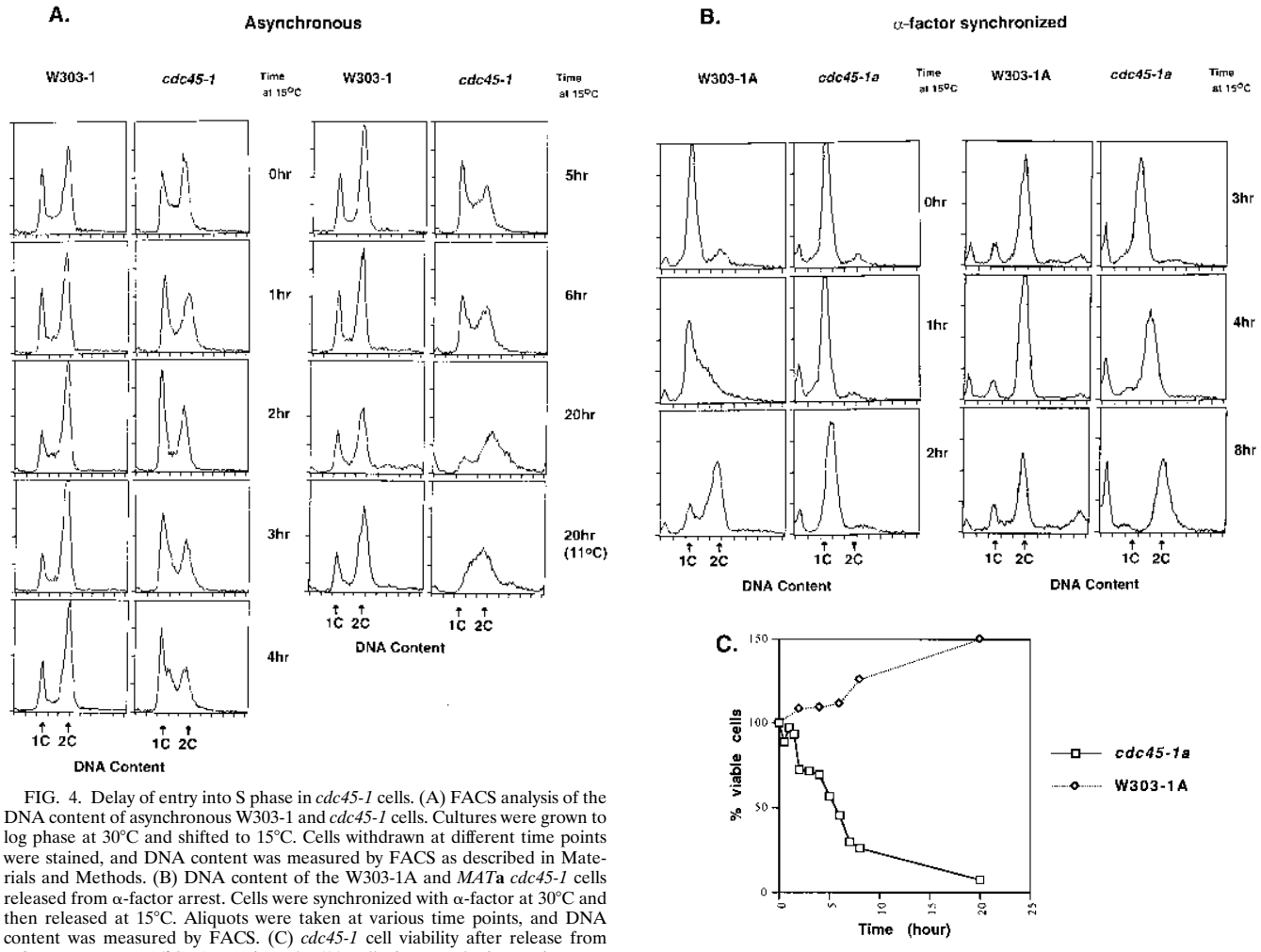

FIG. 4. Delay of entry into S phase in *cdc45-1* cells. (A) FACS analysis of the DNA content of asynchronous W303-1 and *cdc45-1* cells. Cultures were grown to log phase at 30°C and shifted to 15°C. Cells withdrawn at different time points were stained, and DNA content was measured by FACS as described in Materials and Methods. (B) DNA content of the W303-1A and *MAT***a** *cdc45-1* cells released from α-factor arrest. Cells were synchronized with α-factor at 30°C and then released at 15°C. Aliquots were taken at various time points, and DNA content was measured by FACS. (C) *cdc45-1* cell viability after release from α-factor arrest at 15°C. Approximately 500 cells from each time point were plated on YPD plates and grown at 23°C. The number of cells able to form colonies is expressed as a percentage of the number of cells able to form colonies at the zero time point.

cdc45-1 cells until 2 to 3 h after release (Fig. 4B). Furthermore, the mutant cells progressed through S phase much more slowly than the wild-type control. Intriguingly, the viability of *cdc45-1* cells started to decline 2 h after release (Fig. 4C), which correlated with the onset of the delayed S phase. The coincidence between the two events suggested that the replication initiated at the nonpermissive temperature was lethal. Moreover, a significant population of *cdc45-1* cells with less than 1C DNA content appeared after 3 h. The signal is likely to be a result of fragmented DNA of dead cells.

The delayed entry into S phase observed in *cdc45-1* cells could be due to a defect in either initiation or elongation. To distinguish between these two possibilities, we analyzed the DNA content of the wild-type and *cdc45-1* cells (YB0298) released from an HU arrest. α-Factor-synchronized cells were first released into HU-containing medium at 30°C and then released from the HU block at 15°C. FACS analysis revealed that both the wild-type and *cdc45-1* cells rapidly replicated their DNA immediately after being released (data not shown). Therefore, the S phase delay observed in *cdc45-1* cells was largely rescued by allowing initiation to take place at the permissive temperature. Nevertheless, the rate of progression through S phase was slightly reduced in *cdc45-1* cells, suggesting either that fewer origins fired in the mutant cells even at the permissive temperature or, alternatively, that the *cdc45-1* mutation also weakly affects elongation.

As shown in Fig. 4B, the FACS peak of *cdc45-1* cells was stalled at the 1C position until 2 h after α-factor release at 15°C. Once shifted to 30°C, the stalled mutant cells were capable of progressing through S phase rapidly (data not shown). When the *cdc45-1* mutant was shifted to 15°C for 2 h and then shifted back to 30°C in the presence of HU, the cells did not replicate their DNA, suggesting that the *cdc45-1* arrest was prior to the HU block.

The high plasmid loss rate of *cdc45-1* can be suppressed by extra origins on a plasmid. Minichromosomes (plasmids with a single ARS, centromere, and selection marker) are lost at high rate from mutant cells in which they cannot be replicated and/or segregated properly. If a mutant carries a loss-of-function mutation in a protein that functions at replication origins, its high plasmid loss rate could be quantitatively suppressed by placing multiple copies of the ARS onto the plasmid. Successful examples of such suppression have been reported for *cdc6*, *orc2*, *orc3*, and *orc5* mutants (26, 29, 41). To test how the *cdc45-1* mutation affects maintenance of minichromosomes, the *cdc45-1* strain (YB0299) was transformed with a plasmids containing either one copy or tandem copies of an ARS, and the stability of both plasmids in dividing cells was assayed at

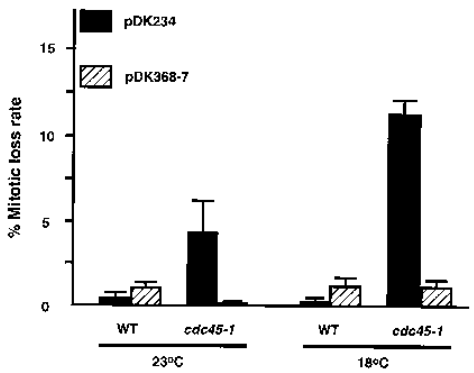

FIG. 5. The high plasmid loss rate of *cdc45-1* can be suppressed by extra origins on the plasmid. pDK243 is a plasmid that contains a single copy of *ARS1* and a *LEU2* selection marker. pDK368-7 is the same plasmid with an additional seven copies of *H4 ARS*. W303-1 and *cdc45-1* cells containing either of the plasmids were grown to early stationary phase in SCM-Leu medium at 23°C and then diluted and grown nonselectively in YPD at either 18 or 23°C. The total plasmid loss was measured after cells underwent 15 doublings, and the mitotic loss rates were calculated as described in Materials and Methods.

various temperatures. At 23°C, a permissive temperature, the single-ARS plasmid pDK234 was lost from *cdc45-1* cells at a rate 10-fold higher than that for the wild-type control. This *mcm* phenotype (high plasmid loss rate) of *cdc45-1* was suppressed by extra origins, as the multiple-ARS plasmid pDK368-7 was stably maintained in the mutant cells (Fig. 5). The loss rate of the single-ARS plasmid observed in *cdc45-1* cells at 23°C was lower than those in *cdc6* or *orc* mutants (26, 29, 41). When the same experiment was repeated at 18°C, a temperature at which *cdc45-1* cells could still grow at a slow rate, the loss rate of single-ARS plasmid pDK234 from mutant cells was elevated to a level more than 30-fold higher than the control level. This more severe defect was also largely rescued by the extra origins on the plasmid (Fig. 5). Therefore, our results suggest that like Cdc6p and ORC, Cdc45p functions at replication origins.

***CDC45* is required for efficient initiation of DNA synthesis at chromosomal replication origins.** The initiation of DNA replication from individual chromosomal origins can be studied by N/N 2D origin-mapping gels (6). All forms of replication intermediate, such as bubble, fork, and linear DNA molecules, are readily separable by this method. In addition, fork migration analysis has been adapted from the 2D origin-mapping gels to study how replication forks pass through a region that does not contain an active origin (7). Therefore, one can address, based on the results of 2D origin and fork migration analyses, whether the genomic fragment being probed is replicated by internally emerged bubbles or by passing replication forks that initiated at an origin located away from the locus being probed.

In our 2D origin analysis, total DNA was prepared from asynchronous cultures of wild-type or *cdc45-1* cells (YB0299) grown at 30°C or from those that had been shifted to 15°C at various time points before harvest. The DNA isolated was subsequently digested with *Nco*I, enriched for replication intermediates, and subjected to N/N 2D gel electrophoresis. Finally, the blots were probed with the 5-kb *Nco*I genomic fragment containing *ARS1* (45).

At both 30 and 15°C, the wild-type strain gave a clear bubble-to-fork transition, that is, intensive bubble arc plus essentially no small forks. This result demonstrated that the probability of initiating at the chromosomal *ARS1* locus was close to 1.0 in wild-type cells (Fig. 6). Similarly, the *cdc45-1* strain also

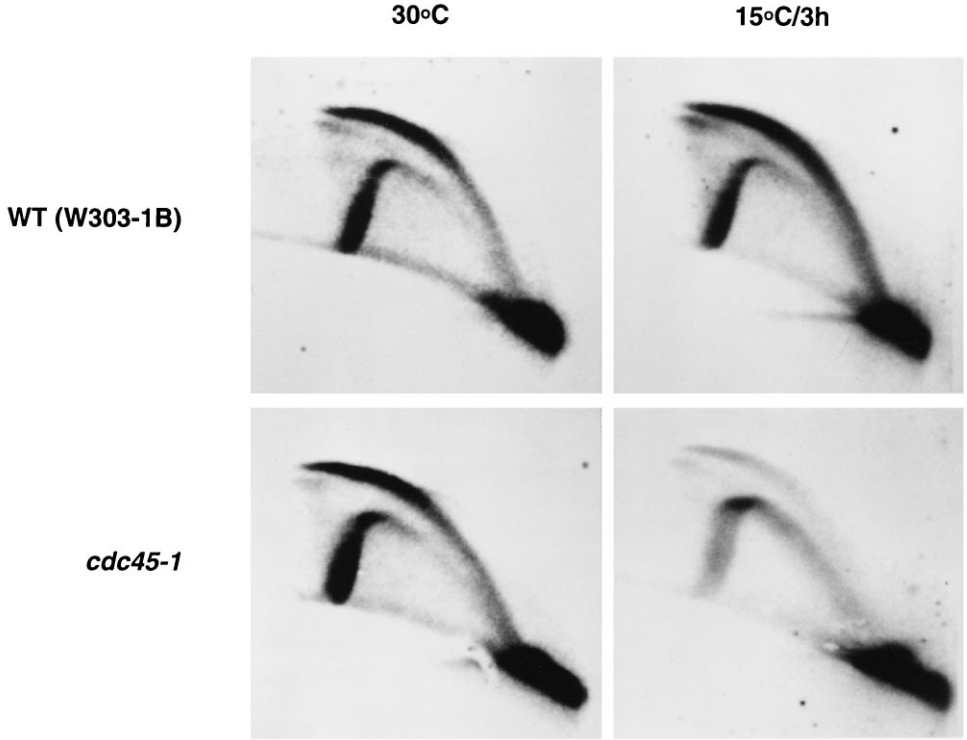

FIG. 6. N/N 2D origin analyses of the chromosomal *ARS1* locus in W303-1 and *cdc45-1* cells. Cells were grown and harvested at 30°C or shifted to 15°C for 3 h prior to harvest. DNA was digested with *Nco*I and probed with the 5-kb *Nco*I-*Nco*I genomic fragment containing *ARS1*.

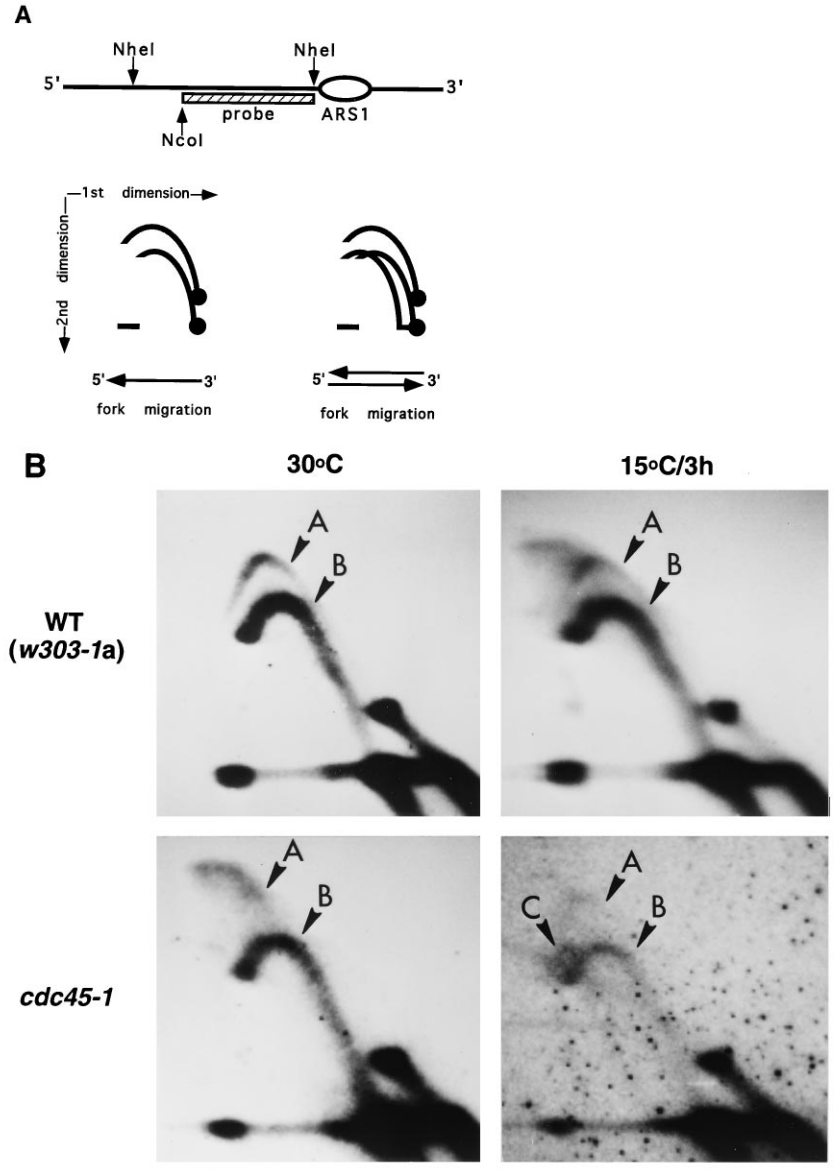

FIG. 7. Fork migration analyses of the 4-kb *Nhe*I-*Nhe*I genomic region adjacent to *ARS1*. (A) Top, schematic representation of the relative positions of *ARS1*, the probe, and the restriction sites used. Bottom, typical 2D gel profiles that result from replication forks passing from the 3′ side (left) or both sides (right). (B) Results of the fork migration analyses. Cells were grown under the conditions described for Fig. 6. DNA was cut with *Nhe*I before the first-dimensional gel electrophoresis and then in-gel digested with *Nco*I prior to the second run. Arc A, the uncut arc resulted from incomplete *Nco*I digestion; arc B, the arc generated by the replication forks from the 3′ side; arc C, the arc generated by the forks from the 5′ side.

showed a bubble-to-fork transition at 30°C, suggesting that the initiation from *ARS1* was not obviously affected by the mutation at this temperature. However, the 2D profile was greatly changed if the cells were shifted to 15°C 3 h before harvest. First, the intensity of the bubble arc was significantly diminished, indicating that *ARS1* was active in only a small fraction of the mutant cells (Fig. 6). Second, the bubble-to-fork transition was replaced by a complete fork arc, suggesting that the *ARS1*-containing region in many mutant cells was passively replicated by forks that had initiated from elsewhere. In longer time points, the 2D gel profile of mutant cells remained largely unchanged except that the intensity of overall signal gradually declined and became barely detectable after 9 h, the time when most of the *cdc45-1* cells were already in late S phase as indicated by FACS (data not shown). To test whether the initiation defect caused by the *cdc45-1* mutation was ARS specific, the blots were stripped and reprobed with a genomic fragment containing *ARS501* (20). In agreement with the results for *ARS1*, *ARS501* could fire in only a small fraction of mutant cells at the nonpermissive temperature (data not shown), supporting the notion that *CDC45* was required generally for efficient initiation of chromosomal replication.

To confirm that the initiation from *ARS1* was reduced in *cdc45-1* cells at the nonpermissive temperature, we analyzed the 4-kb *Nhe*I-*Nhe*I genomic region immediately adjacent to *ARS1* by using a fork migration assay (Fig. 7A). Genomic DNA was cut with *Nhe*I, and the resulting fragments were separated in the first dimension. Before being subjected to gel electrophoresis in the second dimension, the replication intermediates were digested in gel with *Nco*I (Fig. 7A). The 3-kb *Nco*I-

*Nhe*I fragment shown in Fig. 7A was used as a probe for the Southern blot. Since the second digestion removes 1 kb from the 5′ ends of the replication intermediates being probed, the position of fork arc would change according to the direction of fork movement (see references 7 for more detailed mechanism of fork migration analysis).

As shown in Fig. 7B (upper panels), the wild-type strain displayed two fork arcs at both 30 and 15°C; the upper one (arc A) resulted from the uncut *Nhe*I-*Nhe*I fragments, and the lower one (arc B) resulted from the shorter *Nco*I-*Nhe*I fragments. Since the lower arc was displaced vertically from the upper one, it suggested that all of the replication forks came from the 3′ side of the fragment, the same side as *ARS1* (see reference 7 for the basis of interpretation). This result is consistent with our earlier finding that the probability of *ARS1* firing is close to 1.0 in wild-type cells at both temperatures. A similar 2D gel profile was also observed in the *cdc45-1* strain at 30°C, confirming that *ARS1* was being actively used at the permissive temperature (Fig. 7B, lower left panel). After 3 h of incubation at 15°C, however, the total amount of replication from *ARS1* was lower than that observed at 30°C. More importantly, the mutant strain exhibited a less intensive arc B and a third fork arc (Fig. 7B, lower right panel, arc C), which was displaced both horizontally and vertically from the arc A. This newly emerged fork arc directly demonstrated that a substantial fraction of the *Nhe*I-*Nhe*I fragment was replicated by forks passing from right to left in Fig. 7A, apparently due to loss of *ARS1* dominance over the region. Furthermore, the decrease in arc B intensity also suggested that the initiation from *ARS1* was reduced.

In both 2D origin and fork migration analyses, the overall fork arc signal of the *cdc45-1* strain was reduced at 15°C. These observations indicated that passive replication cannot completely compensate for the reduction of *ARS1* usage at the nonpermissive temperature. As suggested by our earlier result with *ARS501*, the adjacent origin responsible for the passive replication was also likely to be weakened along with *ARS1* under this condition. This results in an overall lower frequency of initiation of DNA replication in the genome and therefore problems with progression through S phase at 15°C.

DISCUSSION

Previous studies of the *cdc45-1* mutant have demonstrated that its cold sensitivity could be suppressed by *cdc46-1* and *cdc47-1*, two mutants of *MCM* genes (48). In addition, the suppression by *cdc46* was found to be allele specific (28). Only *cdc46-1*, not *cdc46-5*, could rescue the mutant phenotype of *cdc45-1* at its nonpermissive temperature. Such allele-specific suppression by *cdc46-1* mutant suggests that the interaction between Cdc45p and Cdc46p, one of the Mcm proteins, is specific and could be direct. In the study reported here, we observed that *cdc45-1* was synthetically lethal with *mcm2-1* and *mcm3-1*. Therefore, the *CDC45-MCM* genetic interaction was extended to all of the five characterized *S. cerevisiae MCM* genes that had been implicated in initiation of DNA replication. Results of all these genetic studies indicate that Cdc45p is functionally related to the Mcm proteins.

Based on the experiments using *Xenopus* egg extracts, Blow and Laskey proposed that rereplication cannot normally occur within a single cell cycle because replication licensing factor (RLF), which is essential for initiation, can be used only once in each cell cycle (5). Several recent studies have shown that RLF is not a single factor as originally proposed but rather a sophisticated activity involving many proteins (11, 42, 43). Mcm proteins have been suggested to be a part of the RLF activity. First, *mcm* mutants are apparently defective in replication initiation (10, 24, 28, 66). Second, the timing of nuclear presence (or chromatin binding) of Mcm proteins during cell cycle coincides with the timing of RLF activity (16, 27, 67). Furthermore, a complex of Mcm homologs has been purified as a component of RLF activity from *Xenopus* egg extracts (11, 36, 42, 43), suggesting that *S. cerevisiae* Mcm proteins might also form a complex and play an essential role in licensing. In support of this hypothesis, the five *S. cerevisiae* Mcm family members involved in replication initiation are not only functionally but also structurally related to each other (16, 65, 66). On the other hand, although *CDC45* is genetically linked to all of these *MCM* genes, its predicted protein product does not show substantial sequence similarity to any Mcm protein. The lack of sequence similarity indicates that Cdc45p might be functionally distinguishable from Mcm proteins. However, as suggested by the genetic interactions, the role of Cdc45p must be closely related to that of the Mcm complex. Thus, Cdc45p could be a part of the RLF activity, as is the Mcm complex, and therefore might be involved in establishing the competent pre-RC. Alternatively, Cdc45p may interact with the Mcm complex at or even after the time when initiation occurs. Such interactions might be required for the final execution of the licensed replication origins.

In support of the idea that Cdc45p contributes to RLF activity either directly or indirectly, *cdc45-1* displays defects in replication initiation. A delayed entry into S phase was observed by FACS analysis in both asynchronous and α-factor-synchronized mutant cells at the nonpermissive temperature. After entering S phase, mutant cells replicated their DNA at a very slow rate and were eventually arrested with a partially duplicated genome. Similar observations have been made for *orc2-1*, *orc5-1*, and *mcm3-1* (3, 24, 41), and they can be explained by fewer active chromosomal origins in the genome. Consistent with this notion, at 15°C, *cdc45-1* cells were temporarily stalled prior to the elongation stage that can be blocked by HU. Interestingly, like *orc2-1* (3), *cdc45-1* cells began to lose viability when replication was initiated at the nonpermissive temperature. These findings indicate that initiation at the nonpermissive temperature irreversibly leads to abortive genome duplication and is lethal to the cells. On the other hand, *cdc45-1* cells appeared to be quite normal in the elongation stage. After being released from an HU block *cdc45-1* cells did not show an obvious defect, suggesting that the S-phase delay was caused by inefficient initiation. That is, the probability of initiation of replication at every origin was very low at the nonpermissive temperature.

The high chromosome loss rate of *cdc45-1* when the plasmid contained a single origin was suppressed by adding extra ARSs onto the plasmid, suggesting that the partial loss of Cdc45p function could be quantitatively compromised by multiple origins. Similar suppression has been reported for *orc2*, *orc3*, *orc5*, *cdc6*, and *cdc14* (26, 29, 41). As shown by many studies using different approaches (3, 13, 21, 37, 40), ORC and Cdc6p play essential roles at individual replication origins. In all of these cases, the probability of initiation is increased if the chromosome contains multiple ARSs rather than a single origin. Therefore, the suppression of *cdc45-1* provides an evidence for the functional connection between Cdc45p and replicators.

The function of Cdc45p at replication origins was directly demonstrated by 2D origin and fork migration analyses. At the nonpermissive temperature, each origin fired in only a fraction of *cdc45-1* cells in the population. Since the two origins tested at random (*ARS1* and *ARS501*) behaved similarly, we suggest that every origin fired at a lower frequency in the *cdc45-1* cell

population at 15°C. Thus, one would expect that in any given mutant cell, only a subset of replicators were active. Based on the different 2D gel profiles, Liang et al. (38, 40) have classified the proteins involved in replication initiation into two groups. Proteins such as Orc2p, Orc5p, and Cdc6p function at individual replicators and affect the efficiency of origin firing in the genome. At the nonpermissive temperatures of the mutants of these genes, only a small fraction of the replication origins fire in S phase. On the other hand, proteins like Cdc28p, Cdc34p, and Cdc7p are global regulators for cell cycle progression. Mutant cells are blocked before the entry into S phase at their nonpermissive temperatures, and therefore no partial firing of individual origins can be observed. Thus, our 2D gel results for *cdc45-1*, from both the origin and the fork migration analyses, suggest that Cdc45p is involved in the initiation from individual origins, just as are ORC and Cdc6p. Nevertheless, we noticed a difference between the 2D gel profile of *cdc45-1* and those of *orc2*,*5* and *cdc6* at the permissive temperature (40). Origin-mapping gels of the latter ones exhibited complete fork arcs even when cells were grown at the permissive temperature, suggesting that origin firing was reduced. In contrast, we did not observe an obvious initiation defect in *cdc45-1* at its permissive temperature. This difference could be due to either the weak penetrance of the *cdc45-1* allele at 30°C or a more indirect interaction between Cdc45p and origins. Consistent with the 2D gel results, the plasmid loss rate of *cdc45-1* at its permissive temperature is lower than those of the *orc* and *cdc6* mutants (26, 39, 41).

The synthetic lethality of *cdc45-1 orc2-1* double mutants and the increased temperature sensitivity of *cdc45-1 orc5-1* double mutants functionally join Cdc45p and ORC together. Combined with the genetic interaction between *CDC45* and *MCM*s as well as that between *ORC* and *MCM*s (38, 40), these results suggest that Cdc45p, ORC, and Mcm proteins act in concert in vivo. According to several recent studies using various model systems, Mcm proteins become chromatin bound in late M phase with the help of yet to be identified loading factors (11, 42, 43). In G_1 phase, the in vivo footprint of the pre-RC appears at replicators (17). Cdc6p has been shown to be required for both formation and maintenance of such a footprint (13). Furthermore, the S-phase-promoting function of Cdc6p is restricted to a time window from late M phase to the G_1/S transition, the period when Clb/Cdc28 activity is absent (52). At the G_1/S transition, nuclear Mcm proteins become partially phosphorylated from their underphosphorylated state (14). After replication is initiated, the pre-RC footprint is dissembled and Mcm proteins are displaced from chromatin (35, 43). As indicated by all of these results, the cell cycle-regulated phosphorylation of Cdc6p and Mcm proteins may be involved in triggering the pre-RC and preventing its reformation after initiation. Since *cdc45-1* cells are defective after being released from α-factor arrest at the nonpermissive temperature, Cdc45p apparently has a role after Start. However, the possibility that Cdc45p is also involved in the loading of the Mcm complex onto chromatin which occurs at the end of mitosis cannot be ruled out. Based on our results, it is reasonable to speculate that Cdc45p is required for interaction and/or function of the pre-RC in initiation. Cdc45p might be a structural and/or functional component of the pre-RC, such as the link between ORC and the Mcm complex. Alternatively, Cdc45p might participate in activating and/or disassembling the pre-RC during initiation. For instance, it may play a role in displacing the Mcm proteins from chromatin. The exact role of Cdc45p in replication initiation remains to be addressed by further biochemical investigation.

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ADDENDUM IN PROOF

B. Hopwood and S. Dalton have also reported the isolation of the gene encoding Cdc45p (Proc. Natl. Acad. Sci. USA **93:**12309–12314, 1996).

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