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Fission yeast Nda1 and Nda4, MCM homologs required for DNA replication, are constitutive nuclear proteins

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SUMMARY

The *nda1*$^+$ and *nda4*$^+$ genes of the fission yeast *Schizosaccharomyces pombe* encode proteins similar to budding yeast MCM2 and MCM5/CDC46, respectively, which are required for the early stages of DNA replication. The budding yeast Mcm proteins display cell-cycle dependent localization. They are present in the nucleus specifically from late M phase until the beginning of S phase, so that they were suggested to be components of a replication licensing factor, a positive factor for the onset of replication, which is thought to be inactivated after use, thus restricting replication to only once in a cell cycle. In the present study, we raised antibodies against Nda1 or Nda4 and identified 115 kDa and 80 kDa proteins, respectively. Their immunolocalization was examined in wild-type cells and in various cell-cycle mutants. Both Nda1 and Nda4 proteins remained primarily in the nucleus throughout the cell cycle. In mutants arrested in G_1, S, and G_2 phases, these proteins were also enriched in the nucleus. These results indicate that the dramatic change in subcellular localization as seen in budding yeast is not essential in fission yeast for the functions of Nda1 and Nda4 proteins to be executed. The histidine-tagged *nda1*$^+$ gene was constructed and integrated into the chromosome to replace the wild-type *nda1*$^+$ gene. The resulting His-tagged Nda1 protein was adsorbed to the Ni-affinity column, and co-eluted with the untagged Nda4 protein, suggesting that they formed a complex.

Key words: MCM protein, *Schizosaccharomyces pombe*, DNA replication, Nuclear protein

INTRODUCTION

In the eukaryotic cell cycle, replication of chromosomal DNA occurs in the S phase. The initiation of DNA replication is controlled by a number of genes. In the budding yeast *Saccharomyces cerevisiae* these genes have been identified using cell division cycle (cdc) mutants which are arrested at the border of G_1 and S phase or within S phase (reviewed by Campbell and Newlon, 1991). Among them, the *MCM* genes constitute a family of genes essential for the initiation of replication (reviewed by Tye, 1994). Two different approaches have led to the isolation of mcm mutants defective in the initiation or progression of S phase. First, the *CDC46* gene has been identified as a suppressor gene for cold-sensitive (cs) cdc mutations *cdc45-1* and *cdc54-1*, and the suppressor alleles of cdc46 make the cell heat sensitive (Moir et al., 1982). Mutations in the *CDC45* and *CDC46* genes block DNA replication, and the arrested mutant cells contain a single-genome equivalent of DNA (1C) (Hennessy et al., 1991). Secondly, the *MCM2*, *MCM3* and *MCM5* genes have been identified as mutations which affect the function of autonomously replicating sequences (ARSs) and, as a consequence, destabilize minichromosomes which carry an ARS and a centromere (Maine et al., 1984). Gene cloning and sequencing have demonstrated that Cdc46, Mcm2, Mcm3 and Mcm5 proteins contain a well conserved domain with the ATPase consensus and that Cdc46 and Mcm5 are identical (Tye, 1994). However, MCM proteins are functionally not redundant, and the cells become inviable if one of the *MCM* genes is disrupted (Hennessy et al., 1991; Yan et al., 1991; Gibson et al., 1990). Proteins similar to the budding yeast MCM have been identified in the fission yeast *Schizosaccharomyces pombe*, *Xenopus* and mammals (Coxon et al., 1992; Thommes et al., 1992; Miyake et al., 1993; Todorov et al., 1994; Kimura et al., 1994; Forsburg and Nurse, 1994; Takahashi et al., 1994; Kubota et al., 1995; Kimura et al., 1995).

The replication licensing factor model (Blow and Laskey, 1988; Blow, 1993; Coverley and Laskey, 1994) has been proposed to explain the observation that the re-replication of chromosomal DNA in *Xenopus* egg extracts requires permeabilization of the nuclear membrane. The proposed licensing factor is a *trans*-acting positive factor required for the initiation of DNA replication, has access to the chromatin only during mitosis, and becomes inactive after replication is initiated. The subsequent round of DNA replication must await a new supply of the licensing factor, thus restricting replication to just once per cell cycle. In *S. cerevisiae*, the behavior of Cdc46 (Hennessy et al., 1990), Mcm2 and Mcm3 (Yan et al., 1993) during the cell cycle follow what is predicted for the licensing factor. They enter the nucleus at late M phase, stay there during

G_1 and disappear from the nucleus upon the onset of S phase. A localization study of P1, a mouse Mcm3 homolog, has recently been reported (Kimura et al., 1994). P1 is localized to the nucleus in interphase cells and dissociated from mitotic chromosomes at M phase. P1 changes its subnuclear distribution in S phase and seems to be easily extracted from the postreplicative chromatin by detergent treatment. These results suggest that MCM proteins are candidates for replication licensing factors and it has recently been shown that, in *Xenopus*, the MCM3 homolog is indeed a component of the replication licensing system (Kubota at al., 1995; Chong et al., 1995; Madine et al., 1995).

In *S. pombe*, the *nda1*$^+$ and *nda4*$^+$ genes (similar to MCM2 and CDC46, respectively) were originally identified as cs mutants showing a cell-cycle block (i.e. cell elongation with a single nucleus) with an alteration in the nuclear chromatin region (Toda et al., 1983). Later, *nda1* and *nda4* mutants were shown to be defective in DNA replication (Miyake et al., 1993) and these genes are essential for viability. The *cdc19*$^+$ gene was recently shown to be identical to *nda1*$^+$ (Forsburg and Nurse, 1994). A novel MCM family gene *mis5*$^+$ which is essential for viability was also recently identified as a mutation which shows high frequency minichromosome loss (Takahashi et al., 1994). We report here an examination of the subcellular localization of Nda1 and Nda4 proteins during the fission yeast cell cycle using antibodies raised against these proteins. In contrast to the localization reported for budding yeast, the Nda1 and Nda4 proteins are constitutively enriched in the nucleus throughout the cell cycle.

MATERIALS AND METHODS

Strains and media

S. pombe 972 *h*$^-$ or HM123 (*h*$^-$ *leu1*) was used as a haploid wild-type strain. Cold-sensitive *nda1-376* and *nda4-108* mutant strains were previously described (Toda et al., 1981, 1983; Miyake et al., 1993). Temperature sensitive *cdc* mutants used were *cdc10-129* (Nurse et al., 1976), *cdc22-M45* (Nasmyth and Nurse, 1981) and *cdc25-22* (Fantes, 1979). Cells were grown in complete medium (YPD: 1% yeast extract, 2% glucose, 2% polypeptone) or in a synthetic medium (EMM2; Mitchison, 1970). The permissive temperature for nda1 and nda4 mutant cells was 33°C while the restrictive one was 20°C. To repress the expression of the genes downstream of the *nmt1*$^+$ promoter (Maundrell, 1990), thiamine-HCl was added to a final concentration of 2 μM for liquid culture and 20 μM for plates.

Preparation of antisera and immunofluorescence microscopy

The carboxy-terminal Nda1 was overproduced in *Escherichia coli* by a plasmid constructed as follows: a *Bam*HI linker was added to the *Xho*I site of the *Xho*I-*Bam*HI fragment containing the *nda1*$^+$ gene, and the resulting fragment was ligated with the *Bam*HI site of pET3 vector (Studier et al., 1990). Plasmid used to overproduce the Nda4 protein in *E. coli* was made in the following way. An *Nde*I site was created at the initiation codon of the *nda4*$^+$ gene by polymerase chain reaction (PCR). The *Nde* I-*Eco*RI fragment containing the amino terminus of the resulting gene was inserted at the *Nde*I-*Bam*HI site on pET3 vector (the *Eco*RI site of the fragment and the *Bam*HI site on the vector had been converted into *Xho*I sites by linker ligation). The truncated Nda1 and Nda4 proteins were made in *E. coli* as described (Studier et al., 1990), purified and used to immunize rabbits (Watt et al., 1985; Hirano et al., 1988). Affinity purification of the antisera was performed essentially as described (Smith and Fisher, 1984). Standard procedures of immunofluorescence for fission yeast cells were employed (Hagan and Hyams, 1988). Monoclonal antibody against tubulin of *Trypanosoma brucei* that crossreacted with the fission yeast tubulin was used to stain microtubules (Woods et al., 1989; Funabiki et al., 1993).

Overproduction of Nda1 and Nda4 in *S. pombe*

To overexpress the Nda1 protein from the inducible *nmt1*$^+$ promoter in *S. pombe* (Maundrell, 1990), plasmid pREP1(*nda1*$^+$) was constructed as follows. An *Nde*I site was made at the initiation codon of the *nda1*$^+$ gene by the PCR method. A cDNA library (Fikes et al., 1990) was used as the template for PCR, so that the resulting PCR fragment did not contain an intron in the amino terminus of *nda1*$^+$. The PCR fragments were sequenced to confirm the absence of mutated residues. The amino terminus of the genomic *nda1*$^+$ gene was replaced by the *Nde*I-*Xho*I PCR fragment; then the *Nde*I-*Sma*I fragment covering the entire Nda1 protein was inserted into the *Nde*I-*Bam*HI site of pREP1. For overproduction of the Nda4 protein, the *Nde* I-*Xho* I fragment covering the entire coding region (see above for the construct for *E. coli*) was inserted into the *Nde*I-*Bam*HI site of pREP1 using *Xho*I linker. The resulting plasmid pREP1(*nda4*$^+$) was used. pREP1(*nda1*$^+$) and pREP1(*nda4*$^+$) complemented the cold-sensitivity of *nda1* and *nda4* mutants, respectively, in the presence of thiamine, under which condition low level expression of genes under the *nmt1*$^+$ promoter occurs.

Construction of a strain expressing histidine-tagged Nda1

pREP2(*wee1*$^+$)HA6His was a gift from P. Russel (The Scripps Research Institute). This plasmid contains the *ura4*$^+$ gene, ars1 and the nmt promoter-*wee1*$^+$ ORF (open reading frame) tagged with the HA epitope and 6 histidine residues at its C terminus. The *wee1*$^+$ ORF was then replaced by the *nda1*$^+$ ORF. From this plasmid (pKA3), ars1, the nmt promoter and the N-terminal region of the *nda1* ORF were removed. The the resulting integration plasmid pKA9 was used for transformation of *h*$^-$ *ura4 nda1* cs cells. One *ura*$^+$ and *cs*$^+$ transformant expressed the Nda1 protein with the HA epitope and 6 histidine residues at its C terminus (Nda1-6His), from its endogenous *nda1*$^+$ promoter. Tetrad analyses confirmed that integration had occurred at the nda1 locus (data not shown). These results also indicate that Nda1-6His is functional in vivo. Cell extracts were prepared essentially as described and fractionated by Ni-NTA agarose (Qiagen) according to the instruction provided by the manufacturer (Uemura et al., 1987).

Other methods

Manipulation of DNA was performed using standard procedures (Sambrook et al., 1989). A Beckton-Dickinson (Lincoln Park, NJ) FACScan was used to monitor the cellular DNA content (Costello et al., 1986; Miyake et al., 1993). SDS gel electrophoresis was performed according to the method of Laemmli (1970). Western blotting with antisera against Nda1 or Nda4 proteins was performed essentially as described by Towbin et al. (1979). Anti-rabbit IgG antibodies conjugated with peroxidase were used as secondary antibodies and were detected by the ECL Western blotting system (Amersham). Pulse field gel (PFG) electrophoresis was performed according to the procedures of Fan et al. (1988) with a 0.6% gel (SeaKem LE; FMC) in 0.5× TBE, at 14°C and 50 V with a pulse time of 3,600 seconds for 160 hours, using a CHEF DR-II (Bio-Rad) apparatus.

RESULTS

Identification of the gene products of nda1$^+$ and nda4$^+$ by immunoblotting

Rabbit polyclonal antibodies were raised against bacterially-

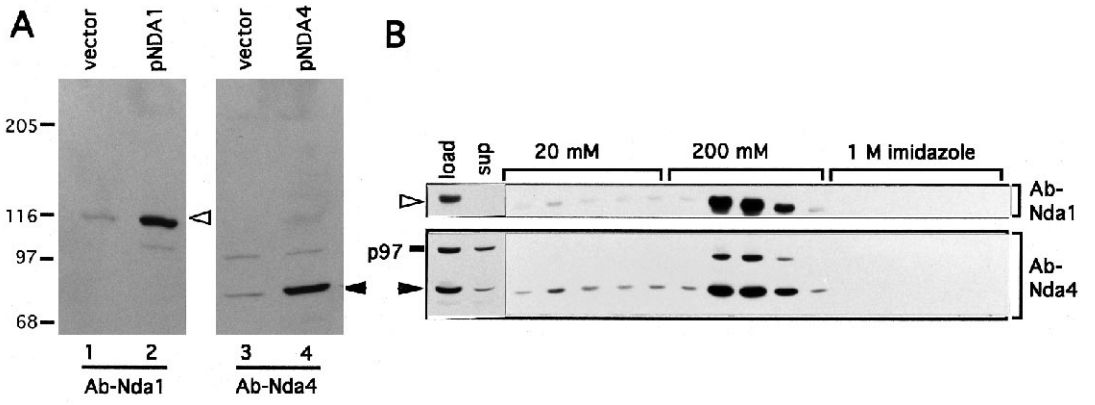

Fig. 1. Immunoblot of *S. pombe* extracts using anti-Nda1 and -Nda4 antibodies. (A) Cell extracts prepared from wild-type cells (h^- leu1) transformed with a vector plasmid (pDB248; lanes 1 and 3) or with plasmids carrying the $nda1^+$ or $nda4^+$ gene (lanes 2 and 4, respectively), subjected to SDS gel electrophoresis and immunoblotted using affinity-purified anti-Nda1 or anti-Nda4 antibodies. Each lane was loaded with the extracts from 10^7 cells. Sizes of molecular mass standards are shown on the left side in kDa. The presumed positions of Nda1 (115 kDa) and Nda4 (80 kDa) proteins are indicated by open and filled arrowheads, respectively. These bands increased in intensity in extracts carrying multicopy plasmid with the $nda1^+$ or $nda4^+$ gene. Cross-reacting antigens (97 kDa for anti-Nda4 antibodies) did not increase in band intensity. (B) Crude extracts of *S. pombe* cells expressing Nda1-6His (load) were fractionated on a Ni-affinity column (see Materials and Methods). sup is the fraction not bound to the resin. Bound proteins were eluted with buffers containing the indicated concentrations of imidazole. These fractions were imunoblotted by the antibodies (Ab) indicated. The 97 kDa protein cross-reacting with anti-Nda4 antibodies is eluted in the same fractions as Nda1-6His and Nda4.

made Nda1 and Nda4 proteins (Materials and Methods). By immunoblotting, affinity-purified anti-Nda1 antibodies detected the 115 kDa protein in the extract of *S. pombe* (Fig. 1A, lane 1), which increased the band intensity in cells carrying multicopy plasmid with the $nda1^+$ gene (Fig. 1, lane 2). The predicted molecular mass of Nda1 was 92.8 kDa (Miyake et al., 1993). This retarded migration might be due to the highly acidic stretches present in the Nda1 protein (calculated pI is 4.99; acidic proteins are thought to bind fewer SDS molecules and to run more slowly in SDS gel). Similar retardation was observed with the MCM2 protein (Yan et al., 1993). Its predicted molecular mass was 101 kDa while it migrated at the 120 kDa position in SDS gel.

Immunoblotting using affinity-purified anti-Nda4 antibodies showed two bands at 80 and 97 kDa in the extracts of *S. pombe* (Fig. 1, lane 3). The 80 kDa band was in good agreement with the predicted size (80.2 kDa; Miyake et al., 1993). The level of this 80 kDa protein increased when extracts of cells transformed with multicopy plasmid carrying the $nda4^+$ gene were employed (Fig. 1, lane 4), although the cross-reacting 97 kDa band did not. This indicates that the 80 kDa protein was the gene product of $nda4^+$ and the 97 kDa protein was not. The cross-reacting 97 kDa protein co-fractionated with Nda1 and Nda4 during several column chromatography steps (Y.A. unpublished results). Fig. 1B shows the results of Ni-affinity column chromatography (Materials and Methods). Not only histidine-tagged Nda1-6His, but also Nda4 and the 97 kDa proteins without His-tags were bound and eluted in 200 mM imidazole. This binding was not observed with normal Nda1 without the His tag (data not shown). Note that the three proteins show exactly the same elution profile, although some Nda4 and 97 kDa protein was not bound and was found in the supernatants (sup). These results suggest that Nda1, Nda4 and the 97 kDa proteins physically interact each other (see Discussion).

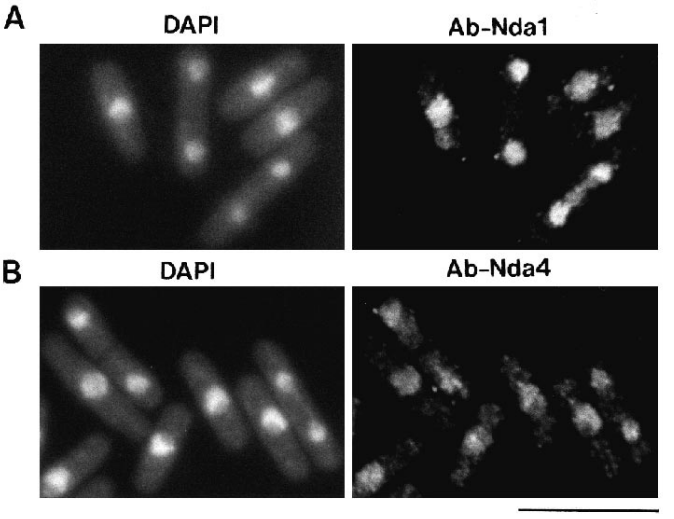

Fig. 2. Nda1 and Nda4 immunolocalize to the nucleus. Asynchronously growing wild-type cells were fixed with formaldehyde and stained with affinity-purified anti-Nda1 (A; Ab-Nda1) or anti-Nda4 antibodies (B; Ab-Nda4). 4′,6-diamidino-2-phenylindole (DAPI) was used to stain DNA. The G_2-phase cells contain a single nucleus while cells with a dividing, elongated nucleus or with two nuclei without a septum are in the M phase. The S phase occurs in cells containing two nuclei with a septum under the culture conditions used (see Fig. 7). Cells show predominantly nuclear staining throughout the cell cycle. Cells stained with anti-Nda4, however, showed significant cytoplasmic staining as well. Bar, 10 μm.

Nuclear localization of Nda1 and Nda4 proteins

We used these affinity-purified antibodies to immunolocalize Nda1 and Nda4 proteins in wild-type fission yeast cells. Exponentially growing cells were fixed with formaldehyde and then

stained with either anti-Nda1 or anti-Nda4 antibodies (Fig. 2). Both antibodies revealed predominantly nuclear staining (right panels, Ab-Nda1 and Ab-Nda4). In cells transformed with a multicopy plasmid containing the *nda1*$^+$ or *nda4*$^+$ gene, nuclear staining with anti-Nda1 or anti-Nda4 antibodies was enhanced (data not shown). Antibodies against Nda4 showed significant cytoplasmic staining, but its intensity did not appear to change during the cell cycle.

In exponentially growing wild-type culture, cells with a single nucleus are in G_2 phase, while cells with two nuclei are in post-M phase. The S phase starts immediately after nuclear division, and replication is complete before cell separation. Small-sized cells immediately after cell division already contain 2C DNA. In each of these cell cycle stages, immunofluorescence for Nda1 and Nda4 was predominant in the nucleus. Nda1 and Nda4 proteins thus do not appear to change their localization during the cell cycle to a visible degree.

Three cdc mutants (*cdc10*, *cdc22* and *cdc25*; Nurse, l990) were employed to examine immunolocalization of Nda1 and Nda4 (Fig. 3A-C; upper panels, DAPI; lower panels, antibody staining). The *cdc10*$^+$ gene is required for START, a commitment to the mitotic cell cycle in G_1 phase (Fig. 3A). The mutant cells are blocked at the G_1 phase. The *cdc22*$^+$ gene encodes the regulatory subunit of ribonucleotide reductase and the mutant cells are arrested at S phase at the restrictive temperature (Fig. 3B). The *cdc25*$^+$ gene encodes the tyrosine phosphatase that removes the phosphate from Tyr-15 of Cdc2. The *cdc25* mutant cells are arrested at the G_2/M boundary at 36°C (Fig. 3C). In all cases, nuclei were stained with antibodies against Nda1 or Nda4. No significant difference was found from the staining patterns of wild-type cells. Nuclear staining in *cdc25* mutant might be somewhat diffuse, but the nuclear chromatin region appeared proportionally extended.

Immunolocalization of Nda1 and Nda4 in nda1 and nda4 mutant cells

We next examined whether nuclear localization was affected in *nda1* or *nda4* mutant cells at the restrictive temperature (20°C). Cells incubated at 20°C were taken at 0, 6 (only for nda4) and 10 hours and subjected to immunofluorescence (Fig. 4). The generation time of *S. pombe* at 20°C is approximately 5 hours. Nuclear immunolocalization of Nda1 and Nda4 was seen at 20°C. Interestingly, the nuclear signal was significantly enhanced in both mutant strains (Fig. 4, 10 hours). This was especially pronounced in *nda4* mutant cells (Fig. 4B).

Effect of overexpression

Wild-type cells carrying multicopy plasmid with the *nda1*$^+$ or *nda4*$^+$ gene produced normal colonies. We then examined whether ectopic overexpression of Nda1 and Nda4 under the *nmt1* promoter (Maundrell, 1990) had any effect. The *nmt1* promoter induces a massive overproduction of protein downstream of the promoter in the absence of thianime. Plasmids pREP1(*nda1*$^+$) and pREP1(*nda4*$^+$) containing the inducible *nmt1*$^+$ promoter (Maundrell, 1989; see Materials and Methods) were constructed. Colony formation of wild-type cells overproducing Nda1 was severely affected, while rather small colonies were made under Nda4 overproduction (Fig. 5A). The levels of overproduced Nda1 and Nda4 are shown by immunoblotting (Fig. 5B). Consistent with the results of colony formation, cell division was severely inhibited when

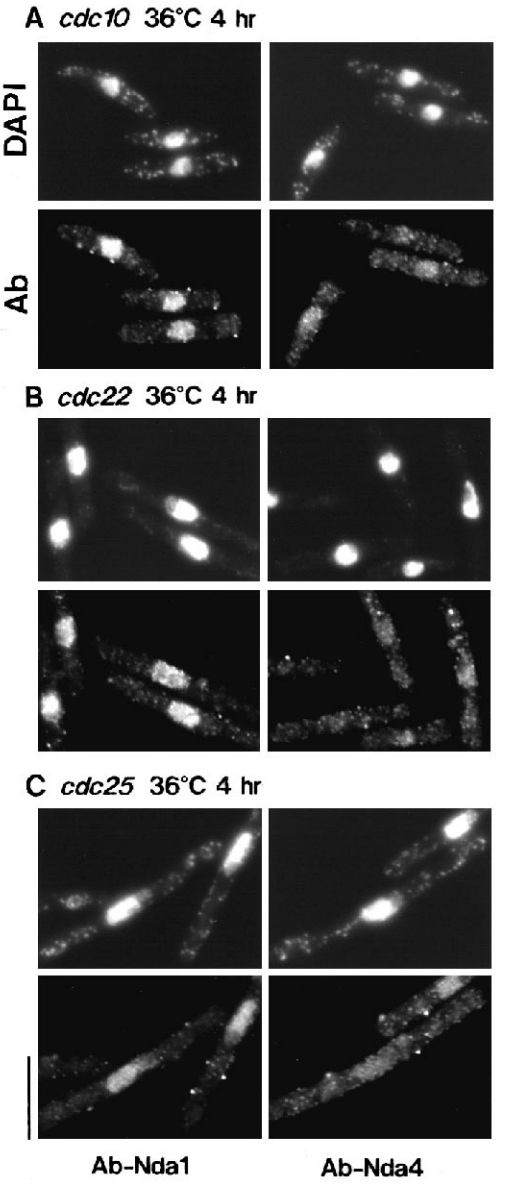

Fig. 3. Immunolocalization of Nda1 and Nda4 in *cdc10*, *cdc22* and *cdc25* mutant cells. Immunolocalization of Nda1 and Nda4 was examined in three cell division-cycle mutants (*cdc10*, *cdc22* and *cdc25*) arrested at the restrictive temperature. DAPI staining of the same cells is shown in the upper panels of A (*cdc10*), B (*cdc22*) and C (*cdc25*). Lower panels, antibody staining: left, anti-Nda1 and right, anti-Nda4 antibodies. In all mutant cells examined, both Nda1 (Ab-Nda1) and Nda4 (Ab-Nda4) were enriched in the nucleus. Bar, 10 μm.

Nda1 was overproduced after 10 hours (Fig. 5C). The frequency of septation index decreased after overproduction, consistent with the blockage of cell division (Fig. 5D).

To determine whether this decrease in septation index was due to the delay at a certain cell-cycle stage, overproducing cells were stained with DAPI. Cells overexpressing Nda1 were arrested with a single nucleus but did not show cell elongation, whereas those overexpressing Nda4 and also containing the single nucleus were somewhat elongated (data not shown). Tubulin immunofluorescence showed typical interphase

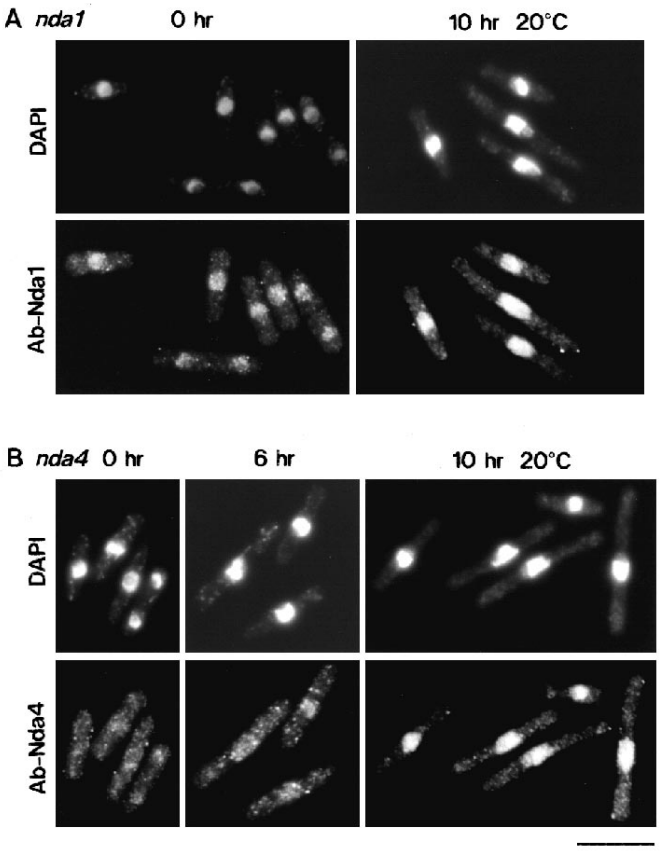

Fig. 4. Localization of Nda1 and Nda4 proteins in mutant cells. Localization of Nda1 in the *nda1* mutant (A) and of Nda4 in the *nda4* mutant (B) was examined at the restrictive temperature for 0-10 hours. The mutant cells were stained with anti-Nda1 (Ab-Nda1) or with anti-Nda4 (Ab-Nda4) antibodies. Chromatin DNA was stained with DAPI. At 10 hours the nuclear signal for Nda1 and Nda4 became much stronger than that at 0 hours. Bar, 10 μm.

microtubule arrays in the cytoplasm, indicating that cells overproducing Nda1 or Nda4 were either arrested or delayed in interphase (data not shown). We used FACScan to examine whether the DNA content of these overproducing cells was affected. In an asynchronous culture of wild-type *S. pombe*, most of the cells are in the G_2 period and have a 2C DNA content. The haploid cells overproducing Nda1 or Nda4 contained 2C DNA (Fig. 6A). Thus DNA replication did not seem to be affected in overproducing cells.

Then, to determine any abnormality at the level of DNA structure in mutant cells, chromosomal DNA was run in pulsed field gel electrophoresis (PFG). In the budding yeast *mcm* mutant (i.e. *cdc46*) larger chromosomes (e.g. chromosome IV) did not enter the PFG gel at the restrictive temperature, probably because of the accumulation of DNA with replication bubbles (Hennessy et al., 1991). Chromosomal DNA in cells treated with hydroxyurea or defective in replication are also unable to enter the gel (e.g. Kelly et al., 1993; Saka et al., 1994). In cs *nda1* and *nda4* mutants at 20°C for 6-12 hours, chromosome DNA entered the gel with greatly reduced efficiency (Fig. 6B); however, this kind of inhibition was not observed in cells overexpressing Nda1 or Nda4 (Fig. 6C).

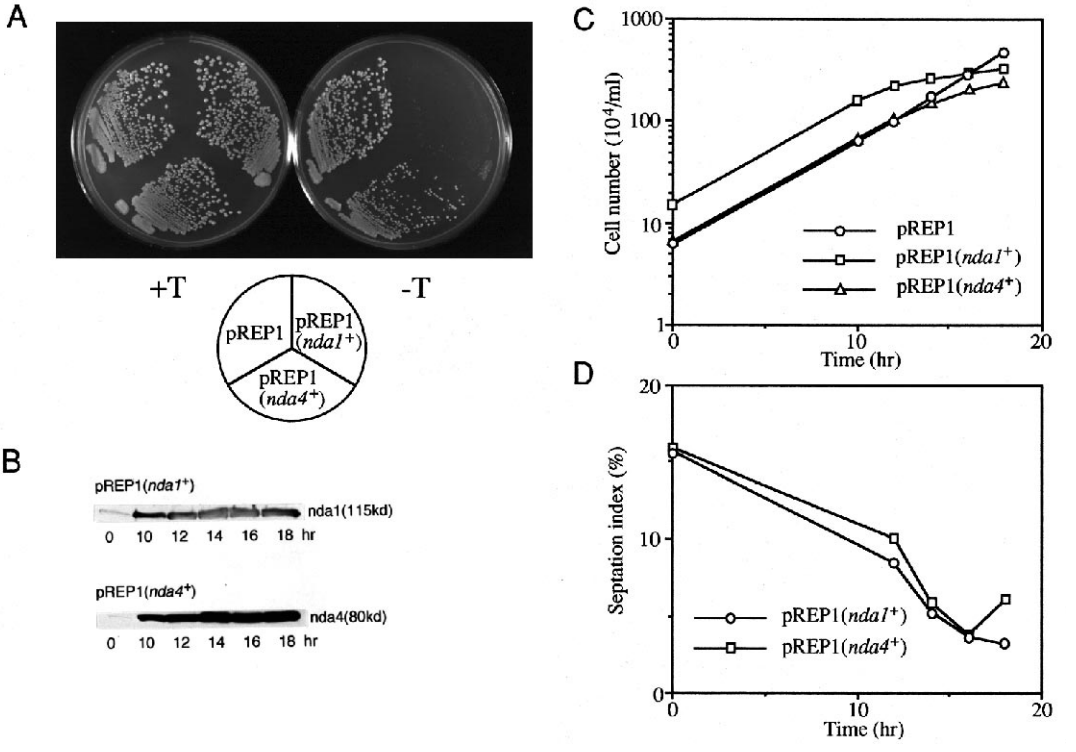

Fig. 5. Overexpression of Nda1 or Nda4 delays cell division. Wild-type cells (HM123) were transformed with pREPl, a vector control, or with plasmids containing the *nda1*$^+$ gene (pREPl(*nda1*$^+$)) or the *nda4*$^+$ gene (pREPl(*nda4*$^+$)) under the inducible *nmt1*$^+$ promoter. (A) Transformants were streaked on minimal EMM2 plates supplemented with (+T) or without (–T) thiamine and incubated for 3 days at 33°C. Expression of the genes under the *nmt1*$^+$ promoter was induced on the plate in the absence of thiamine. Overexpression of the Nda1 protein severely inhibited colony formation. (B) Overproducing cells in the liquid culture after the removal of thiamine were immunoblotted using anti-Nda1 (upper panel) or anti-Nda4 (lower panel). Overproduction started approximately 10 hours after the removal of thiamine. Cells were taken at 10, 12, 14, 16 and 18 hours after the removal of thiamine. (C and D) Transformants were first grown in EMM2 containing thiamine and then transferred to EMM2 lacking thiamine. After 10 hours the gene under the nmt1$^+$ promoter was derepressed. The cell number and septation index were measured.

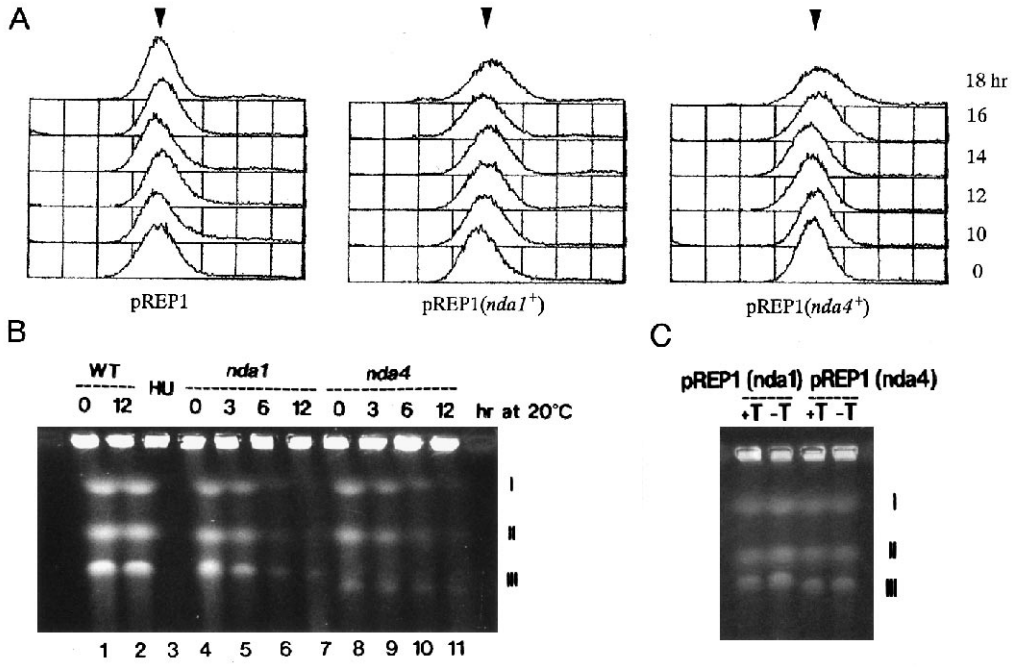

Fig. 6. FACScan and PFG analyses. (A) Wild-type cells carrying plasmids (left, the vector; middle pREP1(*nda1*$^+$); right pREP1(*nda4*$^+$)) were cultured in EMM2 liquid without thiamine. Samples were taken at the time points indicated and the DNA content of cells was examined by FACScan. Arrowheads represent the position of 2C. Note that asynchronously growing wild-type cells normally contain 2C DNA. (B) Wild-type (WT) cells and cold-sensitive nda1 or nda4 mutants were grown at 20°C, and aliquots of the cultures were taken at the time points indicated. Chromosomal DNA was prepared and subjected to pulse-field gel (PFG) electrophoresis. Each lane was loaded with DNA from the same number of cells. Chromosome DNA prepared from wild-type cells grown in the presence of hydroxyurea does not enter the gel (HU). I, II and III indicate the band positions of chromosomes I, II and III. (C) Wild-type cells carrying plasmid pREP1(*nda1*$^+$) or pREP1(*nda4*$^+$) were first grown in EMM2 in the presence of thiamine (+T), then transferred to medium without thiamine and cultured for 16 hours at 33°C (–T). The band intensities remained approximately the same as for wild-type cells.

Judging from the PFG and FACScan patterns, *nda1* and *nda4* mutant cells at 20°C have defects in DNA replication, while overproducing cells appear to contain normal G_2-like chromosome DNA and are delayed in the G_2 stage.

DISCUSSION

In the present paper, we have examined the immunolocalization of Nda1 and Nda4 proteins, the fission yeast MCM homologs (Mcm2 and Mcm5/Cdc46, respectively). We have identified the gene products of *nda1*$^+$ and *nda4*$^+$ as 115 kDa and 80 kDa proteins, respectively, by immunoblotting. Affinity purified anti-Nda4 antibodies cross-react with a 97 kDa antigen. This protein seems to be physically associated with Nda1 and Nda4 (discussed below).

Immunofluorescence microscopy indicated that antibody staining was enriched in the nucleus for both Nda1 and Nda4 proteins. Staining with anti-Nda4 antibodies probably detected not only Nda4 but also the 97 kDa protein. Similar localization of Nda1 and Nda4 plus the 97 kDa protein is consistent with the physical interaction between these proteins. Nuclear staining was enhanced by a multicopy plasmid carrying the *nda1*$^+$ or *nda4*$^+$ gene. Furthermore, nuclear staining was enhanced in both *nda1* and *nda4* mutants at the restrictive temperature. These results indicate Nda1 and Nda4 are nuclear proteins and are consistent with the nuclear localization of MCM homologs in other organisms. Nda1 staining is more exclusively in the nucleus and is very low in the cytoplasm. Staining with anti-Nda4 shows a significant level of speckled signals in the cytoplasm. But the staining patterns of Nda1 and Nda4 are indistinguishable between cells at different cell-cycle stages in an asynchronous culture. The predominance of nuclear staining is constitutive throughout the cell cycle and no sign of any drastic change in their distribution was observed at the level of light microscopy.

An immunolocalization pattern similar to that in wild-type cells was obtained in *cdc10*, *cdc22* and *cdc25* mutant cells at the restrictive temperature. These results are in striking contrast to the behavior of the budding yeast Mcm2, 3, and 5

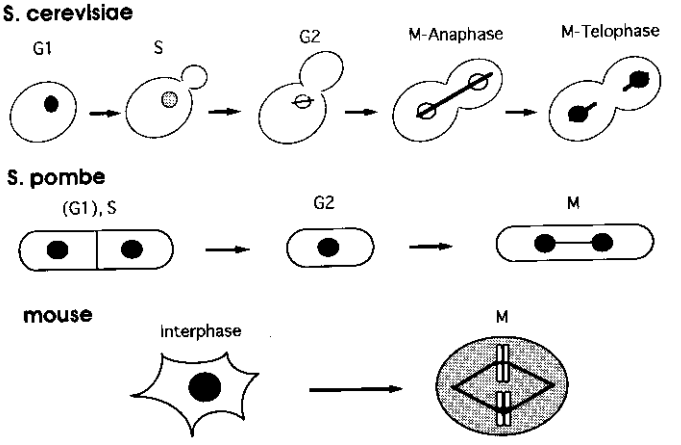

Fig. 7. Immunolocalization of MCM-related proteins in yeasts and mouse. In *S. cerevisiae* the Mcm proteins locate in the nucleus during G_1 phase (shaded) and disappear with the onset of S phase. The proteins enter the nucleus at the end of M phase. In contrast, in *S. pombe* Nda1 and Nda4 proteins are enriched in the nucleus (shaded) throughout the cell cycle. In mouse cells, P1 protein, a homolog of MCM3 is confined to the nucleus in interphase. In the M phase, P1 is dissociated from mitotic chromosomes and dispersed into the cytoplasm.

proteins. They enter the nucleus at the end of M phase and disappear from the nucleus at the beginning of S phase (summarized in Fig. 7; for a review, see Tye, 1994). In the case of Nda4, there might be some reduction of its nuclear signals in *cdc22* and *cdc25* mutant cells and this might imply some physiological significance. However the reduction does not lead to the complete loss of the nuclear signal as is observed in budding yeast and the Nda1 signal is clearly still nuclear. Thus the apparent behavior of Nda1 and Nda4 does not fulfill the basis for the model of replication licensing factors, which can access the chromatin only during mitosis (Blow and Laskey, 1988; Blow, 1993; Coverley and Laskey, 1994). Our results indicate that, in fission yeast, the change in localization between cytoplasm and the nucleus is not essential for executing the functions of Nda1 and Nda4.

Kimura et al. (1994) reported a detailed immunolocalization study of P1 protein, a mouse homolog of MCM3. P1 is dissociated from mitotic chromosomes and localized to the nucleus constitutively in interphase. If the interphase cells are treated with detergent prior to fixation, a fraction of nuclear P1 can be extracted. Presumably the extractable fraction of P1 is not tightly associated with chromatin. This procedure reveals the cell-cycle dependent association and dissociation of P1 to chromatin. In G_1 phase, P1 is not extractable from the nucleus. During S phase, P1 is first dissociated from euchromatin and then later from heterochromatin as though ongoing replication dissociates P1 from chromatin. The change in intranuclear distribution of P1 may be correlated to the degree of phosphorylation; a hyperphosphorylated form of P1 is easily extracted by detergent treatment of the nucleus (Kimura et al., 1994; Todorov et al., 1995). Nda1 and Nda4 proteins might behave similarly to P1. If Nda1 and Nda4 participate in the early stages of DNA replication, this can be accomplished by bringing the proteins from one nuclear compartment to another in order to interact with DNA or particular chromatin proteins, but not by moving through the nuclear envelope. It is possible that the Nda1 and Nda4 proteins stay within the nucleus during M phase simply due to the absence of nuclear envelope breakdown in *S. pombe*. It is of interest to examine whether the extractability of Nda1 or Nda4 from the nucleus varies with the cell cycle.

MCM proteins constitute a family that can be classified into 6 members (Koonin, 1993; Hu et al., 1993; Kimura et al., 1995). In *Xenopus*, immunoabsorption experiments have shown that MCM2, 3 and 5 are in the same complex of 490-660 kDa (Kubota et al., 1995; Chong et al., 1995; Madine et al., 1995). A similar interaction is also observed in mice (Kimura et al., 1995). In the present study, we have shown that the histidine-tagged Nda1 is coeluted with the untagged Nda4 and a 97 kDa protein from a Ni-affinity column, indicating that the MCM proteins were in an oligomeric form in fission yeast as well. Furthermore, immunoprecipitation experiments showed that Nda1 and Nda4 co-precipitate (Y.A., unpublished results). The 97 kDa protein that cross-reacts with anti-Nda4 is found in the same fractions as the Nda1-6His and Nda4 proteins during several chromatography steps including the Ni-column (Fig. 1B; Y.A., unpublished results). Given the similarlities of the primary structures among MCM proteins, we speculate that the 97 kDa protein may be another member of the MCM family.

We note that a minor fraction of Nda4 and the 97 kDa protein does not bind to the Ni-column. This fraction might not bind to Nda1, leading to different antibody staining patterns by anti-Nda1 (exclusively nuclear) and anti-Nda4 antibodies (nuclear and some cytoplasmic). The MCM proteins have well conserved ATPase domains but their molecular functions in replication are still unclear. It will be essential to study biochemically the behavior of the MCM protein complex during the cell cycle.

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