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Characterization of Cdc47p-Minichromosome Maintenance Complexes in *Saccharomyces cerevisiae*: Identification of Cdc45p as a Subunit

STEPHEN DALTON* AND BLAIR HOPWOOD

Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5005, Australia

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Cdc47p is a member of the minichromosome maintenance (MCM) family of polypeptides, which have a role in the early stages of chromosomal DNA replication. Here, we show that Cdc47p assembles into stable complexes with two other members of the MCM family, Cdc46p and Mcm3p. The assembly of Cdc47p into complexes with Cdc46p does not appear to be cell cycle regulated, making it unlikely that these interactions per se are a rate-limiting step in the control of S phase. Cdc45p is also shown to interact with Cdc47p in vivo and to be a component of high-molecular-weight MCM complexes in cell lysates. Like MCM polypeptides, Cdc45p is essential for the initiation of chromosomal DNA replication in *Saccharomyces cerevisiae*; however, Cdc45p remains in the nucleus throughout the cell cycle, whereas MCMs are nuclear only during G_1. We characterize two mutations in *CDC47* and *CDC46* which arrest cells with unduplicated DNA as a result of single base substitutions. The corresponding amino acid substitutions in Cdc46p and Cdc47p severely reduce the ability of these polypeptides to assemble in a complex with each other in vivo and in vitro. This argues that assembly of Cdc47p into complexes with other MCM polypeptides is important for its role in the initiation of chromosomal DNA replication.

Eukaryotic DNA replication is controlled through the activity of replication origins, which initiate new rounds of DNA synthesis once per S phase in each cell cycle (12). Although little is known about how the initiation of chromosomal DNA replication is controlled, it is clear that once DNA replication is initiated, negative controls block the reestablishment of the prereplicative state at replication origins. These negative controls persist throughout G_2 and mitosis but are removed as cells enter G_1 (34), thus ensuring that initiation events at origins are restricted to only once per cell cycle. An activity of *Xenopus* egg extracts known as replication licensing factor (RLF) has previously been implicated in the establishment of the prereplicative state of cells in G_1 (1, 7). RLF activity is thought to function by establishing functionally competent preinitiation complexes at replication origins after breakdown of the nuclear envelope. After performing its licensing role and immediately after an initiation event, the model predicts that RLF is inactivated or destroyed, leaving replication origins in a postreplicative state (1). This provides a simple framework to explain how S phase and mitosis are maintained in the correct order and how replication origins function only once per cell cycle.

The initiation of DNA replication in the budding yeast *Saccharomyces cerevisiae* requires the minichromosome maintenance (MCM) family of polypeptides, which include Mcm2p and Mcm3p (38), Cdc54p/Mcm4p (37), Cdc46p/Mcm5p (18), Mcm6p (5), and Cdc47p/Mcm7p (10). The six members of the MCM family carry a highly conserved motif (the MCM box) which resembles a conserved domain associated with transcription and replication factors with known or assumed DNA-dependent ATPase activity (24). Strains carrying loss-of-function *mcm* mutations typically arrest with unreplicated DNA, a duplicated spindle pole body, and a large bud at the last genetically definable point in G_1. Their role as direct regulators in the initiation of DNA replication is suggested by a number of criteria (18, 27), including observations that they are necessary for the maintenance of autonomously replicating sequence-containing plasmids (3, 14, 38) and in the activity of chromosomal origins of replication (39). The recent demonstration that members of the *MCM* gene family interact with components of the origin recognition complex (29, 35) indicates that MCM polypeptides may play an important role in controlling origin activity through direct recruitment into preinitiation complexes (11).

The subcellular localization of MCM polypeptides in budding yeast changes with the cell cycle (10, 17, 39). Toward the completion of mitosis, these polypeptides are rapidly transported from the cytoplasm into the nucleus, where they remain until the beginning of S phase. As DNA replication begins, MCM polypeptides disappear from the nucleus and relocalize to the cytoplasm, where they remain until the next round of cell cycle-regulated nuclear import (10). Although MCM polypeptides do not shuttle in and out of the nucleus in metazoans, it appears that their ability to bind chromatin in G_1 and then to become displaced from chromatin during S phase (6, 16, 21, 32) is conserved between species. This behavior is consistent with MCM polypeptides playing a role in establishing the prereplicative state and in restricting origin activity to once per S phase, as predicted in the RLF model (1).

Recently, MCM polypeptides have been identified as part of the RLF activity (RLF-M) originally identified in *Xenopus* egg extracts (4, 25, 30). This activity consists of MCM polypeptides assembled into higher-order complexes with each other as well as with other unidentified components, collectively known as RLF-B (4). The ability of at least some MCM polypeptides to assemble together in complexes has also previously been demonstrated in *S. cerevisiae* (26), *Drosophila melanogaster* (36), *Xenopus* egg extracts (4, 30), mice (22), and humans (2), although the exact nature of these complexes is not fully under-

* Corresponding author. Phone: 011 61 8 303 3793. Fax: 011 61 8 303 4348. E-mail: sdalton@biochem.adelaide.edu.au.

stood. Genetic evidence suggests a role for Cdc45p in the control of MCM activity in *S. cerevisiae* (18). *CDC45* is essential for the initiation of DNA replication (18, 19, 40); it genetically interacts with *CDC46* and *CDC47*, but unlike the MCM polypeptides in budding yeast, Cdc45p is localized to the nucleus throughout the cell cycle (19). Cdc45p does, however, physically interact with Cdc46p (19). This raises the possibility that Cdc45p is an accessory component of MCM complexes and part of the RLF system, which restricts DNA replication to once per cell cycle. Here, we characterize the assembly of Cdc47p/Mcm7p into complexes with two other MCM polypeptides from *S. cerevisiae* and identify Cdc45p as a subunit of these complexes.

MATERIALS AND METHODS

Yeast strains, plasmids, expression constructs, and two-hybrid analysis. Cells were routinely grown in YEPD medium (1% yeast extract, 2% Bacto Peptone, 100 μg of adenine per ml, 2% glucose). All *cdc* strains used in this study were backcrossed to the W303 background (*MAT***a**/α *ura3-52 trp1-1 ade2-1 his3-11,15 can1-100* [*psi*$^+$]). All the other strains have been described previously (31). The LexOp-LacZ reporter strain S330 [(*lexOp*)$_6$-*lacZ*::*ura3*] was made by inserting the reporter into the *URA3* locus as described previously (9). To construct a strain conditionally depleted of Cdc46p (S339), we used the heat-sensitive degron approach (13) by replacing the *CDC46* gene with a temperature-sensitive (ts) *ubi-CDC46* fusion by a one-step recombination event. Strains expressing polypeptides with hemagglutinin (HA; 12ca5) or c-myc (9e10) epitope tags were constructed by replacing the untagged gene with the tagged version also by one-step gene replacement. Two-hybrid assays were performed by growing cells to an optical density at 600 nm (OD_{600}) of 0.8 in yeast extract-peptone medium supplemented with 2% galactose (YEPG medium) and then assessing reporter gene activity by a quantitative β-galactosidase assay (9).

The following constructs were used: YEpCDC46, *URA3*$^+$ 2μm YEp24 backbone; YEpCDC47, *URA3*$^+$ 2μm YEp24 backbone; YEpMCM3, *URA3*$^+$ 2μm YEp24 backbone; YLexA(2-202), ATGGGATCC LexA (2-202); YLexCDC47, LexA (2-202)-Cdc47 (1-845); YLexCDC46, LexA (2-202)-Cdc46 (1-775); YLexMCM3, LexA (2-202)-Mcm3 (1-971); pSD.06, Vp16 (412-490); pVCDC46, Vp16 (412-490)-Cdc46 (1-775); pVCDC47, Vp16 (412-490)-Cdc47 (1-845); pVMCM3, Vp16 (412-490)-Mcm3 (1-971); YVCDC45, CDC45 (1-650)-Vp16 (412-845); YLexCDC45, LexA (2-202)-CDC45 (1-650); YCpCDC46.Tag, Cdc46 (1-775)-YPYDVPDYA.stop codon; YCpCDC47.Tag, Cdc47 (1-845)-YPYDVPDYA.stop codon; and YCpMCM3.Tag, Mcm3 (1-971)-YPYDVPDYA.stop codon. Further details of constructs are available on request.

Characterization of *cdc47-1* and *cdc46-1* mutations. Mutations were mapped by a series of targeted recombination events, designed to repair gapped plasmids in vivo (38). This method allowed us to initially map the positions of mutants to specific restriction fragments within the *CDC47* and *CDC46*/*MCM5* open reading frames. After recovery of gap-repaired plasmids carrying either the *cdc47-1* or *cdc46-1* mutant allele, restriction fragments carrying each mutation were sequenced, which led to the identification of single base substitutions. Two plasmids, YCpCDC47 and YCpCDC46, rescue the *cdc47-1* and *cdc46-1* ts mutants at 37°C, respectively. To test whether each point mutation identified by the gap repair strategy was responsible for the original *cdc* defect, *cdc46-1*^{G549A} and *cdc47-1*^{C857A} mutations were introduced into the YCp plasmid carrying the corresponding wild-type gene and then transformed back into the *cdc46-1* or *cdc47-1* strain as a functional test. From a total of more than 400 transformants expressing mutant MCM polypeptides, none grew during replica plating at 37°C (data not shown). In contrast, all the colonies that were transformed (>500 tested) with wild-type YCpCDC46 and YCpCDC47 and grew at 25°C were also viable at 37°C. The *cdc46*^{G549A} and *cdc47*^{C857A} mutations therefore account for the *cdc* phenotype of *cdc46-1* and *cdc47-1* alleles, respectively.

Antibodies. Polyclonal antibodies against purified antigen synthesized in bacteria and consisting of Cdc46p(1-352)-His_6 were raised in rabbits. Antigen was sent to the Pocono Rabbit Farm and Laboratory (Canadensis, Pa.), where rabbits were immunized by their standard protocol. Rabbit polyclonal anti-LexA antibodies have been described previously (33). The immunoglobulin G (IgG) fractions in preimmune and immune sera were purified with caprylic acid (15), and antibodies were affinity purified on a column where antigen was coupled to Affi-Gel (Bio-Rad) according to the manufacturer's directions. HA- and c-myc epitope-tagged polypeptides were detected with affinity-purified 12ca5 (Berkeley Antibody Co.) and 9e10 (Gerard Evan, ICRF, London, United Kingdom), monoclonal antibodies, respectively. In vitro translations in rabbit reticulocyte lysates were performed as described previously (9).

Cell lysates, gel filtration, immunoprecipitations, and immunoblotting. Cells were grown to exponential phase (OD_{600} of 0.8), washed three times in 3 volumes of ice-cold lysis buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone], 25 mM TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone]), and finally resuspended in 0.3 ml of lysis buffer. Cells were lysed at 4°C with 0.5-μm-diameter glass beads in a multibead beater (BioSpec products, Bartlesville, Okla.) by three 30-s bursts at maximum intensity. Lysates (2 to 5 mg of protein/ml) were clarified by centrifugation in a microcentrifuge for 15 min at 4°C and used immediately or snap frozen under liquid N_2. For the fractionation of crude cell lysates by gel filtration, fresh unfrozen extract was used. Total cell lysate (200 μg of total protein) in lysis buffer was clarified through a 0.4-μm-pore-size filter and loaded on a calibrated Superdex 200 (Pharmacia) gel filtration column (flow rate, 1 ml/min) equilibrated and run in lysis buffer at 4°C. Immunoprecipitations were performed directly from column fractions, where appropriate, with affinity beads after preclearing with bovine serum albumin-coupled beads as described below.

For immunoprecipitations from crude cell lysates, extracts were diluted to 0.5 mg of protein/ml with lysis buffer and absorbed against 30 μl of bovine serum albumin-Sepharose beads (50% [vol/vol] slurry) by tumbling for 30 min at 4°C. Beads were pelleted, and the supernatant was tumbled with 20 μl of antibody-Sepharose affinity beads (10 mg of affinity-purified antibody per ml of cyanogen bromide-activated Sepharose beads [15]) for a further 2 h. After the affinity beads were washed four times with cold lysis buffer, immunoprecipitated polypeptides were analyzed directly on a denaturing sodium dodecyl sulfate (SDS)–8% polyacrylamide gel. Immunoblotting and the conditions for SDS-polyacrylamide gel electrophoresis (PAGE) were described previously (10). The detection of pyruvate carboxylase (PC) after immunoblotting was carried out as previously described (28).

TABLE 1. *MCM3* is a high-copy-number suppressor of *cdc47-1* and *cdc46-1* mutants[a]

Strain	*cdc* mutation	Viability[b]
S129 (W303 1a)	*CDC*$^+$	+
S220	*cdc47-1*	+
S102	*cdc6-2*	−
S103	*cdc23-1*	−
S104	*cdc13-1*	−
S105	*cdc15-2*	−
S134	*cdc7-2*	−
S218	*cdc46-1*	+
S219	*cdc45-1*	−
S221	*cdc54-1*	−
S253	*cdc4-2*	−
S254	*cdc34-2*	−

[a] Isogenic strains carrying different *cdc* mutations were transformed with YEpMCM3, a 2μm-based high-copy-number plasmid carrying the entire *MCM3* gene. All *cdc* mutants are ts at 37°C, with the exception of *cdc45-1* and *cdc54-1* mutants, which are cold sensitive at 12°C.

[b] +, mutant grows at a rate comparable to that of the *CDC*$^+$ strain at the normal restrictive temperature; −, mutant whose restrictive temperature remains unchanged.

RESULTS

***CDC47* genetically interacts with *CDC46* and *MCM3*.** *CDC47* genetically interacts with at least two other members of the *MCM* family, *CDC46* and *CDC54* (18). In addition to these interactions, *CDC47* genetically interacts with a non-*MCM* gene, *CDC45*, that also performs an essential role in the initiation of chromosomal DNA replication (18, 19, 40). To identify additional regulators of DNA replication, high-copy-number suppressors of the ts *cdc47-1* mutation were identified. In this screen, *MCM3* was identified as a dosage-specific suppressor of the *cdc47-1* mutation (8), demonstrating further genetic interactions among *MCM* genes. To determine whether the effect of *MCM3* overexpression was specific to the *cdc47-1* mutant, a high-copy-number plasmid carrying the *MCM3* gene was transformed into *cdc* strains, including some with replication initiation defects (*cdc6*, *cdc7*, *cdc45*, *cdc46*, and *cdc54*). The only mutation to be suppressed, however, was *cdc46-1*; like *cdc47-1*, it corresponds to a defect in a member of the MCM family of polypeptides (Table 1).

Cdc47p assembles into complexes with MCM polypeptides, Cdc45p, and itself in vivo. Genetic analysis (18) (Table 1) indicates that Cdc47p may be recruited into complexes with

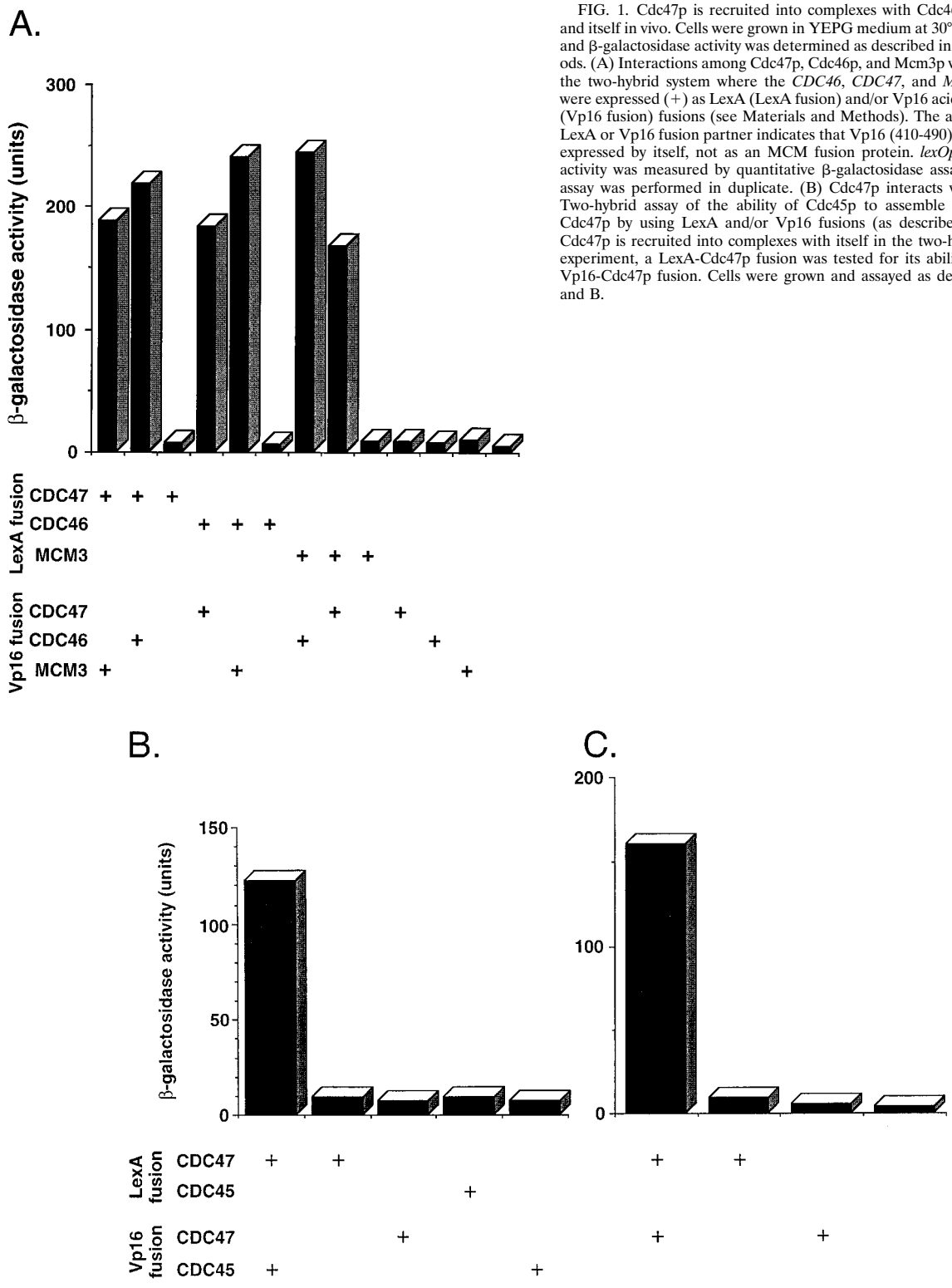

FIG. 1. Cdc47p is recruited into complexes with Cdc46p, Mcm3p, Cdc45p, and itself in vivo. Cells were grown in YEPG medium at 30°C to an OD_{600} of 0.8, and β-galactosidase activity was determined as described in Materials and Methods. (A) Interactions among Cdc47p, Cdc46p, and Mcm3p were assayed by using the two-hybrid system where the *CDC46*, *CDC47*, and *MCM3* gene products were expressed (+) as LexA (LexA fusion) and/or Vp16 acidic activation domain (Vp16 fusion) fusions (see Materials and Methods). The absence of a specified LexA or Vp16 fusion partner indicates that Vp16 (410-490) or LexA (2-202) was expressed by itself, not as an MCM fusion protein. *lexOp-lacZ* reporter gene activity was measured by quantitative β-galactosidase assays. Each two-hybrid assay was performed in duplicate. (B) Cdc47p interacts with Cdc45p in vivo. Two-hybrid assay of the ability of Cdc45p to assemble into complexes with Cdc47p by using LexA and/or Vp16 fusions (as described for panel A). (C) Cdc47p is recruited into complexes with itself in the two-hybrid system. In this experiment, a LexA-Cdc47p fusion was tested for its ability to interact with a Vp16-Cdc47p fusion. Cells were grown and assayed as described for panels A and B.

Cdc46p and Mcm3p. While this work was in progress, the assembly of some MCM polypeptides into complexes in *S. cerevisiae* was reported (26). To characterize the assembly of Cdc47p into complexes, we tested its ability to interact with Cdc46p and Mcm3p by using the two-hybrid system. A summary of the pairwise protein interactions among Cdc47p, Cdc46p, and Mcm3p tested in this study is shown in Fig. 1A. In each case, quantitative results for liquid culture β-galactosidase assays are shown. Using pairwise combinations of Vp16 and LexA fusion proteins, strong activation of the *lexOp-lacZ*

reporter gene was detected when interactions among Cdc46p, Cdc47p, and Mcm3p were tested. We conclude from the results of this set of experiments that Cdc47p can be recruited into complexes with Cdc46p and Mcm3p whether it is fused to a LexA DNA-binding domain or a Vp16 activation domain. We also show that Cdc46p and Mcm3p interacted in the same assay.

Cdc45p has been previously implicated as a component of the RLF complex in yeast, based on its ability to physically associate with Cdc46p (19). To establish whether Cdc45p interacts with other MCM polypeptides, such as Cdc47p, we again used the two-hybrid assay. The data in Fig. 1B show that Cdc47p and Cdc45p interacted in the two-hybrid assay as LexA or Vp16 fusion proteins. We consistently observed that activation of the *lacZ* reporter gene was lower for Cdc47p-Cdc45p interactions than for interactions between Cdc47p and Cdc46p or Mcm3p, altough the reason for this is unclear. These results confirm the genetic analysis prediction of Cdc45p interaction with Cdc47p in vivo (18). During characterization of interactions involving Cdc47p, we observed that a strain expressing the LexA-Cdc47p and Vp16-Cdc47p fusions registered activation of the *lacZ* reporter gene, suggesting a strong interaction (Fig. 1C). The ability to self-interact, or more precisely the ability of more than one Cdc47p polypeptide to be recruited into the same complex, has previously been observed for other members of the MCM family, including Mcm2p, Mcm3p, and Mcm5p/Cdc46p (26). It is, however, unclear whether these interactions are direct or indirect.

Replication-defective forms of Cdc47p and Cdc46p do not interact in vivo. The *cdc47-1* and *cdc46-1* ts mutants arrest at 37°C with largely unreplicated DNA in S phase (18) and, like other *mcm* mutants, show an obvious MCM defect (3, 19, 38). We characterized *cdc47-1* and *cdc46-1* mutations and showed that they arise from single amino acid substitutions at positions that are conserved among Cdc46p/Mcm5p (Fig. 2A) and Cdc47p/Mcm7p (Fig. 2B) family members, respectively. Both mutations lie outside the conserved MCM box that is common to all six members of the MCM family (5).

To determine whether the phenotype of *cdc47* and *cdc46* mutants was related to the abilities of mutant Cdc47p (Cdc47-1p) and Cdc46p (Cdc46-1p) to be assembled into complexes, we again used the two-hybrid system. We were particularly interested in comparing the abilities of mutant Cdc47p and Cdc46p to interact with each other under permissive (25°C) and restrictive (37°C) conditions. When either Cdc47-1p or Cdc46-1p fusions were tested in the two-hybrid assay at 37°C, a reduction of at least fourfold in reporter gene activity, in comparison to those for interactions between nonmutant MCM polypeptides, was observed (Fig. 3A). In contrast, only slight differences were detected when the same experiments were performed at 25°C. Interactions between these polypeptides were further compromised when both fusion proteins were mutants (Fig. 3A). This result correlates with observations that a haploid strain carrying both mutations (*cdc46-1 cdc47-1*) is inviable at all temperatures (18). Hence, the abilities of Cdc46-1p and Cdc47-1p to recruit each other into complexes at 37°C was severely decreased and thus correlated with the inability of these polypeptides to perform their essential cell cycle function. It is unlikely that this result was due to differential protein stability, as the relative levels of wild-type and mutant polypeptides did not vary significantly at either 25 or 37°C (Fig. 3B) (see Fig. 7) (8).

Coimmunoprecipitation of Cdc46p with Cdc47p and Mcm3p. To further characterize the assembly of Cdc46p into complexes with Cdc47p and Mcm3p, we investigated interactions among these polypeptides in crude cell extracts. Affinity-purified HA

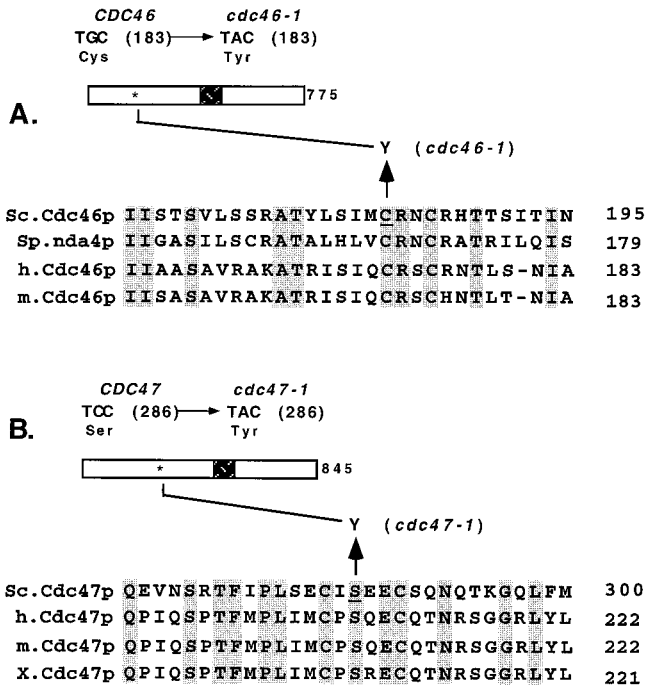

FIG. 2. Identification of single amino acid substitutions in mutant Cdc46p ($Cdc46p^{C183Y}$) and Cdc47p ($Cdc47p^{S286Y}$) polypeptides. The mutations in *cdc46-1* and *cdc47-1* alleles were mapped by the gap repair method (see Materials and Methods for details) and identified by sequencing of recovered mutant clones on both strands. The positions of mapped nucleotide and amino acid substitutions are indicated by asterisks. A patterned box represents the conserved MCM box (flanked by RGDIN and LSRFD motifs). (A) Alignment of Cdc46p/Mcm5p polypeptides from budding yeast (Sc.Cdc46p [17]), fission yeast (Sp.nda4p [5]), humans (h.Cdc46p [20]), and mice (m.Cdc46p [22]) in the region corresponding to the $Cdc46p^{C183Y}$ amino acid substitution. Sequence identities are shown in shaded boxes, with the position indicated at the end of each amino acid sequence. The alignment shows the replaced cysteine at position 183 (underlined) that is conserved among Cdc46p/Mcm5p family members. Dashes indicate gaps. (B) Alignment of Cdc47p/Mcm7p members from budding yeast (10), humans (h.Cdc47p; GenBank accession no. D55716), mice (m.Cdc47p; GenBank accession no. D26091), and *Xenopus* eggs (X.Cdc46p [35]), showing the serine at position 286 (underlined).

monoclonal antibody was used in the detection of epitope-tagged MCM polypeptides in addition to polyclonal antibodies directed against Cdc46p. These polyclonal antibodies were shown to be specific and not to cross-react with other MCM polypeptides by a number of criteria. Immune serum, but not preimmune serum, recognized a polypeptide of 95 kDa in crude cell extracts, corresponding to the predicted mobility of Cdc46p (Fig. 4A). Moreover, affinity-purified antibodies recognized a single polypeptide of 95 kDa (Fig. 4B) not detected in an isogenic strain expressing ubi-cdc46p, a chronically unstable form of Cdc46p (13). These antibodies also immunoprecipitated in vitro-translated Cdc46p, but not Cdc47p and Mcm3p (Fig. 4C).

Strains expressing HA-tagged Cdc46p, Cdc47p, or Mcm3p were grown in liquid culture, and immune complexes were precipitated from crude cell extracts with either anti-HA monoclonal antiserum or anti-Cdc46p polyclonal antiserum. Immunoprecipitates were washed, resolved on a denaturing gel, and transferred to a membrane filter, which was reciprocally probed with either anti-Cdc46p or -HA antibody. We first tested whether Cdc46p was associated with epitope-tagged Cdc47p by probing HA immunoprecipitates with anti-Cdc46p antibodies. Figure 5A (lanes 3 and 4) shows that Cdc46p was

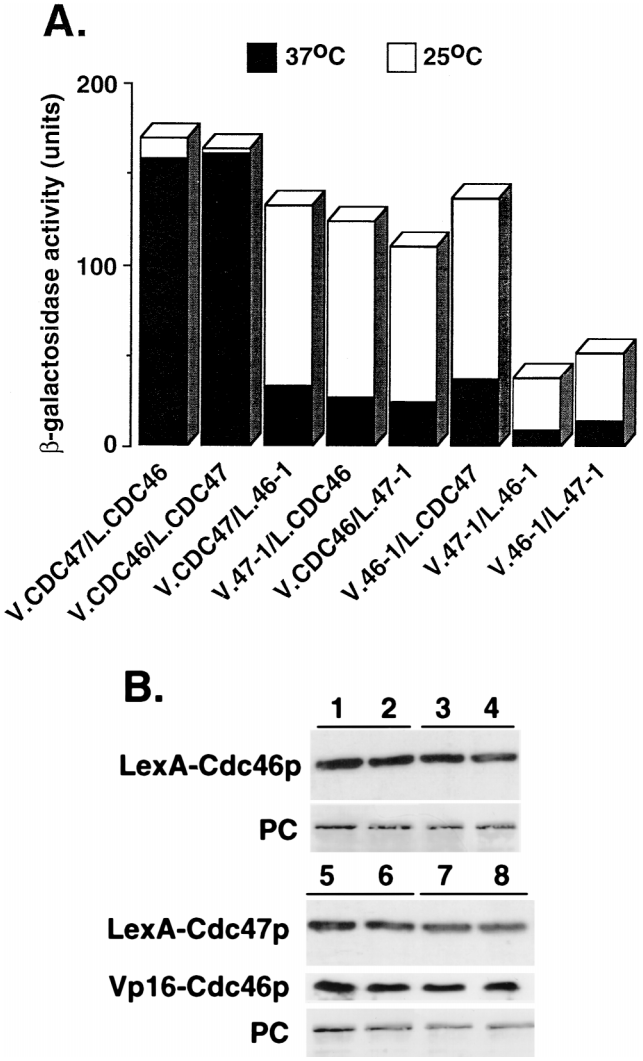

FIG. 3. (A) Two-hybrid analysis of interactions between wild-type and ts mutant forms of Cdc46p and Cdc47p. Cells were grown to an OD_{600} of 0.2 in YEPG medium and then for a further 3 h at either 25 or 37°C. Cell extracts were prepared, and quantitative β-galactosidase assays were performed in duplicate. The reporter gene activity in the two-hybrid assay was determined by quantitative β-galactosidase assay. (B) Levels of wild-type and ts mutant LexA and Vp16 fusion proteins are unaltered at 25 and 37°C. The levels of wild-type (Cdc46p and Cdc47p [lanes 1, 2, 5, and 6]) and mutant (Cdc46-1p and Cdc47-1p [lanes 3, 4, 7, and 8]) LexA and Vp16 fusion proteins tested in the two-hybrid assay were determined by immunoblot analysis. Extracts (20 μg of total cell protein) prepared from cells grown on galactose at either 25°C (lanes 1, 3, 5, and 7) or 37°C (lanes 2, 4, 6, and 8) were resolved on an 8% denaturing polyacrylamide gel, transferred to a membrane filter, and probed with polyclonal antiserum raised against LexA or Cdc46p. As a control, the levels of the biotin-binding enzyme PC (28) were determined in extracts by probing immunoblots with avidin alkaline phosphatase.

precipitated from extracts only when HA-tagged Cdc47p was present. Hence, Cdc46p coprecipitated in a complex with Cdc47p. Similar experiments also showed that Cdc46p physically interacts with Mcm3p (Fig. 5A, lanes 5 and 6) (26), confirming data from two-hybrid analysis. As a second approach, Cdc46p immunoprecipitates from the same extracts were probed with anti-HA antibody. Tagged Cdc47p and Mcm3p were detected in Cdc46p immunoprecipitates by this method (Fig. 5B), confirming previous experiment showing that Cdc46p associates with Cdc47p and Mcm3p in vitro.

Analysis of Cdc47p-MCM-Cdc45p complexes by gel filtration. Gel filtration analysis of MCM complexes demonstrated that the vast majority of Cdc46p and Cdc47p in crude extracts coelute. Both polypeptides began to elute in the same fraction as the 232-kDa marker, considerably later than did monomeric recombinant Cdc46p (rCdc46p), which coeluted with the 158-kDa marker (Fig. 6A) (8). Although only one elution peak for both MCM polypeptides was detected, this peak seemed to be broader than that expected for a monomeric polypeptide or a single homogeneous complex. This may indicate the existence of multiple complexes of various sizes that are not adequately resolved by gel filtration (see below). An analysis of Cdc45p in these fractions revealed it to be in two distinct populations, differing significantly in size (Fig. 6A). The smaller form of Cdc45p is most likely to be a monomer, although we cannot exclude the possibility that it is part of a low-molecular-weight complex since the elution profile of this class of Cdc45p is relatively broad.

Based on our earlier demonstrations that Cdc47p associates with Cdc46p (Fig. 1 and 5) and that Cdc45p interacts genetically with Cdc47p (Fig. 1B) and physically with Cdc46p (19), it was predicted that Cdc47p and Cdc45p were components of the Cdc46p complexes identified (Fig. 6A). To address this possibility, Cdc46p was immunoprecipitated from fractions recovered after gel filtration of crude cell lysate (Fig. 6A). After

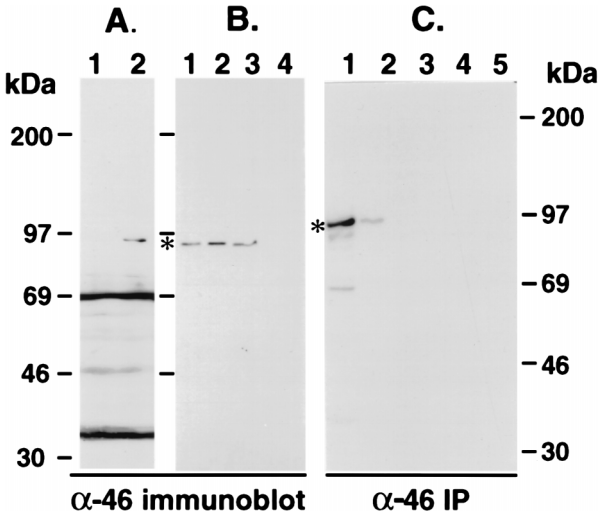

FIG. 4. Characterization of polyclonal anti-Cdc46p antibodies (α-46). (A) Twenty micrograms of W303 1a crude cell extract was resolved by SDS-PAGE on an 8% running gel, blotted, and probed with either caprylic acid-purified preimmune IgG (lane 1) or immune IgG (lane 2), each at a dilution of 1:1,000. A polypeptide specifically recognized by IgG from a rabbit immunized with Cdc46p antigen was detected at 95 kDa (indicated by an asterisk). (B) Crude cell extracts (20 μg of total protein) from W303 1a and an isogenic strain (S339) grown under conditions where Cdc46p is chronically unstable (ubi-Cdc46p; see Materials and Methods) were electrophoresed on an 8% denaturing polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with affinity-purified anti-Cdc46p antibody (1:1,000 dilution). Lanes: 1, log-phase W303 1a; 2, α-factor (G_1) arrested; 3, nocodazole (metaphase) blocked; 4, Cdc46p-depleted extract from ts *ubi-cdc46* strain (S339) arrested at 37°C. A single band corresponding to the predicted mobility of Cdc46p (95 kDa) is indicated by an asterisk. (C) Rabbit reticulocyte lysate was programmed with either Cdc46, Cdc47, or Mcm3 mRNA and translated in the presence of [^{35}S]methionine. Equal amounts of in vitro-translated proteins (judged by autoradiography of ^{35}S-labelled proteins after electrophoresis on an SDS-polyacrylamide gel) were tumbled with (lanes 1, 2, 4, and 5) or without (lane 3) affinity-purified anti-Cdc46p antibody (1:1,000 dilution) and protein A-Sepharose beads. Immunoprecipitates (IPP) were washed, run on a denaturing polyacrylamide gel, and analyzed directly from the gel after drying. Lanes: 1, 1 μl of Cdc46 translate, 2, 0.25 μl of Cdc46 translate; 3, 1 μl of Cdc46 translate; 4, 1 μl of Cdc47 translate; 5, 1 μl of Mcm3 translate. The position of full-length immunoprecipitated Cdc46p is indicated by an asterisk.

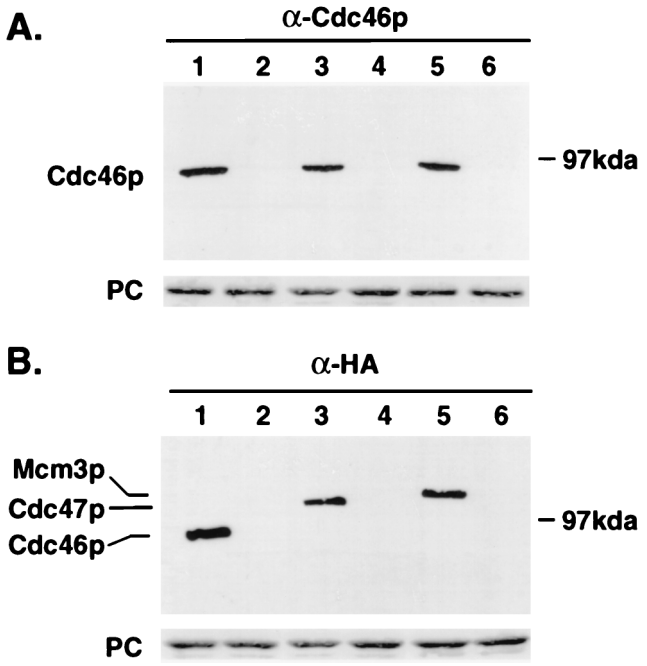

FIG. 5. Interactions among Cdc46p, Cdc47p, and Mcm3p in crude cell lysates. Cell lysates were made from W303 1a cells expressing HA-tagged or untagged MCM polypeptides from CEN plasmids. (A) Extracts were prepared from cells expressing HA-tagged Cdc46p, Cdc47p, and Mcm3p (lanes 1, 3, and 5, respectively) and untagged Cdc46p, Cdc47p, and Mcm3p (lanes 2, 4, and 6, respectively). Polypeptides were immunoprecipitated (200 μg of total protein) with affinity beads (prepared by coupling affinity-purified HA monoclonal antibody to Sepharose beads). Immunoprecipitates were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with affinity-purified anti-Cdc46p polyclonal antibodies (α-Cdc46p; 1:1,000 dilution). For comparison, the relative amounts of total input protein used for immunoprecipitation were assessed by probing immunoblots (20 μg of input protein) with avidin alkaline phosphatase to detect PC (28). (B) Extracts were prepared from cells expressing HA-tagged Cdc46p, Cdc47p, and Mcm3p (lanes 1, 3, and 5, respectively) and untagged Cdc46p, Cdc47p, and Mcm3p (lanes 2, 4, and 6, respectively) as described for panel A, but polypeptides were immunoprecipitated with Cdc46p affinity beads (prepared by coupling affinity-purified anti-Cdc46p polyclonal antibodies to Sepharose beads). Immunoprecipitates were resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with affinity-purified HA monoclonal antibody (α-HA). The relative amounts of total input protein were assessed as described for panel A with PC as the control.

SDS-PAGE and blotting, immunoprecipitates were probed with monoclonal antibodies which recognize epitope-tagged versions of Cdc47p (HA tag; 12ca5) and Cdc45p (c-myc tag; 9e10). Cdc47p was found to be a component of Cdc46p immunoprecipitates in all fractions where Cdc46p was originally detected by immunoblotting (Fig. 6B). The relative amount of Cdc47p in each fraction was similar to that for Cdc46p. Hence, Cdc47p is a component of at least some of the Cdc46p complexes detected by gel filtration. A similar analysis revealed that Cdc45p was a component of these precipitates but, in contrast to Cdc47p, in only the largest subset of Cdc46p complexes (see Discussion). It was also clear that only a relatively small amount of Cdc45p detected in the 440-kDa fraction by immunoblotting was associated with Cdc46p (compare the amount of Cdc45p in the 440-kDa fraction in Fig. 6A with that in Fig. 6B). This suggests that Cdc45p is assembled into MCM complexes which do not contain Cdc46p.

Cdc46p and Cdc47p form stable complexes throughout the cell cycle. Because of our observations that Cdc46p and Cdc47p associate in vivo and in vitro, we reasoned that their role in the initiation of DNA replication could be regulated by assembly and disassembly of complexes at different points of the cell cycle. Using a series of mutants and inhibitors which arrest cells at well-defined stages of the cell cycle, we showed that Cdc46p and Cdc47p associate in early G_1, late G_1, S phase, metaphase, and anaphase (Fig. 7A). In *cdc46-1* and *cdc47-1* mutants, which arrest in a partially replicated S-phase state (18), interactions between the corresponding polypeptides (Cdc46-1p–Cdc47p and Cdc46p–Cdc47-1p interactions, respectively) were not observed. This raised the possibility that Cdc46p-Cdc47p complexes transiently dissociate during late G_1 or S phase or that the mutant polypeptides do not assemble into complexes under restrictive conditions (37°C), which may be related to the cell cycle arrest of *cdc46-1* and *cdc47-1* mutants. The latter possibility is more likely, as the abilities of Cdc46-1p and Cdc47-1p to assemble into complexes were severely compromised at 37°C in the two-hybrid assay (Fig. 3). To unequivocally resolve this issue, we tested the abilities of Cdc46p and Cdc47p to associate at different points of the cell cycle in wild-type (*CDC46*$^+$) and *cdc46-1* cells arrested with cell cycle inhibitors at 25°C and then either maintained at 25°C or shifted to 37°C. The ability of Cdc46p to interact with Cdc47p was determined by probing HA immunoprecipitates with anti-Cdc46p antibodies. In the *CDC46*$^+$ strain, coimmunoprecipitation of Cdc46p and Cdc47p was seen regardless of temperature and cell cycle position. In the *cdc46-1* strain, however, Cdc46p and Cdc47p assembled into complexes at 25 not 37°C, regardless of cell cycle position (Fig. 7B). This correlates with the two-hybrid analysis (Fig. 3) in which the corresponding in vivo interactions were compromised at 37°C. The inabil-

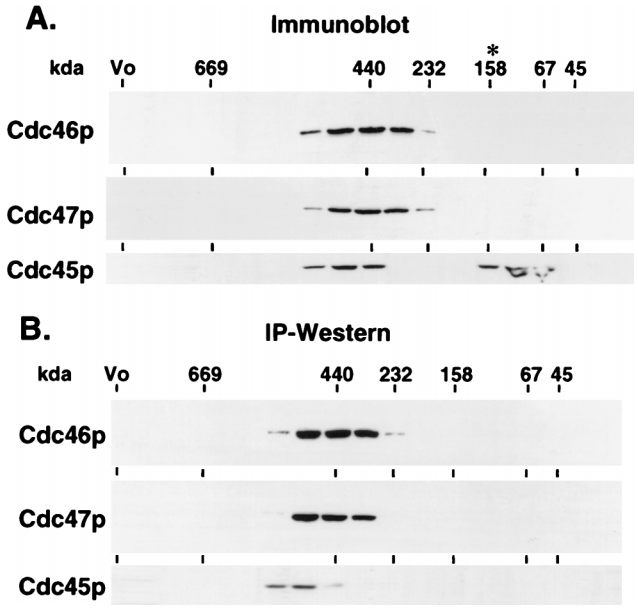

FIG. 6. Cdc45p is a subunit of MCM complexes. Crude cell lysate (0.4 mg) (*MAT***a** *CDC46 CDC47-HA*$_3$*::cdc47 CDC45–c-myc*$_9$*::cdc45*) prepared from cells in exponential-growth phase (OD_{600} of 0.8) was clarified by filtration through a 0.4-μm-pore-size filter and loaded on a calibrated Superdex 200 gel filtration column. (A) Fractions were collected, and equal volumes were resolved on an 8% polyacrylamide gel, transferred to nitrocellulose, and probed separately with affinity-purified anti-Cdc46p antibodies or affinity-purified HA (12ca5) or c-myc (9e10) antibody (each at a 1:1,000 dilution). The position at which native rCdc46p (unpublished data) eluted from the column is indicated by an asterisk. V_o, voided volume. (B) Clarified cell lysate (0.4 mg) was fractionated as described for panel A, and Cdc46p was immunoprecipitated (IP) from fractions with Cdc46p affinity beads, immunoblotted (Western), and then probed with HA and 9e10 monoclonal antibodies and Cdc46p polyclonal antibody to detect Cdc47p-HA, Cdc45p–c-myc, and Cdc46p, respectively. V_o, voided volume.

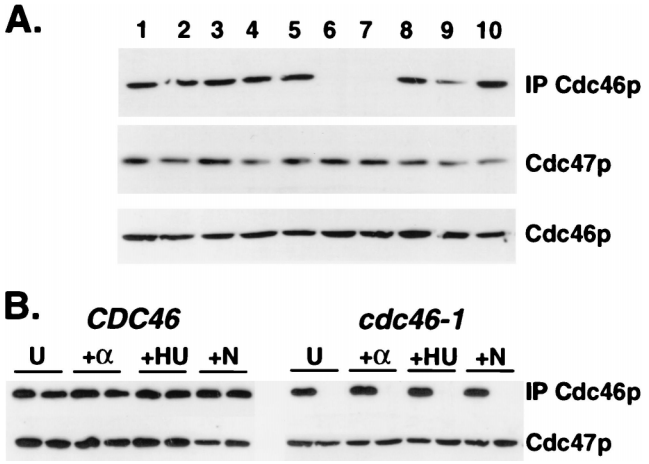

FIG. 7. Cdc47p and Cdc46p form stable complexes throughout the cell cycle. (A) Extracts were prepared from cycling cells in exponential-growth phase and cells arrested at various points of the cell cycle. (Top) Lane 1, cycling cells; lane 2, early G_1, elutriated cells (>90% unbudded); lane 3, α-factor (G_1) block; lane 4, *cdc34*; lane 5, *cdc7*; lane 6, *cdc46-1* (S-phase block); lane 7, *cdc47-1* (S-phase block); lane 8, hydroxyurea (S-phase block); lane 9, nocodazole (metaphase); lane 10, *cdc15* (anaphase). Cell cycle arrest was confirmed by microscopic examination. Cdc47p-HA was immunoprecipitated (200 μg of total protein) with HA affinity beads, and immunoprecipitates were probed with anti-Cdc46p polyclonal antibody. (Middle and bottom) Equal amounts of input protein (30 μg) were immunoblotted and probed for HA-Cdc47p and Cdc46p to ensure that loading levels were comparable. (B) Failure of Cdc46p and Cdc47p to associate in the *cdc46-1* mutant is independent of cell cycle position. Cells were grown at 25°C to mid-log phase, and cell cycle inhibitors were added for 90 min before a shift to 37°C (when appropriate). Cells were harvested after a further 90 min. HA-Cdc47p immunoprecipitates (IP) from cells arrested with α-factor (+α), hydroxyurea (+HU), and nocodazole (+N) and from unsynchronized cells (U) were immunoblotted and probed with anti-Cdc46p antibodies. Two lanes are shown for each treatment; extracts were prepared from cells at 25 (left) and 37°C (right). (Bottom) Immunoblot analysis showing the relative amount of input HA-Cdc47p in each extract. Epitope-tagged Cdc47p was expressed from a CEN-based plasmid.

ity of mutant MCM polypeptides to interact in vitro is therefore cell cycle stage independent but temperature dependent. Hence, there is a correlation between the abilities of Cdc46p and Cdc47p to interact and their abilities to support normal cell cycle progression.

DISCUSSION

Cdc47p-MCM complexes. This report describes the assembly of Cdc47p-Mcm7p into high-molecular-weight complexes with two other MCM polypeptides, Cdc46p and Mcm3p. These biochemical interactions were originally implicated through genetic analysis of *mcm* mutants (18). Interactions between Cdc47p and Cdc46p are stable throughout the cell cycle, making it unlikely that their assembly per se is a rate-limiting step in the formation of preinitiation complexes at replication origins. We do not, however, rule out the possibility that other MCM subunits undergo regulated assembly-disassembly at different points of the cell cycle. Our observations are consistent with other reports that MCM complexes remain intact during the early cell cycles of the *Drosophila* embryo (36) and in the early cleavage divisions of the *Xenopus* egg (16).

Characterization of MCM complexes by gel filtration revealed that they elute in a relatively broad, high-molecular-weight range. This is slightly different from what was reported previously for Mcm2p and Mcm3p complexes in *S. cerevisiae*, where two discrete populations of complex were identified (26). We suspect that the complexes detected in our studies by gel filtration are similar to the larger class (443 to 669 kDa) of assemblies described previously (26). However, further characterization is required to verify this. Why the lower-molecular-weight forms of MCM complex were not prominent in our experiments is not clear, as the MCM complexes described in this report are quite stable, but it could be due to different protein extraction conditions, the method of biochemical analysis (gradient versus gel filtration), or different MCM polypeptides being analyzed.

Although Cdc47p is clearly assembled into complexes with Cdc46p (and Mcm3p), we cannot at this time speculate on the exact compositions or stoichiometries of these complexes. For example, we do not know whether all three polypeptides are assembled into the same complex together or there are unidentified components in addition to those described in this report. It appears that most Cdc47p is assembled into high-molecular-weight complexes (Fig. 6) and that at least some of this is in association with Cdc46p. MCM complexes, including those containing Cdc46p, have been reported previously in the range of 443 to 669 kDa. To account for the existence of high-molecular-weight complexes observed by us and others (26), it is likely that they are composed of more than two polypeptides. The existence of tripartite MCM complexes composed of Cdc47p, Cdc46p, and Mcm3p is therefore a strong possibility. Polypeptides other than Cdc45p may also contribute to the apparent largeness of MCM complexes. Experiments in this laboratory aimed at defining the composition of MCM complexes have been made difficult by our inability to reassemble MCM complexes in vitro by using purified components or mixing together in vitro-translated MCM polypeptides. This may be due to a requirement for posttranslational modification or for an additional accessory protein(s) involved in complex stabilization and/or assembly.

Heterogeneity of MCM complexes. Multiprotein MCM complexes of 490 to 660 kDa have previously been biochemically characterized in *D. melanogaster* (36), *Xenopus* egg extracts (4, 16, 30), and humans (32). However, detailed biochemical descriptions of their compositions and how these complexes are assembled have not been reported. In *D. melanogaster* (36) and *S. cerevisiae* (26), for example, MCM complexes composed of different MCM subtypes have been identified but no biochemical activity has been assigned. The significance of each type of MCM complex is therefore yet to be established, but biochemical and genetic evidence suggests that their role in the early stages of chromosomal DNA replication is conserved between species. We suspect that high-molecular-weight MCM complexes defined by density gradients (26, 36), gel filtration (32), and native gel electrophoresis (16, 36) are more heterogeneous than previously believed. This is supported by observations that MCM complexes are distributed over a relatively large size range (26, 32, 36) and by the identification of different subtypes of MCM complexes in various species (26, 36). The demonstration that Cdc45p is a subunit of some, but not all, MCM complexes further highlights this point (discussed below).

What is the biochemical role of MCM complexes? No demonstrable biochemical activity associated with the assembly of MCM polypeptides into complexes has yet been reported. We assume, however, that the assembly of MCM polypeptides into higher-order complexes is linked to and essential for their role in the initiation of replication. To establish this as a possibility, we characterized Cdc46-1p and Cdc47-1p mutants and their assembly into complexes with each other. We attribute the cell cycle defect associated with these *mcm* mutants to single amino acid substitutions (C183 to Y183 for Cdc46-1p and S286 to Y286 for Cdc47-1p) which map to a region N-

terminal to the conserved MCM box. In the case of *cdc46-1*, the replaced cysteine lies in a region conserved among other Cdc46p/Mcm5p subtypes, indicating that MCM polypeptides may have conserved subtype-specific interaction surfaces for protein-protein contacts (Fig. 2A). The replaced cysteine in $Cdc46p^{C183Y}$ and its flanking residues are also conserved, although to a lesser extent, in Mcm2p, where cysteine residues are proposed to be part of a Zn finger structure involved in DNA recognition (38). In Mcm5p family members, however, the resemblance to Zn finger proteins is more tenuous (23). The replaced serine residue at position 286 in Cdc47p is also conserved between species in a highly conserved Mcm7p subtype-specific domain (Fig. 2B) at the N-terminal side of the MCM box. This again suggests the existence of MCM subtype-specific domains which are required for protein-protein interactions. The inability of $Cdc46p^{C183Y}$ and $Cdc47p^{S286Y}$ to interact correlates with a cell cycle defect which blocks progression through S phase (18). This is consistent with the idea that the assembly of MCM polypeptides into complexes is important for the initiation of DNA replication.

Cdc45p is a subunit of MCM complexes. Previous studies have characterized MCM complexes in yeast, *D. melanogaster*, *Xenopus* eggs, mice, and humans as being composed of multiple MCM subunits. In this report, we have shown that Cdc45p is also a component of MCM complexes. Although functional Cdc45p homologs in metazoans have yet to be described, we have identified a number of expressed sequence tags derived from human and *Drosophila* cDNAs which potentially encode polypeptides with high degrees of amino acid identity to Cdc45p. Together with Tsd2, a polypeptide resembling Cdc45p from *Ustilago maydis* (19), this suggests that Cdc45p is conserved throughout evolution and raises the possibility that it is also a component of RLF in vertebrates.

We have previously shown that approximately 40% of Cdc45p can be detected in complexes with Cdc46p, but only a small proportion of Cdc46p is associated with Cdc45p (19). This is consistent with our observations that like Cdc47p and other MCM polypeptides, Cdc46p is an abundant polypeptide (in contrast to Cdc45p) (8, 26). This indicates that only a small proportion of MCM complexes have Cdc45p as a subunit (Fig. 6). The same is likely to be true for Cdc47p, as gel filtration analysis of complexes indicates that only a small fraction of the total pool of Cdc47p cofractionates with Cdc45p. It is possible that the assembly of MCM polypeptides into higher-order complexes with rate-limiting subunits, such as Cdc45p, is an important step in the full establishment of preinitiation complexes. For example, the assembly of Cdc45p into complexes with preassembled MCM polypeptides may facilitate their recruitment to replication origins during G_1 (11). As the requirements for recruitment of MCM complexes to chromatin and/or replication origins are unknown, this is a distinct possibility. Alternatively, Cdc45p recruitment into MCM complexes may be dependent on and follow the binding of MCM polypeptides to origins. The second possibility is less likely, as the interactions described in this report did not involve the assembly of components at replication origins. At this stage, we do not know whether Cdc45p is associated with MCM polypeptides throughout the cell cycle or there is regulated assembly-disassembly. In this regard, it is interesting that Cdc45p is nuclear throughout the cell cycle, whereas MCM polypeptides (in budding yeast) are nuclear only during G_1 and perhaps for part of S phase. One possibility is that Cdc45p interacts with MCM polypeptides only after their entry into the nucleus at the completion of mitosis. This would invoke a model where MCM polypeptides and Cdc45p assemble at some early stage in G_1 and then dissociate during S phase. Work is in progress to test this idea.

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