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Fission Yeast Genes *nda1*$^+$ and *nda4*$^+$, Mutations of Which Lead to S-Phase Block, Chromatin Alteration and Ca^{2+} Suppression, Are Members of the *CDC46/MCM2* Family

Sanae Miyake,*† Nobuyuki Okishio,‡ Itaru Samejima,‡ Yasushi Hiraoka,‡§ Takashi Toda,‡ Izumu Saitoh,* and Mitsuhiro Yanagida‡‖

*Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, P.O. Takanawa, Tokyo 108, Japan; and ‡Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Fission yeast cold-sensitive mutants *nda1-376* and *nda4-108* display a cell cycle block phenotype at the restrictive temperature (cell elongation with the single nucleus) accompanied by an alteration in the nuclear chromatin region. DNA content analysis shows that the onset of DNA synthesis is blocked or greatly delayed in both mutant cells, the block being reversible in *nda4-108*. Upon release to the permissive temperature, *nda4-108* cells resumed replicating DNA, followed by mitosis and cytokinesis. The *nda4* phenotype was partly rescued by the addition of Ca^{2+} to the medium; Ca^{2+} plays a positive role in the *nda4*$^+$ function. The predicted protein sequences of *nda1*$^+$ and *nda4*$^+$ isolated by complementation are similar to each other and also, respectively, to those of the budding yeast, *MCM2* and *CDC46*, both of which are members of the gene family required for the initiation of DNA replication. The central domains of these proteins are conserved, whereas the NH_2- and COOH- domains are distinct. Results of the disruption of the *nda1*$^+$ and *nda4*$^+$ genes demonstrates that they are essential for viability.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe nda* mutants were isolated on the basis of cell division cycle arrest phenotype that produced elongated cells with a single nucleus under the restrictive condition (Toda *et al.*, 1981, 1983; Yanagida *et al.*, 1986). Mutations *nda2* and *nda3* exhibited condensed chromosomes reminiscent of mitotically arrested cells. The *nda2*$^+$ gene was isolated by complementation of the *nda2-52* mutant and was found to encode one of the two α-tubulins present in fission yeast (Toda *et al.*, 1984; Adachi *et al.*, 1986). Subsequently, the *nda3*$^+$ gene was isolated by complementation of the *nda3-ben1* mutant and was discovered to code for the other tubulin, that is, β-tubulin (Hiraoka *et al.*, 1984). Both *nda2* and *nda3* mutants were shown to be defective in the spindle formation so that the progression of mitosis is blocked (Toda *et al.*, 1983; Umesono *et al.*, 1983; Hiraoka *et al.*, 1984; Kanbe *et al.*, 1990).

We report here characterizations of two other *nda* mutants, *nda1-376* and *nda4-108*, that show the altered nuclear chromatin region but not the condensed individual chromosomes (Toda *et al.*, 1983; Yanagida *et al.*, 1986). Fluorescence-activated cell sorter (FACScan) analysis and immunofluorescence microscopy using anti-tubulin antibodies established that both *nda1* and *nda4* mutants are defective in DNA synthesis and arrested in the interphase. The *nda1*$^+$ and *nda4*$^+$ genes were isolated by transformation of the mutants. The

‖ Corresponding author. Present addresses: †Department of Biochemistry, Toho University School of Medicine, Ohmori-Nishi 5-21-16, Ota-ku, Tokyo 143, Japan; §Kansai Advanced Research Center, Communications Research Laboratory, 588-2 Iwaoka, Nishi-ku, Kobe 651-24, Japan.

gene products of *nda1*$^+$ and *nda4*$^+$ predicted by their nucleotide sequencing showed that they are similar to each other and belong to the same gene family recently identified and believed to play an important role in the onset of DNA replication (Hennessy *et al.*, 1991; Yan *et al.*, 1991). Gene disruption experiments indicated that these are essential genes for viability.

MATERIALS AND METHODS

Strains and Media

S. pombe haploid strains, derivatives of *h*$^-$ *leu1*, were used. Mutant strains *nda1-376* and *nda4-108* used in the present study were previously described (Toda *et al.*, 1981, 1983; Yanagida *et al.*, 1986). Culture media for *S. pombe* were YPD (complete rich medium: 1% yeast extract, 2% polypeptone, 2% glucose; 1.6% agar was added for plates), SD (minimal medium: 0.67% yeast nitrogen base without amino acid, 2% glucose; 1.7% agar was added for plates), and EMM2 (minimal medium) (Mitchison, 1970). *Escherichia coli* was grown in LB (0.5% yeast extract, 1% polypeptone, 1% NaCl [pH 7.5]; 1.5% agar was added for plates).

Indirect Immunofluorescence Microscopy

The procedure described by Hagan and Hyams (1988) was followed for preparing and fixing cells. For microtubule staining the monoclonal antibody raised against *Trypanosoma brucei* α-tubulin TAT-1 (Woods *et al.*, 1989) and Texas Red-conjugated goat anti mouse IgG and IgM antibodies (EY Laboratories, San Mateo, CA) were used as primary and secondary antibodies, respectively.

Transformation, Integration, and Gene Disruption

The lithium acetate method (Ito *et al.*, 1983) was used to transform *S. pombe* cells. Integration of cloned sequences onto the chromosome was performed by homologous recombination (Rothstein, 1983). For disruption of the *nda4*$^+$ and *nda1*$^+$ genes, plasmids carrying these genes replaced with the *S. pombe ura4*$^+$ gene were constructed, linearly cleaved, and used for transformation. Stable transformants obtained were analyzed by tetrads and Southern hybridization.

Southern Hybridization and Nucleotide Sequence Determination

The standard procedures for Southern blotting (Maniatis *et al.*, 1982) were employed. The 4.3-kilobase (kb) *Bam*HI-*Sma* I fragment that is able to complement cold-sensitive (cs)*nda1-376* was sequenced by the dideoxy method (Sanger *et al.*, 1977). Nucleotide sequence of the 3.6-kb *Hin*dIII fragment that complemented *nda4-108* was also determined. To establish putative introns in the *nda1*$^+$ gene, the region was cloned from an *S. pombe* cDNA library (the gift of Dr. J. Fikes, Massachusetts Institute of Technology) by the polymerase chain reaction (PCR) method.

FACScan Analysis

A Becton-Dickinson (Lincoln Park, NJ) FACScan apparatus was used to monitor the cellular DNA contents. Procedures employed were similar to those described previously (Costello *et al.*, 1986). Cells (1–5 × 10^7) were collected, washed twice with 1 ml distilled water, and then resuspended in 3 ml distilled water. Ethanol (7 ml) was added with vigorous agitation, and cells were stored at 4°C for >12 h. After washing once with 5 ml 50 mM sodium citrate (pH 7.0) and resuspension in 1 ml of the same buffer, RNase A (preboiled for 15 min, Sigma, St. Louis, MO) was added to a final concentration of 1 mg/ml. After a 2-h incubation at 37°C, propidium iodide (Sigma) was added (final concentration, 12.5 μg/ml), and the resulting stained cell suspensions were analyzed.

RESULTS

Cell Division Arrest Phenotypes of nda1 and nda4 Mutants

Both *nda1-376* and *nda4-108* strains (Toda *et al.*, 1981, 1983) are cs, and neither produce colonies at the restrictive temperature (20°C). In liquid cultures, the cell number of both strains increased approximately twofold, and cell division was arrested after 8–10 h at 20°C (Figure 1A). The generation time for wild-type at 20°C is ~5 h. Cells stained by a DNA-specific fluorescent probe diamidino-phenylindole (DAPI) (Toda *et al.*, 1981) are shown in Figure 2, A and B, and Figure 2, E and F for *nda4-108* and *nda1-376*, respectively. The ar-

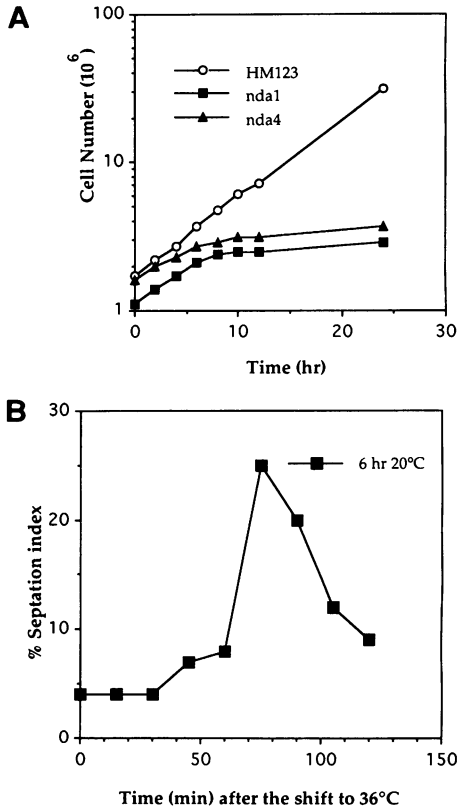


Figure 1. The cell cycle block of *nda4-108* at 20°C is reversible. (A) The cell number of *nda1-376* and *nda4-108* mutants incubated at the restrictive temperature increased approximately twofold before their cell division was arrested. HM123 was wild-type control. (B) *nda4-108* mutant cells exponentially grown at 36°C were blocked by the temperature shift to 20°C for 6 h. The resulting arrested cells were then released by changing the temperature back to 36°C. The percentage septation index of the cultures was measured at 15-min intervals (~500 cells were counted for each time point). Cells treated for 6 h at 20°C (■) resumed dividing after 75 min at 36°C.

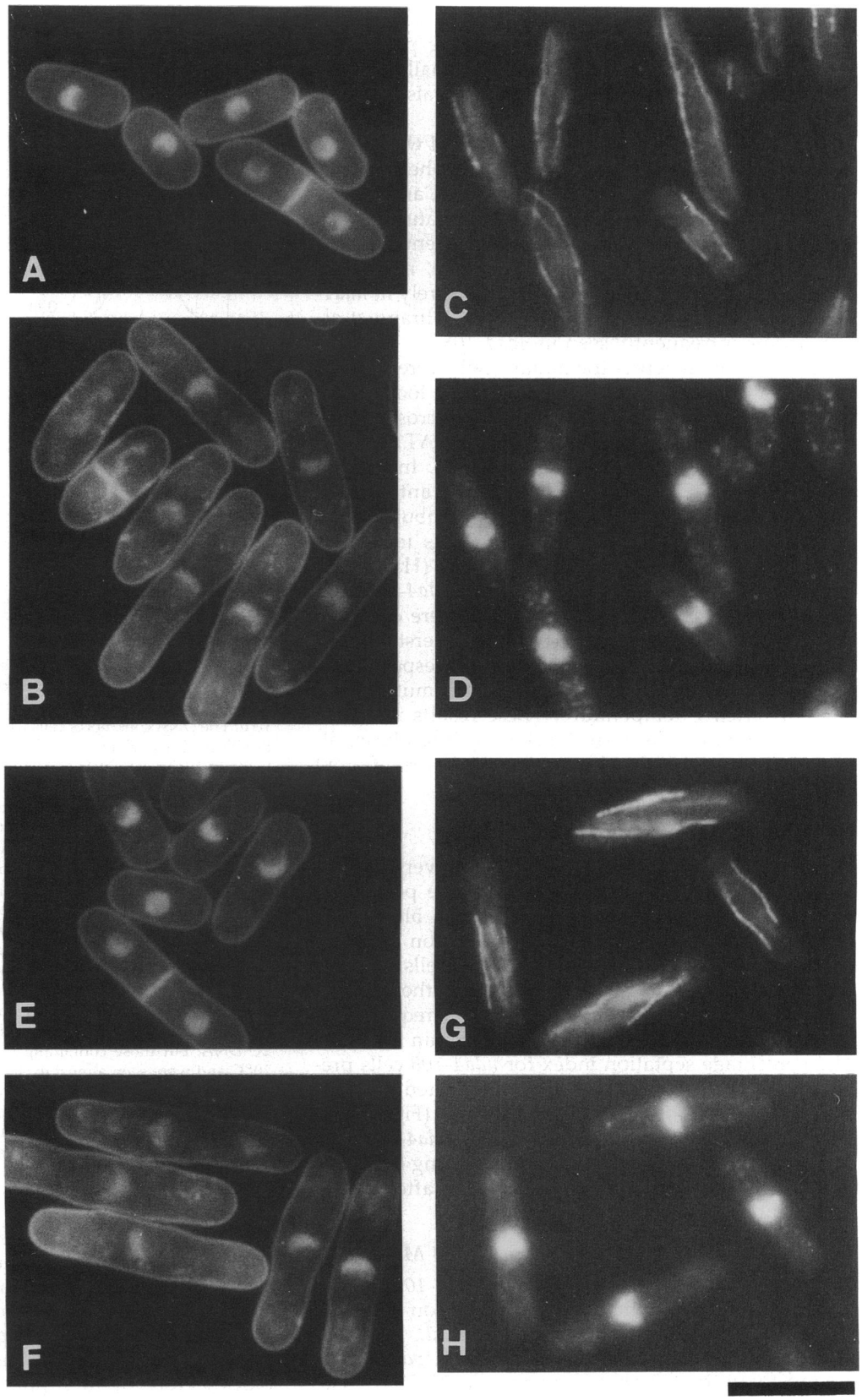


Figure 2. Phenotypes of *nda1-376* and *nda4-108* mutant cells as seen by DAPI and anti-tubulin staining. Cultures of *nda4-108* and *nda1-376* were incubated at the restrictive temperature (20°C) for 0–10 h. Cells were then observed by a DNA-specific fluorescent dye, DAPI, or immunofluorescence microscopy using monoclonal antibody against tubulin. DAPI stain: *nda4-108* for 0 h (A) and 10 h (B) at 20°C, *nda1-376* for 0 h (E) and 10 h (F). Anti-tubulin stain: *nda4-108* (C) and *nda1-376* (G) for 10 h at 20°C. The same cells of the two strains counterstained by DAPI are shown in D and H, respectively. Cells were elongated and the occurrence of the mitotic figures was low in both mutant cells after 10 h. The nuclear chromatin regions of these mutant cells seen by DAPI were altered from the normal hemispherical (Toda *et al.*, 1981) to crescent-like shape after 10 h at 20°C. They are occasionally irregularly shaped in *nda4-108* cells (B). The cytoplasmic granules intensely stained by DAPI after 10 h (B and F) might represent the mitochondrial DNAs that were apparently separated to the two ends of the cells. In C and G, the cytoplasmic microtubule arrays are seen in abundance. In the specimens prepared for immunofluorescence microscopy, the shape of the nuclear chromatin regions stained by DAPI became obscure and somewhat deformed. Bar, 10 μm.

rested cells were elongated and contained a single nucleus (Figure 2, B and F), whereas at the permissive temperature (33 or 36°C), cells grew normally and the shape of nuclear chromatin region was also normal (Figure 2, A and E).

The chromatin region visualized by DAPI was altered in the mutant cells from the normal hemispherical form at the permissive temperature (Figure 2, A and E) to a crescent-like shape at the restrictive temperature (Figure 2, B and F). Alteration was more prominent in *nda4-108* than in *nda1-376* cells. The fiber-like, irregularly shaped region was seen in *nda4-108* but rarely in *nda1-376*. Cells displaying the *cut* phenotype (Hirano *et al.*, 1986) were infrequently seen in *nda4-108*.

To determine whether the mutant cells were arrested in mitosis or interphase, their microtubule localization was visualized by immunofluorescence microscopy using the monoclonal anti-tubulin antibody TAT1 (Woods *et al.*, 1989; Hagan and Yanagida, 1990). Interphase and mitotic cells can be distinguished by anti-tubulin staining. In wild-type interphase, microtubule arrays were observed in the cytoplasm, whereas in mitosis spindle microtubules existed in the nucleus (Hagan and Hyams, 1988). As shown in Figure 2, C (*nda4-108*) and G (*nda1-376*), the mutant microtubules were observed only in the cytoplasm (the same cells counterstained by DAPI are shown in Figure 2, D and H, respectively). No spindle structure was observed in the mutant cells at the restrictive temperature. These results indicated that the mutant cells were blocked in interphase, although the shape of the chromatin region was different from that of interphase.

Reversible Cell Cycle Block of nda4-108

To investigate whether the arrest was reversible, the blocked mutant cells were released to the permissive temperature (Hiraoka *et al.*, 1984). If the block were reversible, cells should resume cell division after the release. Exponentially growing mutant cells at 36°C were first incubated at 20°C for 6 h, and then shifted back to 36°C. The septation index (the frequency of septated cells) was then measured at 15-min intervals. The percentage septation index for *nda4-108* cells previously incubated at 20°C for 6 h (indicated by filled squares) peaked 75 min after the release (Figure 1B). These results showed that the block of *nda4-108* cells was reversible. A similar experiment using *nda1-376* failed to show the reversible cell division after the release.

DNA Synthesis Defects in nda1 and nda4 Mutants

The DNA contents in *nda1-376* and *nda4-108* mutant cells were analyzed with a Beckton-Dickinson FACScan by the procedures described (Costello *et al.*, 1986; Kinoshita *et al.*, 1991). Haploid wild-type *S. pombe* cells are mostly in the G2 phase and contain 2C DNA. The

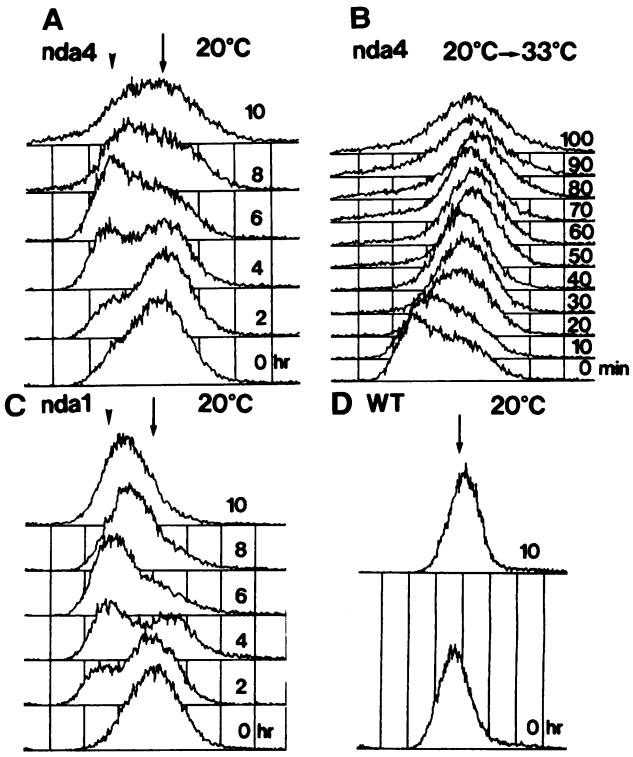


Figure 3. Defects of DNA synthesis in *nda4-108* and *nda1-376* cells. (A) Cells of *nda4-108* cells were first grown at 33°C then transferred to 20°C, and aliquots of the cultures were taken at 2-h intervals for 10 h. The DNA contents of cells were analyzed by a Beckton-Dickinson FACScan. The peak positions for the cellular DNA contents equivalent to 2C and 1C were obtained by the haploid wild-type and G1-arrested *cdc10* mutant cells (Costello *et al.*, 1986; Kinoshita *et al.*, 1991) and are indicated by the arrows and arrowheads, respectively. Cells of *nda4-108* initially contained the 2C DNA, but those containing the 1C DNA appeared after 2 h and were prominent after 4–6 h. After 8–10 h, cells containing 2C DNA increased, indicating that the block of DNA synthesis in *nda4-108* was not tight. (B) *nda4-108* cells incubated at 20°C for 6 h were released by the shift to 33°C. Portions of the cells were taken every 10 min and analyzed by FACScan. Cells containing the 2C DNA began appearing rapidly after 10–20 min and by 30 min after the shift most contained the 2C DNA. (C) *nda1-376* cells were first grown at 33°C and then transferred to 20°C. Portions of the cells were taken at 2-h intervals for 10 h followed by the analysis of DNA content with FACScan. *nda1-376* cells initially contained the 2C DNA, but those containing the 1C DNA appeared after 2 h at 20°C and were seen almost exclusively after 6 h. Most cells after 10 h contained the 1C DNA, indicating that the block of DNA synthesis in *nda1-376* was tight. (D) Wild-type HM123 (h^- *leu1*) were grown at 33°C and then shifted to 20°C. FACScan analysis was done for cells after 0 and 10 h at 20°C. Cells containing 2C DNA were present.

2C peak position is indicated by the arrow in Figure 3, A and C, whereas the position for 1C DNA determined by employing *cdc10* mutant cells (Nurse *et al.*, 1976; Costello *et al.*, 1986) is indicated by the arrowhead. Mutant cells defective in S-phase will pass through G2- and the subsequent M-phase and be followed by the block before or in S-phase. Resulting arrested cells

should display 1C or an intermediate level between 1C and 2C DNA.

We found that both *nda4-108* and *nda1-376* mutants are defective in the onset of DNA synthesis, as shown in Figure 3. Cells of *nda4-108* grown at 33°C were transferred to 20°C, and aliquots of the culture were taken at 2-h intervals and analyzed by FACScan (Figure 3A). Large fractions of *nda4-108* cells contained the 1C DNA after 4–6 h at 20°C. Roughly two-thirds of the cells after 6 h at 20°C were found at the 1C DNA position. The block of DNA synthesis in *nda4-108*, however, was not tight. Cells containing the 2C DNA or intermediary levels between 1C and 2C DNA increased after 6–8 h. The delayed DNA synthesis became prominent after 10 h.

When the *nda4-108* cells arrested at 20°C for 6 h were liberated by the shift to the permissive temperature, those containing the 2C DNA rapidly appeared, together with the disappearance of cells containing 1C DNA (Figure 3B). The DNA synthesis seemed to occur rapidly within 20 min after the release. The septation index of these released cells reached the maximum after 75–90 min (Figure 1B) so that the nuclear division has been completed within this period between the completion of DNA synthesis and the septum formation.

The FACScan analysis using *nda1-376* mutant cells is shown in Figure 3C. *nda1-376* cells are blocked at the onset of S-phase. Cells containing the 1C DNA appeared 2 h after the shift to 20°C and were abundant after 6 h. The block of DNA synthesis in *nda1-376* seems to be tighter than that in *nda4-108*. Wild-type control is shown in Figure 3D. No change was observed before and after the shift to 20°C for 10 h. Cells contained 2C DNA.

nda4-108 Was Suppressed by Ca^{2+}

We examined various chemical and physical agents for their effect on the growth phenotype of mutant cells and found that the calcium ion concentration in the culture medium had a profound influence on the phenotype of *nda4-108* (Figure 4A). No colony of *nda4-108* was formed at 26°C, which is less restrictive than the regularly employed restrictive temperature of 20°C. Small colonies, however, were produced on the plates when 100 mM $CaCl_2$ was added. This striking complementation was specific to calcium ion because the addition of 100 mM $MgCl_2$ had no effect. Other cations such as Zn^{2+}, Mn^{2+}, Na^+, and K^+ also showed no effect.

Lower concentrations of Ca^{2+} (25 and 50 mM) showed positive but weaker effects on the colony formation (Figure 4B) with 10 mM $CaCl_2$ being the minimal concentration detected as having any enhancing effect. Larger colonies were made by raising the concentrations of $CaCl_2$. Because the colony was produced at 26°C but not at 20°C in the presence of 100 mM $CaCl_2$, there was only partial suppression of *nda4-108* by the calcium ion. Such positive effect of the calcium ion was not found for the colony formation of *nda1-376*.

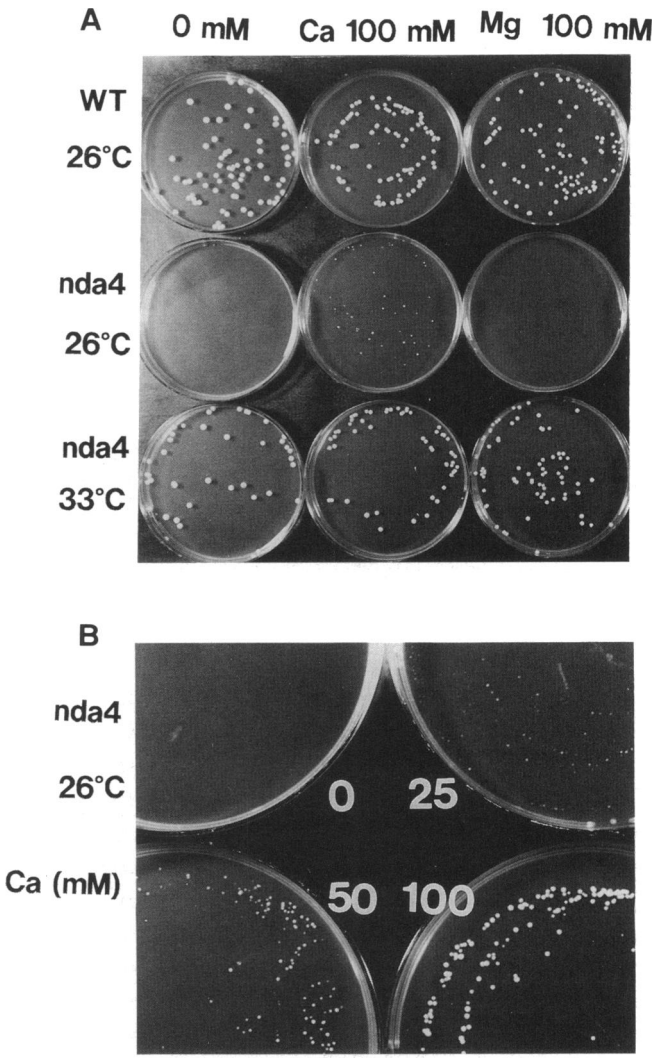
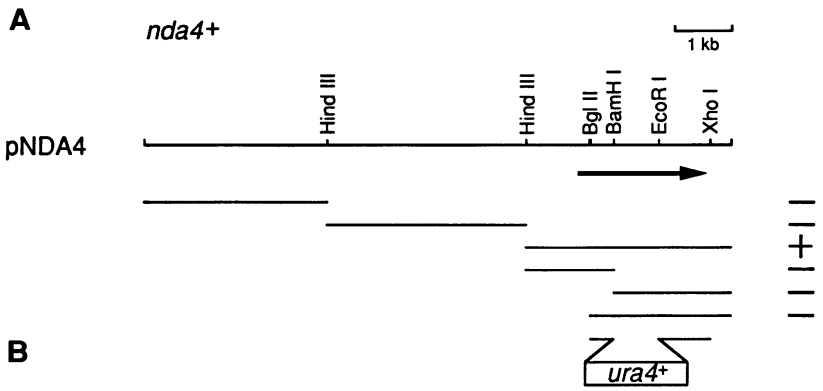


Figure 4. Partial rescue of the cs phenotype of *nda4-108* by Ca^{2+}. (A) *nda4-108* cells were plated on rich YPD medium with or without Ca^{2+} at a restrictive temperature, 26°C, that was less restrictive than that (20°C) used for regular experiments. Small colonies were formed at 26°C on the plates containing 100 mM $CaCl_2$, but no colony was formed on the plates not containing Ca^{2+} or containing 100 mM $MgCl_2$. In the wild-type plated at 26°C or *nda4-108* cells plated at the permissive temperature (33°C), normal colonies were formed on the plates with or without 100 mM $CaCl_2$ or $MgCl_2$. This complementation of *nda4-108* by Ca^{2+} was not observed when the temperature used was 20°C. (B) *nda4-108* mutant cells were plated on rich medium containing 0, 25, 50, or 100 mM $CaCl_2$ at 26°C. Larger colonies were made at 26°C on the plate containing 100 mM $CaCl_2$ than those on 25 or 50 mM $CaCl_2$. No colony was formed on the plate without $CaCl_2$ at this temperature.

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 636   L  F  L  T  S  T  L  A  A  A  T  Q  S  S  P  E  V  T  E  E  V  K  K  I  E  A  S  L  R  K
1995  AAGGTTACCTATAGGGTTTCAGGCGAGTTACCGTATGCTTATACGAGAATACGTAAATGGGGTATGTTTATTGATTAAATCTAAGTCTAA
 666   R  L  P  I  G  F  Q  A  S  Y  R  M  L  I  R  E  Y  V  N  G  ^^^^^  Intron (40 bp)   ^^  ^
2085  CATAATTTTAGCATGGATATTCACAACACGCGTTAGAAATGGCTTTACAAATTCGTTCGTCGAAGGAAACAATTCAACTCAGAAATGGCG
 686          ^^H  G  Y  S  Q  H  A  L  E  M  A  L  Q  I  R  S  S  K  E  T  I  Q  L  R  N  G  G
2175  GACAAACGGTGTATCGATCAGGAGTATGAAATTTAGCGAGCGTACTATTTATTAGATGATATAATTTACAAATATAATACATGATCGTGA
 713   Q  T  V  Y  R  S  G  V  *
2265  TAAGAAATATCAGTCTCGAG
```

Figure 5. Cloning and sequencing of the *nda4*$^+$ gene. (A) Plasmid pNDA4 containing the *nda4*$^+$ gene (the coding region indicated by the arrow with the direction of transcription) was obtained by complementation (+). Derivatives of pNDA4 made by deletion were examined for their ability to complement *nda4-108* mutant. Some restriction sites are indicated. Plasmid carrying the *ura4*$^+$ gene as the marker and used for gene disruption is also shown. (B) The nucleotide sequence containing the *nda4*$^+$ gene is shown with the predicted amino acid sequence. A putative intron has the consensus sequence GTANG– –CTNA– –AG.

Isolation of the nda4$^+$ *Gene by Transformation*

Genomic DNA clones that complemented the cs phenotype of *nda4-108* were isolated from an *S. pombe* DNA library using the *Saccharomyces cerevisiae LEU2* as the selection marker. Plasmids recovered from three independent transformants were found to be identical and designated pNDA4 (Figure 5A). The minimal 3.6-kb clone could fully suppress the cs phenotype of *nda4-108* (+ indicates complementation), whereas the same clone did not complement *nda1-376*. The arrow indicates the coding region of the *nda4*$^+$ gene with the direction of transcription. Genomic Southern hybridization probed with the cloned DNA showed a set of hybridization bands with the

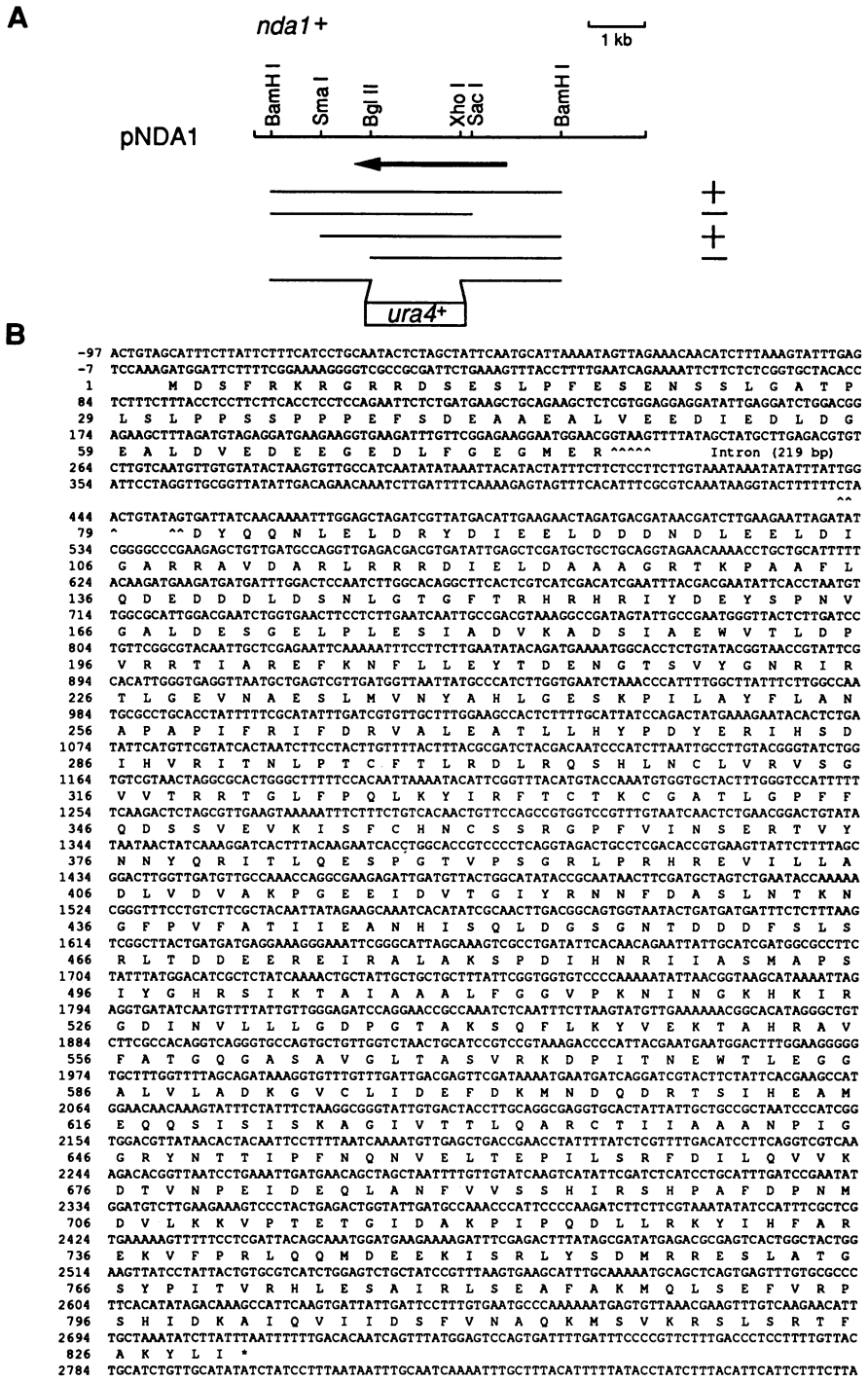

Figure 6. Cloning and sequencing of the *nda1*$^+$ gene. (A) Plasmid pNDA1 containing the *nda1*$^+$ gene (the coding region indicated by the arrow with the direction of transcription) was isolated by complementation of cs *nda1-376* (+). Some restriction sites are shown. Plasmid used for gene disruption is also indicated. (B) The nucleotide sequence of the *nda1*$^+$ gene is shown with the predicted amino acid sequence. The putative intron has the consensus sequence.

expected sizes, indicating that the cloned sequence is unique in the genome. The 3.5-kb *Hin*dIII located adjacent to the complementable DNA fragment was integrated on the chromosome of a fission yeast strain HM123 (*h*$^-$ *leu1*) by homologous recombination. The resulting stable Leu$^+$ transformant was crossed with *nda4-108*. Tetrad analysis of the resulting spores showed the 2$^+$:2$^-$ segregation for the Leu$^+$ marker, demonstrating that the cloned sequence was integrated on the chromosome. The cs marker was found

A

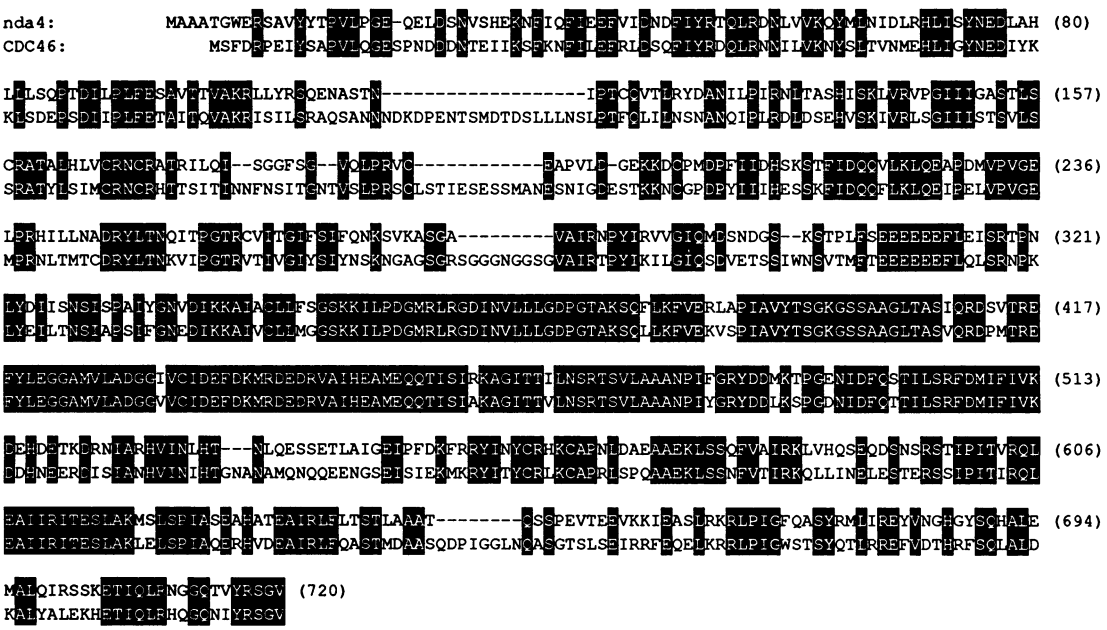

Figure 7. Comparison among nda4, CDC46, nda1, and MCM2. (A) Amino acid sequence comparison between nda4 and CDC46. Identical amino acids are boxed. (B) Sequence comparison between nda1 and MCM2. + and − represent the basic and acidic residues, respectively. * indicates the stretch PPSSP. (C) Sequence comparison among MCM2, nda1, nda4, and CDC46. The identical amino acids are boxed.

to cosegregate with the Leu$^+$ marker so that the cloned DNA was integrated on the chromosome by homologous recombination. Thus, the DNA sequence complementing *nda4-108* must be derived from the *nda4*$^+$ gene.

The Predicted Amino Acid Sequence of nda4$^+$ *Resembles That of CDC46*

An ~2.6-kb long nucleotide sequence containing the *nda4*$^+$ gene was determined. A large open reading frame was found, and its predicted amino acid sequence is shown (Figure 5B). A short intron having the consensus GTANG– –CTNA– –AG was presumed in the C-terminal region. PCR cloning from a *S. pombe* cDNA library using the primers that surrounded the hypothetical intron produced a DNA fragment of expected size, supporting the presence of this intron. The hypothetical nda4 protein thus obtained contains 720 amino acids (calculated molecular weight [MW], 80.2 kDa).

Database search demonstrated that the *nda4*$^+$ gene is closely similar to the *S. cerevisiae CDC46* gene that is required for the initiation of DNA replication (Hennessy *et al.*, 1990, 1991). The amino acid comparison between nda4 and CDC46 proteins is depicted in Figure 7A. Similarity between them spans the entire coding region except for several short stretches. The greatest similarity was found in the central domain, and conservation of a highly acidic stretch EEEEEEFL is conspicuous. No definitive calcium binding motif was found in the protein sequences, nor has any functional protein motif been found. Whether the budding yeast *CDC46* can suppress the cs phenotype of *nda4-108* mutant remains to be determined.

Isolation of the nda1$^+$ *Gene by Complementation*

DNA clones that fully complemented *nda1-376* mutation were isolated from the *S. pombe* genomic DNA library by procedures similar to those used for the *nda4*$^+$ gene. Plasmid pNDA1 thus obtained contained a 7-kb long genomic DNA insert (Figure 6A). By subsequent subcloning, the 4.3-kb long *Bam*HI-*Sma* I fragment was shown to be the minimal complementable fragment; this fragment did not complement *nda4-108*. The sequence was integrated onto the chromosome by homologous recombination. The Leu$^+$ stable transformants of *nda1-376* simultaneously became Cs$^+$, suggesting that an integration rescue occurred. Tetrad dissection of a cross between the integrant and a marker strain HM124 (*h*$^+$ *leu1 ade7 ben1*) confirmed that the sequence was integrated on the *nda1* locus, the 2$^+$:2$^-$ segregation for the Leu$^+$ marker and the cosegregation for *nda1* and Leu$^+$ markers. The cloned sequence thus must be derived from the *nda1*$^+$ gene.

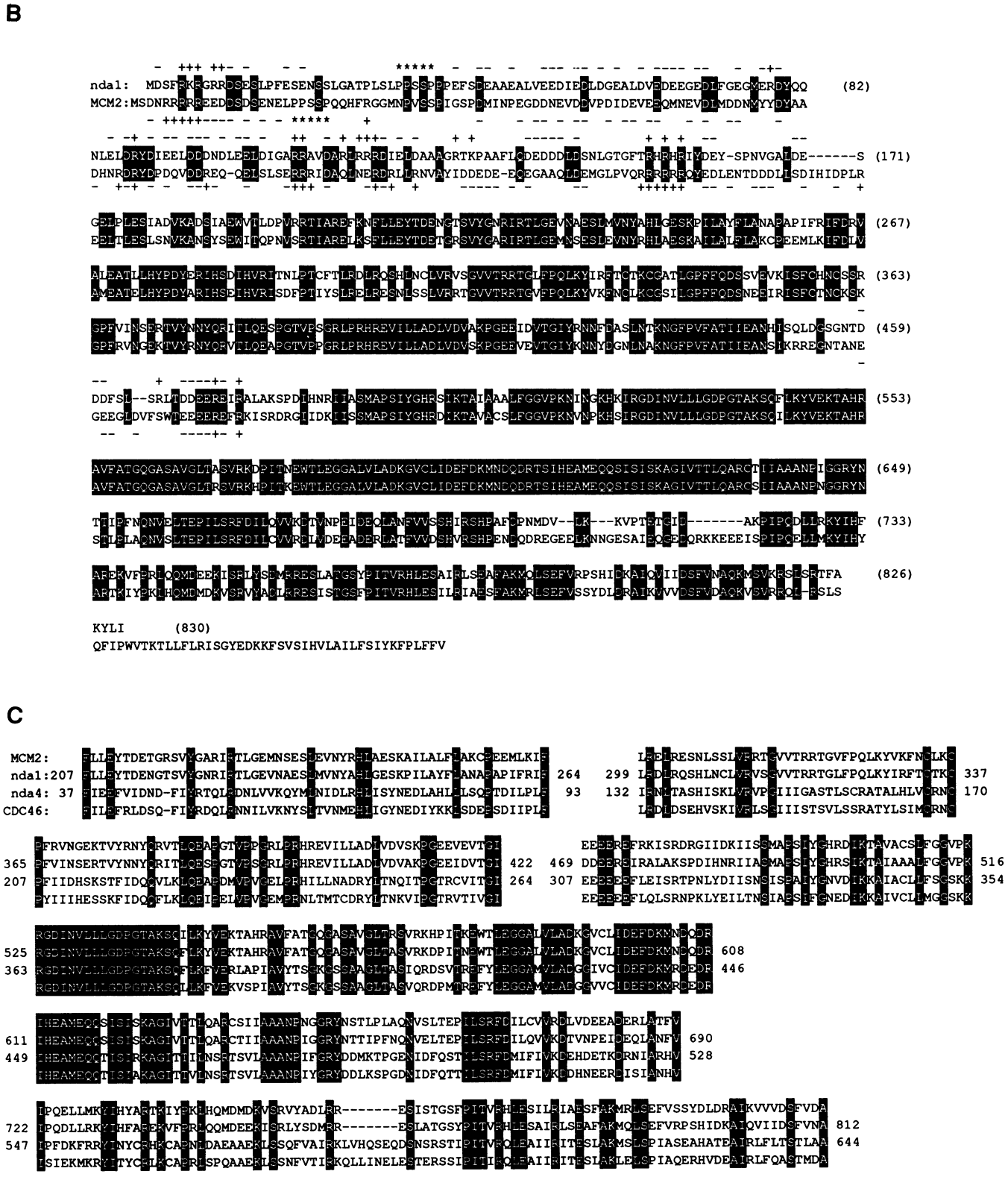

Figure 7. (Continued)

Predicted Amino Acid Sequence of nda1$^+$ Resembles That of MCM2

Nucleotide sequencing of the fragment containing the *nda1*$^+$ gene is shown with the predicted amino acids (Figure 6B). An intron containing the consensus GTANG– –CTNA– –AG was presumed at the 78th codon. To establish this intron, a clone was obtained by the PCR method from an *S. pombe* cDNA library using the primers near the putative intron. The resulting cDNA sequence determined was identical to that of genomic DNA except for the region of the presumed intron. The predicted nda1 protein sequence thus contains 830 amino acids (calculated MW, 92.8 kDa).

Database search revealed that the presumed nda1 polypeptide is similar to the budding yeast MCM2 (Yan *et al.*, 1991) that is required for the maintenance of autonomously replicating sequence (ARS) activity and the initiation of DNA replication. The MCM2 amino acid sequence is similar to that of the CDC46, and these sequences belong to the same gene family that includes the budding yeast MCM3 (Yan *et al.*, 1991) and the fission yeast cdc21 amino acid sequences (Coxon *et al.*, 1992). Similarity of nda1 protein to CDC46, MCM3, and cdc21 is much lower than that to MCM2. Sequence identity between nda1 and MCM2 spans most of the coding region except the NH_2-terminal domain (Figure 7B). Hydrophilic residues are abundant in the N-termini. Although the frequency of identical amino acids is low, the distributive modes of charged residues (indicated by + and −, respectively, for the basic and acidic residues) are surprisingly well conserved in ~170-residue long NH_2-domains. The terminal cluster is basic, followed by acidic residues and the proline and serine-rich stretch and so on. Central to the near C-terminus of nda1 and MCM2 is well conserved. The zinc finger motif postulated for MCM2 (Yan *et al.*, 1991) is also found in nda1. Four important Cys and Phe residues upstream of the zinc finger motif are preserved. In MCM2, the essential role of this zinc finger motif was established (Yan *et al.*, 1991).

Amino Acid Sequence Similarity Between nda1 and nda4

Identical amino acids among nda1, MCM2, nda4, and CDC46 are illustrated in Figure 7C. Approximately 600–amino acid long regions are similar to each other. The degree of conservation is illustrated in Figure 8. The darkly hatched regions have the highest conservation among four proteins. Frequent conservation of Pro and Gly suggested that these proteins may have similar configurations rich in turns. The 18-residue long identical stretch RGDINVLLLGDPGTAKSQ exists in four of the proteins.

Gene Disruption of nda1$^+$ and nda4$^+$

The chromosomal *nda4*$^+$ gene was disrupted by one step gene replacement (Rothstein, 1983). The *Bgl* II-*Xho* I fragment in the *nda4*$^+$ gene was cloned, and the internal *Bam*HI-*Eco*RI was removed from the fragment and replaced with the *S. pombe ura4*$^+$ gene (Figure 5A). The resulting plasmid was linearized and used for transformation of a diploid JY765 (*h*$^+$/*h*$^-$ *leu1/leu1 ura4/ura4 ade6-210/ade6-216*). Heterozygous Ura$^+$ transformants obtained were examined by genomic Southern hybridization that confirmed the gene disruption of *nda4*$^+$. Tetrad dissection of the heterozygous diploid transformants showed only two viable haploid segregants that were always Ura$^-$, showing that *nda4*$^+$ is essential for viability. The gene-disrupted haploid spores of *nda4*$^+$ were germinated and divided once or failed to divide. Examples of cells are shown in Figure 9A. Cells that failed to divide were often elongated.

The gene disruption of *nda1*$^+$ was similarly done. The 5-kb *Bam*HI fragment was subcloned into pUC118, and the resulting plasmid was double digested with *Bgl* II and *Xho* I that cleaved off an internal fragment that was replaced with the *S. pombe ura4*$^+$ gene (Figure 6A). Resulting plasmid was linearized and introduced into a diploid JY765 (*h*$^+$/*h*$^-$ *leu1/leu1 ura4/ura4 ade6-210/ade6-216*). Heterozygous diploids thus obtained were examined by Southern hybridization and dissected by tetrad. Results similar to those for disruption of the *nda4*$^+$ gene demonstrated that the *nda1*$^+$ was also essential for viability. The disrupted haploid spores of *nda1*$^+$ showed a disruption phenotype similar to that of *nda4*$^+$. That is, the spores germinated, but most cells remained undivided or divided once or twice (Figure 9B).

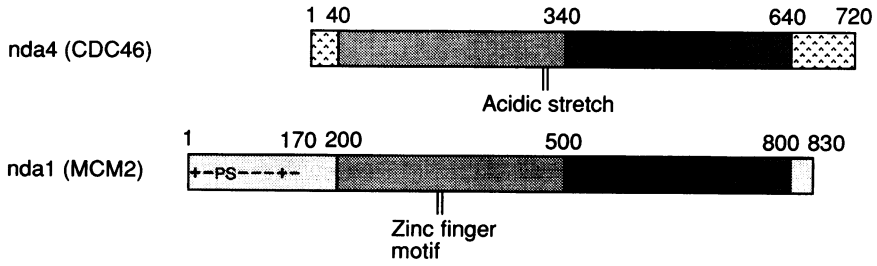

Figure 8. Schematic representation of nda4 (CDC46) and nda1 (MCM2). The light and dark hatched regions correspond to low and high homology among four proteins. The approximate locations of the acidic stretch and zinc finger motif are indicated by the two short lines.

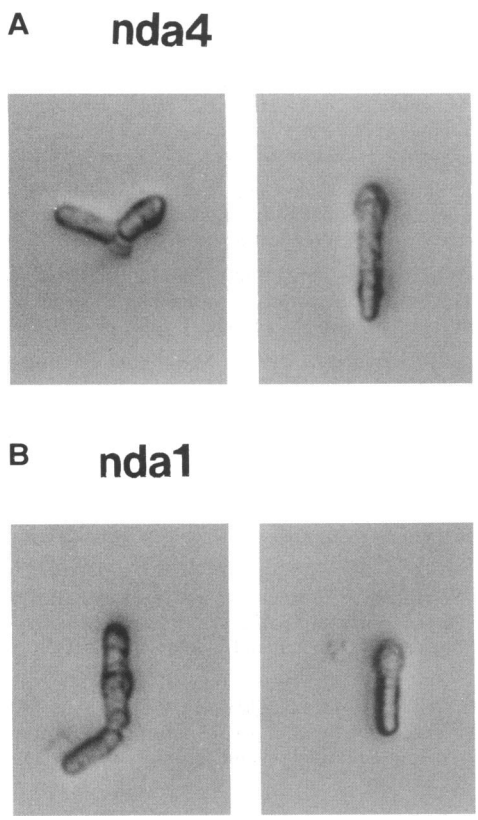

Figure 9. The *nda1*$^+$ and *nda4*$^+$ genes are essential for viability. The haploid spores with gene disruption of the *nda4*$^+$ (A) or the *nda1*$^+$ (B) gene were germinated. Some germinated cells divided once, but no further cell division took place.

Chromosome Mapping of nda1-376 and nda4-108

The chromosomal locus of *nda1-376* was previously determined to be in the left arm of chromosome II, tightly linked to *glu1* (0.48 cM) (Toda *et al.*, 1983). They are not identical, however, as the cloned *nda1*$^+$ gene isolated in the present study complemented *nda1-376* but did not complement *glu1*.

The genetic locus of *nda4-108* was allocated in chromosome I by haploidization (Toda *et al.*, 1983). Tetrad analysis was conducted to map *nda1* using various genetic markers in chromosome I. A close linkage was found to *his6* (the map unit distance was 3.4 cM; PD: NPD:TT = 41:0:3) and to *crm1* (1.1 cM) (Adachi and Yanagida, 1989). The *nda4*$^+$ gene was found to be about 10 kb apart from *crm1*$^+$.

DISCUSSION

We show in the present paper that fission yeast mutants *nda1-376* and *nda4-108* are defective in DNA synthesis. Consistent with this, we found that the *nda1*$^+$ and *nda4*$^+$ genes cloned by complementation encoded polypeptides closely similar to those of the budding yeast genes *MCM2* and *CDC46*, respectively, both of which were previously reported to be required for the initiation of replication (Hennessy *et al.*, 1991; Yan *et al.*, 1991). It remains to be determined whether the *nda1*$^+$ and *nda4*$^+$ genes, which are essential for viability, are functional homologues for *MCM2* and *CDC46*, respectively.

Do the products of the *nda1*$^+$ and *nda4*$^+$ genes function solely at the onset of DNA replication? The complete S-phase block in *nda1-376* cells supports a hypothesis that the *nda1*$^+$ gene product is essential for replication. A significant fraction of *nda4-108* cells, however, was not blocked but retarded in the DNA synthesis. The *nda4*$^+$ gene may not be absolutely required for replication. Because *nda4-108* does not form a colony even at 26°C, mutation itself is unlikely to be leaky. Effect of *nda4* mutation on cell growth is pleiotropic, and the *nda4*$^+$ gene product may be multifunctional.

The *CDC46* gene (Hennessy *et al.*, 1990, 1991) is involved in an early step of DNA replication. An important property of CDC46 protein is that its subcellular localization varies with the cell cycle (Hennessy *et al.*, 1990). The CDC46 protein is transported quickly from the cytoplasm into the nucleus as mitosis is completed and remains there until the next round of division is initiated. The behavior of this protein resembles that of the "licensing factor" postulated in a model that explains the once-per-cell-cycle replication of DNA (Blow and Laskey, 1988). We have raised anti-nda4 antiserum and plan to investigate the localization of the nda4 protein in cell division cycle.

The phenotype of *nda4-108* was partially rescued by a high concentration of Ca^{2+}, which appears to have a positive effect on the *nda4*$^+$ gene function. The functional implication for the suppression by Ca^{2+}, however, is not understood, though two possibilities are considered. nda4 protein directly interacts with Ca^{2+} and is activated; alternatively, nda4 protein is indirectly modulated by other components that are regulated by Ca^{2+}, and nda4 protein might be activated by Ca^{2+}-interacting proteins such as calmodulin, Ca^{2+}-dependent kinase, or phosphatase. A potential site for the Ca^{2+} binding consensus sequence exists in nda4 but is not experimentally examined. In any case, the relationship between Ca^{2+} and nda4 protein seems to be an important aspect in understanding the *nda4*$^+$ gene function.

The amino acid sequence similarity between nda4 and CDC46 predicted protein spans nearly the entire region. No obvious nuclear location signal (NLS) was found for nda4/CDC46, but a highly acidic stretch EEEEEEFL, which can serve as the domain for binding to a basic protein, is conserved.

We found that the sequences of nda1 and MCM2 proteins are closely similar except for the first 170 amino acids, where the charged and polar residues are abun-

dant and present as alternating clusters. They may act as the NLS or the degradation signal Proline-rich (PEST) sequences. The highly charged NH_2-domains and the zinc finger motif postulated in MCM2 (Yan *et al.*, 1991), which is also perfectly conserved in nda1, are the two most prominent features for MCM2/nda1. They are thus likely to be DNA binding proteins.

The *S. cerevisiae mcm2* and *mcm3* mutations have been extensively studied (Sinha *et al.*, 1986; Gibson *et al.*, 1990; Yan *et al.*, 1991). They exhibit an increase in chromosome loss and recombination (Yan *et al.*, 1991) but fail to activate the ARS of minichromosomes (Maine *et al.*, 1984). *MCM2* functionally overlaps with *MCM3* as the double mutant is lethal (Yan *et al.*, 1991). We have not examined these properties in *nda1-376* mutant. A putative mammalian homologue P1 has been found for MCM3 (Thömmes *et al.*, 1992). In addition to *CDC46/nda4*$^+$, *MCM2/nda1*$^+$, *MCM3*/P1, the fission yeast *cdc21*$^+$ required for DNA replication (Nasmyth and Nurse, 1981) is now added to the same gene family (Coxon *et al.*, 1992). Members of the gene family hence are conserved from yeast to mammal (Hennessy *et al.*, 1991; Thömmes *et al.*, 1992).

The roles of individual members in the *CDC46/MCM2* gene family are largely unknown. Knowledge essential for understanding them is that of the molecular functions of structural subdomains, the presence of which has been recognized from the comparison of nda1, nda4 and CDC46, and MCM2. The similarity between nda4 and nda1 resides within the central domain of ~600 amino acids. The 300-residue long domain is the highly homologous core sequence for the gene family. The conserved region in MCM3 and cdc21 is also restricted within this central domain. Molecular and biochemical studies are needed to understand the role of these proteins in DNA replication.

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