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Schizosaccharomyces pombe Mcm3p, an essential nuclear protein, associates tightly with Nda4p (Mcm5p)

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ABSTRACT

MCM proteins are required for the proper regulation of DNA replication. There are six MCM proteins in all eukaryotes which interact to form a large complex. We report the cloning of fission yeast *mcm3*$^+$. *mcm3*$^+$ is essential and spores carrying a Δ*mcm3* disruption arrest with an apparently replicated DNA content. The protein is found constitutively in the nucleus and levels remain constant throughout the cell cycle. Mcm3p binds particularly tightly to Nda4p (Mcm5p), but is loosely associated with the other *Schizosaccharomyces pombe* MCM proteins. Thus, Mcm3p is a peripheral MCM subunit.

INTRODUCTION

The MCM proteins, named for the original yeast minichromosome maintenance mutants (1), are found in all eukaryotes. There are six members of the MCM family, MCM2–7, each of which is essential for regulation of DNA replication. These proteins decorate the chromatin prior to initiation of DNA replication and are displaced as DNA replication progresses (reviewed in 2–4). A large complex containing all six of the MCM proteins has been identified in several systems, including human, mouse, *Xenopus* and *Schizosaccharomyces pombe* (5–9). The exact function of the MCM proteins is still unclear, although recent reports suggest that a subset of these proteins has *in vitro* helicase activity (10,11). Biochemical and genetic experiments have implicated MCM proteins in selection and activation of replication origins, facilitating loading of the replication machinery, and in licensing unreplicated chromatin for replication (reviewed in 2–4).

Recently, we showed that the relative affinity of different MCM proteins for the complex is not uniform. In particular, we showed that the complex contains a tightly associated core, including Cdc21p (Mcm4p) and Mis5p (Mcm6p), and loosely associated subunits, including Cdc19p (Mcm2p) and Nda4p (Mcm5p; 12). A similar architecture has been observed for MCM complexes from mouse, *Xenopus* and human (6–8,10,13–16). We have now extended our study by cloning and characterizing *S.pombe* Mcm3p. We show that *mcm3*$^+$ encodes an essential nuclear protein. Cells lacking *mcm3*$^+$ arrest after undergoing a severely delayed S phase. The Mcm3p subunit forms a tight interaction with Nda4p (Mcm5p), but is only loosely associated with other members of the family.

MATERIALS AND METHODS

Yeast strains and plasmids

Schizosaccharomyces pombe strains were grown in Edinburgh minimal medium (EMM) and supplemented with adenine, histidine, leucine and uracil as required (17). All strains were derived from *972 h*$^-$. Cell cycle mutant strains were grown at 25°C and shifted to 36°C for 4 h to impose the cell cycle block. All other strains were grown at 32°C.

Nitrogen-starved cells were prepared by harvesting exponentially growing wild-type cells, washing and inoculating the cells into EMM lacking nitrogen (NH_4Cl). Cells were grown at 25°C and harvested after 12 h.

Construction of the *nda4-HA* strain (FY803) was described previously (12). We constructed the *mcm3-myc* strain (FY916) using PCR to amplify the *mcm3*$^+$ open reading frame from the *mcm3*$^+$ genomic clone (pDS62) and cloned the product into pBluescriptSK+ to make pDS89, containing an in-frame *Not*I site. The *Not*I fragment from pDS89, when cloned into expression vector pDS672 (Sherman, Pasion and Forsburg, in preparation), gains two copies of the c-*myc* tag at the 3′-end of the gene to create pDS94. We placed the *Nsi*I–*Sal*I fragment of pDS94, containing the 3′-end of the tagged gene, into pJK148 (*leu1*$^+$; 18). Following linearization, this plasmid (pDS98) was integrated into the *mcm3*$^+$ locus, creating a partial tandem duplication of the gene and expressing the tagged form under the endogenous promoter (Fig. 3A).

Cloning of *mcm3*$^+$

Using a primer based on the previously published sequence of the *mcm3* central core (5′-CAAATCTAAGAAGTTGAGATTTTG-CAGTGG-3′) (19) and a degenerate primer based on a region highly conserved between MCM3 family members (5′-CAGGA-GAWGCCIGAGATGGCWCCHGC-3′), we amplified an *mcm3*$^+$-specific probe by PCR and screened an *S.pombe* genomic plasmid library (20) and an *S.pombe* cDNA library (21) by hybridization.

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The entire *mcm3*$^+$ gene and flanking sequences (pDS62) was sequenced on both strands (Salk Institute Sequencing Facility). The RNA start was determined by sequencing the 5′-end of a partial *mcm3* cDNA (pDS88) and is 224 bp upstream of the *mcm3*$^+$ ATG. The GenBank accession no. is AF063864.

mcm3 disruption

We amplified the 5′- and 3′-ends of the *mcm3* genomic clone by PCR and cloned the fragments into pBluescriptSK+. We inserted the *his3*$^+$ cassette from pAF1 (22) to create pDS84 (see Fig. 1A for a schematic), disrupting amino acids 90–719 of Mcm3p. The disruption cassette was excised with *Pst*I and *Kpn*I and transformed into a diploid strain (*h*$^-$/*h*$^+$ *ura4/ura4 leu1/leu1 his3/his3 ade6/ade6*) to create FY909. We confirmed the disruption by Southern blot.

Spore germination

We analyzed strain FY909 (Δ*mcm3*::*his3*$^+$/*mcm3*$^+$ *his3/his3*) and a diploid heterozygous at the *his3* locus (*his3*$^+$/*his3-D1*) by spore germination (21). Spores were washed and partially purified by centrifugation through a 25% glycerol cushion.

For the spore germination time course, spores were inoculated into EMM plus adenine, leucine and uracil to a final concentration of 1×10^8 spores/ml. Cultures were grown, with shaking, at 32°C. Every hour, spore samples were harvested by centrifugation and fixed for flow cytometry as in Sazer and Sherwood (23), except that we stained the cells with 2 µM Sytox Green (Molecular Probes). Data were collected on a Becton Dickinson FACScan and analyzed using Cell Quest software for the Macintosh.

Antibodies

We raised polyclonal rabbit antibodies to the N-terminal 300 amino acids of Mcm3p, purified from bacteria as a 6×His-tagged fusion protein, as described previously (12). The expression plasmid, pDS91, contains the 5′ *Xho*I–*Msc*I fragment from pDS89 in pRSETB (Invitrogen). Antibodies were affinity purified from western blots using purified protein.

Antibodies to Nda4p and Mis5p have been described previously (12). Monoclonal anti-HA 12CA5 antibody was a kind gift of Jill Meissenholder and Tony Hunter. Monoclonal anti-α-tubulin antibody was purchased from Sigma (T5168). Purified monoclonal anti-c-*myc* 9E10 antibody was purchased from BAbCO (MMS-150P). Donkey-anti-mouse::Cy3 conjugated secondary antibody used for indirect immunofluorescence was purchased from Jackson ImmunoResearch Laboratories (715-165-150).

Protein extracts, immunoblotting and immunoprecipitation

Cell lysates were prepared, immunoblotted and used for immunoprecipitation as described previously (12). Buffers and specific experimental details are as follows. Cell lysis buffer contained 20 mM HEPES, pH 7.0, 50 mM potassium acetate, 5 mM magnesium acetate, 100 mM sorbitol, 0.1% Triton X-100, 1 mM ATP, 1 mM DTT and protease inhibitors. SDS sample buffer contained 100 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 200 mM dithiothreitol and 0.02% bromophenol blue.

SDS–PAGE was performed using 7% gels (National Diagnostic Protogel), except for the Mcm3 antibody characterization, which was performed using a 6% gel.

Immunoprecipitations were performed with 300 µg total protein. Buffers used for washing immunoprecipitates were as follows. Gentle wash buffer was lysis buffer (see previous). Harsh wash buffer was modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate). Western blot detection was by enhanced chemiluminescence (Amersham). Films were scanned and assembled using Adobe Photoshop and Canvas for the Macintosh.

Microscopy and indirect immunofluorescence

Germinating spores that had been fixed for flow cytometry were rehydrated in 50 mM sodium citrate and stained with DAPI.

Indirect immunofluorescence was performed essentially as described (24). Briefly, after fixation, cell walls were digested with NovoZym 234 (BiosPacific) and Zymolyase 20T (Seikagaku) and incubated with anti-*myc* antibody in PEMBAL. Cells were then incubated with donkey-anti-mouse::Cy3 secondary antibody and stained with DAPI.

Cells were visualized using a Leitz fluorescence microscope and photographed on Kodak Ektachrome 400. Film was scanned into Adobe Photoshop for the Macintosh using a Nikon slide scanner.

RESULTS

Isolation of the *S.pombe mcm3*$^+$ gene

We cloned the *mcm3*$^+$ gene from an *S.pombe* genomic library using a hybridization strategy. Previously, a PCR fragment corresponding to the conserved central domain of fission yeast *mcm3*$^+$ was isolated (19). We used a specific oligonucleotide based on this sequence and a degenerate oligonucleotide based on a region of high protein sequence similarity between MCM3 homologs of *Saccharomyces cerevisiae*, *Xenopus*, human and mouse (25–29) to amplify a probe. We used this fragment to screen an *S.pombe* genomic library (20) by hybridization and isolated four overlapping clones, one of which contained the entire *mcm3*$^+$ gene with flanking sequences (shown schematically in Fig. 1A). We identified the 5′-end of the mRNA, at nt –224 relative to the ATG, by sequencing an *mcm3*$^+$ cDNA clone isolated by screening an *S.pombe* cDNA library (21).

The predicted amino acid sequence of the *S.pombe* Mcm3 protein (Mcm3p) shows a high similarity to Mcm3 proteins from other organisms (Fig. 1C). Mcm3p displays ~43% identity and 55% similarity to each of the other Mcm3 proteins over the entire lengths of the proteins. Typical of other MCM proteins, there is a central core of homology that has a much higher sequence identity.

Closer analysis of the protein sequence of Mcm3p reveals a number of potential functional domains (Fig. 1B). A putative leucine zipper domain is located near the N-terminus of the protein. As with other MCMs, the core MCM homology domain of Mcm3p contains sequences reminiscent of DNA-dependent ATPases (30). In the C-terminal half of the protein, there is one putative cyclin-dependent kinase (CDK) phosphorylation site and an SV40 large T antigen-type nuclear localization sequence (NLS), followed by a large acidic domain. The Mcm3 homologs from other species also have NLS and ATPase domains, however, the putative leucine zipper domain is unique to *S.pombe* Mcm3p. In other proteins, leucine zipper domains have been implicated in protein–protein interactions (31–33).

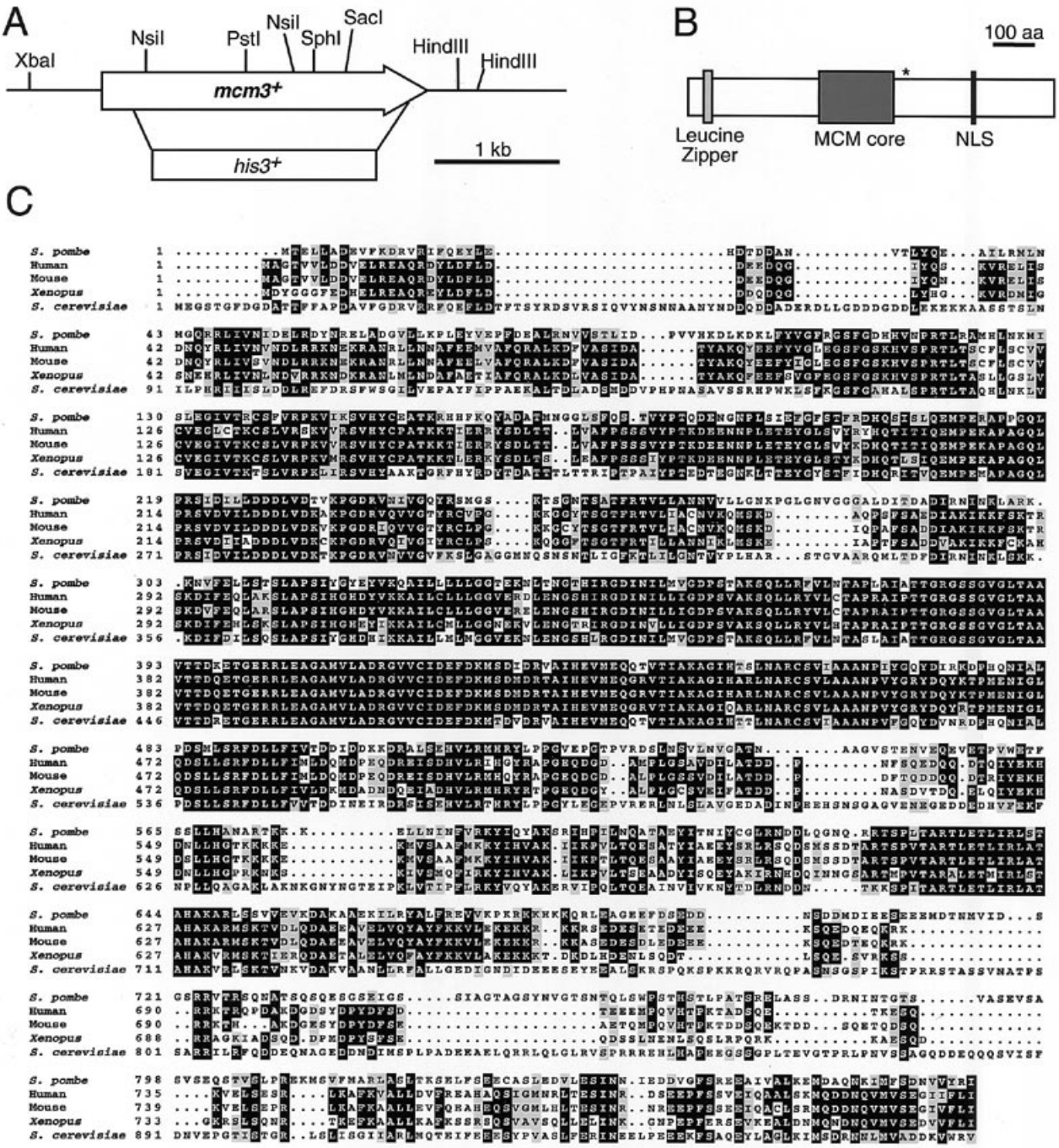

Figure 1. Cloning of *S.pombe mcm3*$^+$. (**A**) Schematic of the *mcm3*$^+$ genomic clone with selected restriction sites. Location of *his3*$^+$ in the Δ*mcm3*::*his3*$^+$ disruption is shown. (**B**) Schematic of Mcm3p. Location of the putative leucine zipper (41-$LX_6LX_6LX_6L$-62) and nuclear localization sequence (NLS) (676-KPKRKK-681) are shown. The asterisk (*) denotes a consensus Cdc2 phosphorylation site (527-TPVR-530). (**C**) Alignment of MCM3 protein sequences (25–29). Sequences were aligned with ClustalW and output generated using MacBoxshade. Identical residues are shown in white text with a black background. Conserved residues are shaded.

mcm3$^+$ is essential

To determine the phenotype of cells lacking *mcm3*$^+$, we constructed an *mcm3* disruption. We replaced most of the *mcm3*$^+$ open reading frame with *his3*$^+$ (Fig. 1A), removing amino acids 90–719 of Mcm3p. We integrated this construct into the *mcm3*$^+$ locus of a diploid *his3-D1/his3-D1* strain and confirmed the structure by Southern blotting (data not shown). Of 10 tetrads dissected, all showed 2:2 segregation of viable:inviable spores and all of the viable spores were *his*$^-$ (data not shown). Additionally, random spore analysis revealed that, of more than 1000 colonies formed, all were *his*$^-$ (data not shown).

We assayed the phenotype of the Δ*mcm3* cells using a bulk spore germination procedure (Materials and Methods). Spores

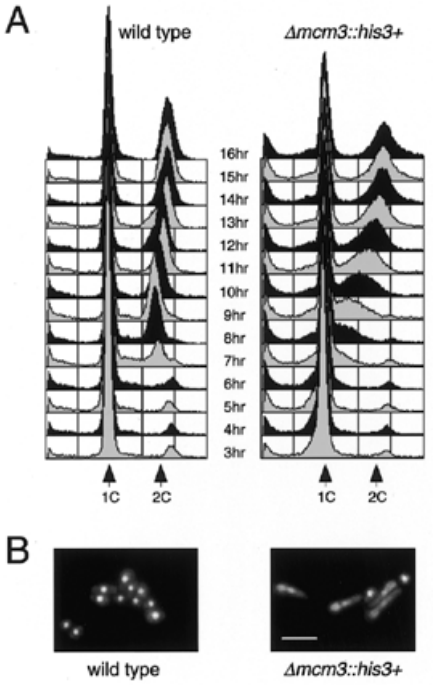

Figure 2. Δ*mcm3* spores arrest with a 2C DNA content. (**A**) Flow cytometry of germinating Δ*mcm3* and wild-type spores. Samples were taken hourly from cells growing in selective media. (**B**) Δ*mcm3* and wild-type spores at the 12 h time point, stained with DAPI. Scale bar represents 10 µm.

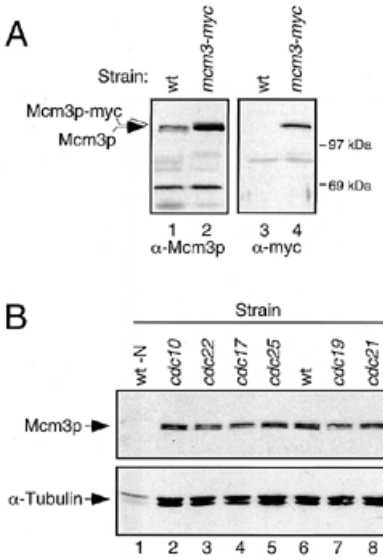

Figure 3. Mcm3p levels are constant during the cell cycle. Samples of 10 µg total protein per lane, immunoblots with antibodies as shown. (**A**) Characterization of anti-Mcm3p antibody. Lanes 1 and 3, wild-type lysate; lanes 2 and 4, lysate from *mcm3-myc* strain. (**B**) Lysates from cells blocked in the cell cycle, immunoblotted with anti-Mcm3p and anti-α-tubulin. Lane 1, wild-type blocked by nitrogen starvation (stationary phase); lane 2, *cdc10-V50* (G_1 phase); lane 3, *cdc22-M45* (early S); lane 4, *cdc17-K42* (late S); lane 5, *cdc25-22* (G_2/M); lane 6, wild-type asynchronous culture; lane 7, *cdc19-P1* (S phase); lane 8, *cdc21-M68* (S phase).

from the *mcm3* disruption strain and from a wild-type strain heterozygous for the *his3-D1* mutation were inoculated into minimal medium lacking histidine at 32°C. Only spores carrying the Δ*mcm3*::*his3*$^+$ disruption (or in the wild-type control, only the spores with a wild-type *his3*$^+$ gene) can germinate. We took hourly samples and fixed them for flow cytometry. FACS analysis revealed that germinating spores lacking the *mcm3*$^+$ gene underwent a delayed S phase and arrested with a 2C DNA content (Fig. 2A). Increased DNA content was first evident at the 7 h time point in both the Δ*mcm3* and wild-type spores. The wild-type spores completed DNA replication by 8 h, while Δ*mcm3* mutant spores accumulated DNA more slowly, taking ~4 h longer than the wild-type to reach a 2C DNA content. Microscopic analysis of spores at the 12 h time point showed that the Δ*mcm3* spores contain intact nuclei and are very elongated in comparison with *his*$^+$ wild-type spores (Fig. 2B). About 10% of the germinating spores were able to divide once, but the rest remained arrested with a *cdc* (cell division cycle) phenotype.

Mcm3p levels are constant through the cell cycle, but Mcm3p is undetectable in nitrogen-starved cells

To investigate the behavior of the Mcm3 protein, we raised polyclonal antibodies to the N-terminal one third of Mcm3p, a region which shares little homology with the other *S.pombe* MCM proteins. On a western blot of wild-type *S.pombe* lysate, affinity-purified antibodies recognize a doublet of ~105 kDa in size, slightly larger than the Mcm3p predicted molecular weight of 97.4 kDa. Specificity of the anti-Mcm3p antibody on western blots was tested by comparing a wild-type cell lysate with a lysate from cells in which the endogenous *mcm3*$^+$ gene was replaced with an epitope-tagged version of the gene. The c-*myc* epitope-tagged protein (Mcm3p-*myc*) has a slightly slower migration than the wild-type protein on an SDS–polyacrylamide gel (Fig. 3A). The presence of multiple isoforms of wild-type Mcm3p could indicate that a portion of the cellular pool of Mcm3p has post-translational modifications.

We examined the levels of endogenous Mcm3p in cell lysates from nitrogen-starved cells, mutants blocked at different stages of the cell cycle and from two *mcm* mutants (12). A western blot with antibodies to Mcm3p (Fig. 3B) showed that, when compared with the α-tubulin control, the level of Mcm3p does not vary significantly during the cell cycle or in *mcm* mutant backgrounds. This is similar to results seen with other *S.pombe* MCM proteins (12). Interestingly, Mcm3p is undetectable in the lysate from nitrogen-starved cells (Fig. 3B, lane 1). Note that the amount of α-tubulin is also reduced in nitrogen-starved cells, so for this experiment, the total protein load in Figure 3B, lane 1, was verified by staining with Coomassie brilliant blue (data not shown).

Mcm3p is in the nucleus throughout the cell cycle

We used indirect immunofluorescence to determine the cellular localization of Mcm3p. Because the antibody to Mcm3p has a high background when used for indirect immunofluorescence, we used a strain in which the endogenous *mcm3*$^+$ gene was replaced with an *myc*-tagged version of the gene. We compared asynchronous cultures of this strain and a strain transformed with an *myc*-tag plasmid (Fig. 4). In the *mcm3-myc* strain, cells in all stages of the cell cycle show nuclear localization of Mcm3p-*myc*.

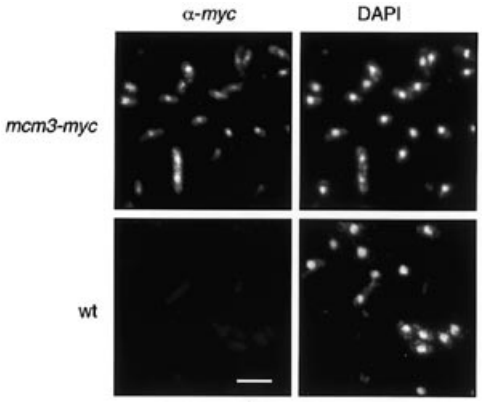

Figure 4. Mcm3p is nuclear throughout the cell cycle. Indirect immunofluorescence of the *mcm3-myc* strain (top) and a wild-type strain carrying the *myc*-tagging plasmid pDS673 (wt, bottom) with antibodies to c-*myc*. (Left) Anti-*myc* immunofluorescence; (right) DAPI staining. Scale bar represents 10 μm.

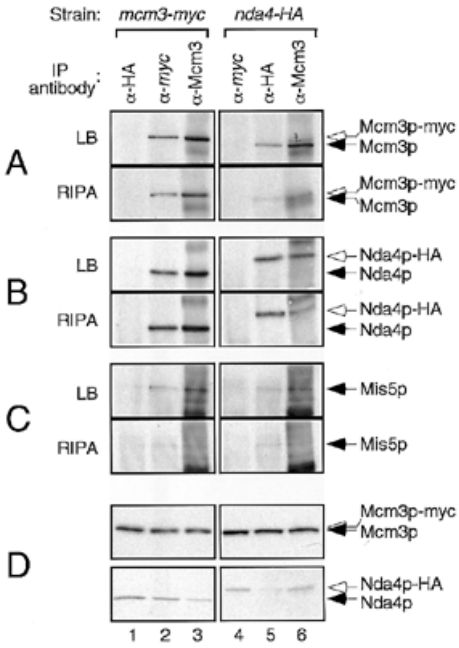

Figure 5. Mcm3p is tightly associated with Nda4p (Mcm5p) and loosely associated with Mis5p (Mcm6p). Aliquots of 300 μg total protein from the indicated strains were immunoprecipitated with the antibody shown. Immunoprecipitates were washed non-stringently with lysis buffer (LB, top) or stringently with modified RIPA buffer (RIPA, bottom). Blots were probed with antibodies to (**A**) Mcm3p, (**B**) Nda4p (Mcm5p) and (**C**) Mis5p (Mcm6p). Anti-Mcm3p immunoprecipitations (lanes 3 and 6) were also done with a wild-type strain with similar results (data not shown). (**D**) Supernatants from immunoprecipitations. Equal amounts of supernatants remaining after immunoprecipitation (~5 μg total protein) were blotted with antibodies to Mcm3p and Nda4p (Mcm5p), as indicated.

Mcm3p forms a tight association with Nda4p (Mcm5p)

The six *S.pombe* MCM proteins, including Mcm3p, form a large complex (9). Recently, we showed that MCMs associate in this complex with different relative affinities (12). In particular, we found that Nda4p (Mcm5p) is very loosely associated with the other MCM proteins. To investigate the strengths of the interactions between Mcm3p and the other MCMs, we used reciprocal immunoprecipitation and washed duplicate immunoprecipitates with either a gentle buffer (low salt, low detergent) or a harsh buffer (moderate salt, high detergent; 12). We used strains in which either the endogenous *mcm3*$^+$ gene or *nda4*$^+$ gene was replaced with *mcm3-myc* or *nda4-HA*, respectively. Equal amounts of lysates from asynchronous cultures were immunoprecipitated with antibodies to the HA epitope, the *myc* epitope and to Mcm3p. As shown in Figure 5D, we were unable to immunodeplete all of the Mcm3p or Nda4p from the lysates.

When the lysate from *nda4-HA* strains was immunoprecipitated with antibodies to the HA-tag, Mcm3p co-immunoprecipitated and was detectable even after stringent washing (Fig. 5A, lane 5; 12). Similarly, when Mcm3p-*myc* or Mcm3p was immunoprecipitated, Nda4p or Nda4p-HA remained strongly associated (Fig. 5B, lanes 2, 3 and 6). Thus there is a tight affinity between Mcm3p and Nda4p (Mcm5p). In contrast, Mis5p (Mcm6p), a core MCM protein, was only weakly associated with Mcm3p or with Nda4p (Mcm5p; Fig. 5C). Similar results were observed for Cdc19p (Mcm2p) and Cdc21p (Mcm4p; data not shown).

DISCUSSION

Thus far, genes encoding all six MCM proteins have been cloned in *S.cerevisiae*, *Xenopus*, mouse and humans (for reviews see 3,4,34). Previously, only four of the six *S.pombe* MCM genes had been cloned (19,35–37). We have now cloned fission yeast *mcm3*$^+$ (this report) and *mcm7*$^+$ (Liang and Forsburg, in preparation).

The *mcm3*$^+$ gene is 2640 bp in length and encodes a protein of 879 amino acids. The predicted Mcm3 protein has a high sequence similarity to other members of the MCM3 class and shares the core MCM homology domain, containing sequences similar to DNA-dependent ATPases (30), and a putative SV40 large T antigen-type NLS. Unlike the other members of this class, Mcm3p has a leucine zipper domain near the N-terminus. This type of domain has been implicated in protein–protein interactions (31–33), and in Mcm3p, could be important in its interactions with other members of the MCM family.

A disruption of *mcm3*$^+$ shows that this gene is essential, as Δ*mcm3* cells arrest in the first cell cycle with a *cdc* phenotype. Spores carrying the disruption begin DNA replication at approximately the same time as wild-type spores, but DNA synthesis is very slow. The Δ*mcm3* spores reach a 2C DNA content by ~12 h, in comparison with 8 h in wild-type spores. This phenotype is identical to that previously reported for Δ*cdc19* (*mcm2*; 36) and suggests that *mcm3*$^+$ may not be essential for initiation at all origins. However, we cannot rule out the possibility that maternal carryover of Mcm3p into the Δ*mcm3* spores allows the limited replication seen in these spores.

Using antibodies to Mcm3p, we showed that the protein level does not vary significantly in lysates from mutant cells blocked at several stages of the cell cycle. Interestingly, no Mcm3p is detected in cells blocked by nitrogen starvation. A similar drop in MCM protein levels has been seen in human cells arrested in G_0 (38).

Indirect immunofluorescence shows that Mcm3p is a nuclear protein and is detected in the nucleus at all stages of the cell cycle. This is consistent with the localization of several other *S.pombe* MCM proteins (39,40) and with MCM3 homologs in higher eukaryotes (27–29,41).

As in other eukaryotes, the six *S.pombe* MCMs associate together in a large complex. Recently, we domonstrated that this complex contains a tightly associated core of at least Cdc21p (Mcm4p) and Mis5p (Mcm6p) that has weak associations with peripheral MCM proteins, including Cdc19p (Mcm2p) and Nda4p (Mcm5p; 12). Here, we have investigated the relative affinity of Mcm3p for the other MCM proteins using reciprocal co-immunoprecipitation and found that it associates very tightly with Nda4p (Mcm5p). The available antibodies were unable to immunodeplete all of the Mcm3p or Mcm4p from the lysates, so we cannot reach any conclusions regarding stoichiometry of the complex. It is worth noting, however, that when Mcm3p is immunoprecipitated there is a slight decrease in Nda4p detected in the remaining supernatant and vice versa (e.g. compare Fig. 5D, lanes 1 and 3). Like Nda4p (Mcm5p), the association of Mcm3p with other MCM proteins is very weak. Thus, fission yeast Mcm3p and Nda4p (Mcm5p) form a dimer that is peripherally associated with the core MCM complex. This is consistent with observations in other systems, in which MCM3 and MCM5 form a tightly associated dimer that is easily removed from other MCMs (7,13–15,42), and suggests that the relative affinity of an MCM protein for the complex is a conserved feature.

From these and other studies, it is evident that there are important differences between individual MCM proteins and their associations with the MCM complex. Recent reports suggest that the core MCM subunits have helicase activity and that MCM2 is a potential inhibitor of this activity (10,11). Thus, perhaps the peripheral MCM proteins, including Mcm3p, are important in regulating this activity or in providing specific interactions with other components of the replication apparatus. With cloning of the last *S.pombe* MCMs, we now have tools to investigate these functions.

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