Higher Order Chromosome Structure Is Affected by Cold-sensitive Mutations in a *Schizosaccharomyces pombe* Gene *crm1* Which Encodes a 115-kD Protein Preferentially Localized in the Nucleus and at Its Periphery

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Abstract. We isolated a novel class of *Schizosaccharomyces pombe* cold-sensitive mutants with deformed nuclear chromosome domains consisting of thread- or rodlike condensed segments at restrictive temperature. Their mutations were mapped in a novel, identical locus designated *crm1* (chromosomal region maintenance). The *crm1* mutants also show the following phenotypes. DNA, RNA, and protein syntheses diminish at restrictive temperature. At permissive temperature, the amount of one particular protein, p25, greatly increases. The mutant growth is hypersensitive to Ca²⁺ and resistant to protein kinase inhibitors. We cloned the 4.1-kb-long *crm1* gene that rescued the above phenotypes by transformation and determined its nucleotide sequence, which predicts a 1,077-residue protein. Affinity-purified antiserum raised against the *crm1* polypeptide expressed in *Escherichia coli* detected a 115-kD protein in *S. pombe* extracts. Genomic Southern hybridization and immunoblotting suggested that the *crm1* product might be highly conserved in distant organisms. Through immunofluorescence microscopy, the *crm1* protein appeared to be principally localized within the nucleus and also at its periphery. We speculate that the *crm1* protein might be one of those nuclear components that modify the chromosome structures or regulate the nuclear environment required for maintaining higher order chromosome structures.

Distinct classes of nuclear proteins and components are thought to affect higher order chromosome structures. Certain DNA-binding proteins or enzymes directly interacting with DNA should be included among these. For instance, histone H1 is required for the formation of 30-nm chromatin filaments (Thoma et al., 1979), and DNA topoisomerase II (topo II) is shown to be essential for chromosome condensation as well as separation (e.g., Uemura et al., 1987; Newport and Spann, 1987). A group of enzymes modifying chromosomal proteins such as acetylases (deacetylases), kinases (phosphatases), methylases, and polyADP-ribosylation enzymes may play important roles in dynamic chromosome structures and functions (Matthews, 1988). These enzymes do not directly interact with chromosomal DNA but modified proteins cause profound chromosomal changes under different cellular conditions. Insoluble nuclear skeletal structures called nuclear matrix (Berezney and Coffey, 1974) or scaffold (Mirkovic et al., 1984) remain after complete extraction of DNA and histones, and are believed to be involved in the formation and maintenance of higher order chromosome structure. One of the two major polypeptides in the scaffold is shown to be topo II (Earnshaw et al., 1985; Gasser et al., 1986). Nuclear components that regulate nuclear environment and physicochemical parameters in the nucleus, such as ionic constituents or pH, also may affect higher order chromosome structures. Little is known of these components, however. Some of them may be situated at the nuclear envelope which separates the nucleus from the cytoplasm. The nuclear pores are essential structures for the import of proteins and the export of RNAs (Newmeyer and Forbes, 1988; Richardson et al., 1988). Identification of these genes and their products, which are required for higher order chromosome structure, is important to understand how the chromosome superstructures are maintained and regulated.

Yeast, a unicellular eukaryote, shares many chromosomal properties with higher eukaryotes. Although nuclear envelope breakdown and the high degree of chromosome condensation comparable to that of higher eukaryotes are not observed during mitosis, yeast and higher eukaryotes may share basically the same principle of chromosome organization. Within the last several years, powerful genetic strategies have been well developed in two different yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. There may be two ways to study the genes implicated in the superstructure of chromosomes using these yeast genetic systems. One way

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1. Abbreviations used in this paper: cs, cold sensitive; DAPI, 4',6-diamidino-2-phenylindole; topo II, DNA topoisomerase II; ts, temperature sensitive.
is to isolate mutants defective in one protein which has a known function and to investigate their chromosomal-defec
tive phenotypes. The other is to search a series of tempera
ture-sensitive (ts) or cold-sensitive (cs) mutants for defects
in chromosome superstructures and to identify their genes
and products. We have been pursuing both approaches using
the fission yeast *S. pombe* as a model organism (Yanagida et
al., 1986).

The former strategy was applied to isolate mutants for
topo II in *S. pombe* (Uemura and Yanagida, 1984). The latter
strategy has been successful for isolation of a number of mu
tants showing defective phenotypes in the chromosome su
perstructure (Hirano et al., 1986, 1988, 1989). The ts cut
mutants show nuclear cytological phenotypes similar to those
to of topo II mutants (Hirano et al., 1986). The ts nuc1 mutant,
showing a defective nuclear phenotype similar to the double
mutant of topo I and II, was recently found to be impaired
in the largest subunit of RNA polymerase I (Hirano et al.,
1989). The other ts mutation nuc2 blocks chromosome separa
ation in mitotic anaphase; the nuc2 gene encodes a nu
clear scaffold-like protein (Hirano et al., 1988) which has
a subdomain capable of binding tightly to DNA (Hirano, T.,
and M. Yanagida, manuscript in preparation).

In the present study, we searched 982 cs mutants of *S.
pombe* for those specifically altered in higher order chromo
some structure when they are incubated at restrictive tempera
ture. Cs mutations often take place in the structural genes
for proteins which exist as a part of larger structures. For ex
ample, many cs but no ts mutants were obtained in the a- and
ß-tubulin genes of *S. pombe*. In bacteriophage T4, cs mu
tants have been useful for analyses of the head assembly and
DNA packaging (Showe and Black, 1973; Yanagida et al.,
1983). We isolated a class of *S. pombe* cs mutants with a
novel nuclear phenotype; an irregularly deformed chromo
some domain consisting of thread-like, condensed segments
at restrictive temperature. Their mutations were mapped in
an identical locus designated *crml* (chromosome region
maintenance). We report here detailed phenotypes of the
*crml* mutants, identification of the gene product, and its cel
lular localization. Our results support a hypothesis that the
*crml* gene product has a role in maintaining the higher or
der chromosome structure.

**Materials and Methods**

**Strains and Media**

Standard genetic procedures for *S. pombe* described by Gutz et al. (1974) were followed. Strains used are listed in Table I. *S. pombe* was grown in YPD (complete rich medium; 2% glucose, 2% polypepton, 1% yeast ex
tract; 1.7% agar was added for plates), EMM2 (minimal medium described by Mitchison, 1970; 2% agar was added for plates), SD (minimal medium; 0.67% yeast nitrogen base without amino acids, 2% glucose; 2% agar was added for plates), and SPA (sporulation plate medium described by Gutz et al., 1974). *Escherichia coli* was grown in LB medium (0.5% yeast ex
tract, 1% polypepton, 1% NaCl, pH 7.5; 1.5% agar was added for plates). Final concentrations of antibiotics used were 100 µg/ml ampicillin and 170 µg/ml chloramphenicol.

**Mutagenesis**

The procedure of mutagenesis was as described by Uemura and Yanagida (1984), with modifications. 1 ml of wild-type *S. pombe* (HM123; h² leu1) culture grown in YPD at 30°C was harvested during the exponential stage of growth (1 × 10⁷ cells per ml), washed once with 50 mM Tris-maleic buffer at pH 6.0, and suspended in 1 ml Tris-maleic buffer. N-Methyl-N'-

nitrosoguanidine was added to the cell suspension (final concentration 300 µg/ml) and the mixture was incubated at 33°C for 30 min. Then the cells were centrifuged, washed three times with Tris-maleic buffer, and once with YPD. The washed cells were suspended in 5 ml YPD, incubated at 36°C overnight, diluted with distilled water, and plated on YPD. The survival ra
tio determined by counting colonies on YPD plates was 0.5–2%. Colonies grown on YPD plates at 36°C were replica plated and then incubated at
22°C for 1 d; 1–2% of the total colonies were unable to grow at this tempera
ture. In total, 982 cs⁻ strains were selected (designated cs⁻-982).

**DAPI Staining and Cytological Screening**

Each of the 982 cs⁻ strains was grown in YPD at 36°C (permissive tempera
ture) to cell concentrations of ~5 × 10⁶ cells/ml, transferred to 20°C (re
strictive temperature), and incubated for 12 h. Cells were fixed with 2.5% glutaraldehyde, washed twice with distilled water, and their chromosomes were stained with 2.5-10 µg/ml 4',6-diamidino-2-phenylindole (DAPI).

They were observed by an epifluorescence microscope to which a television camera was connected (Toda et al., 1981).

**Determination of Cell Number, DNA, RNA, and Protein Contents**

The cells were exponentially grown in a YPD medium at 36°C, shifted to
20°C at a cell concentration of 5 × 10⁶/ml and incubated for 15 h with
shaking. Aliquots of the cultures were taken at 3-h intervals. Cell number was determined by a counting chamber (Kayagaki Seisakusho Co., Tokyo). DNA and RNA were determined as described (Bostock, 1970). Protein was determined by the method of Lowry et al. (1951).

**Procedures for DNA Manipulation and Yeast Transformation**

Standard techniques for DNA manipulation (Maniatis et al., 1982) were used throughout. Yeast transformation was performed by the lithium acetate method described by Ito et al. (1983). Plasmids were recovered from *S. pombe* transformant as described by Beach et al. (1982).

**Preparation of *S. pombe* Genomic DNA**

The method described by Rothstein (1985) was followed. The cells were digested with zymolyase 100T (Seikagaku Kogyo Co., Japan) at 36°C for 30 min.
DNA Sequencing

DNA to be sequenced was cloned into Bluescribe M13 (Vector Cloning Systems, San Diego, CA) and the recombinant plasmids were subjected to the stepwise-deletion method described by Yanisch-Perron et al. (1985). For preparation of single strand phages, JM101 was used as a host (Messing and Vieira, 1982). Chain-termination sequencing was essentially the same as described by Sanger et al. (1977) using a 17-nucleotide synthetic primer (Takara Shuzo Co, Japan).

Expression and Partial Purification of a Part of the crml+ Gene Product

The 2.5-kb Bgl II fragment of the crml+ gene that contains a coding region for the 722 amino acid residues of the COOH domain was cloned into the Barn Hi site of the bacterial expression vector pET-3a (Rosenberg et al., 1987), which carries a promoter of gene φ10 of the T7 bacteriophage (Studier and Moffatt, 1986). Resulting plasmid pFA280 encodes φ10–crml+ fused protein which contains the NH2-terminal 12 amino acid residues of the T7 gene φ10 protein and the COOH-terminal 722 amino acid residues of crml+ protein. The φ10–crml+ fused protein was expressed in the bacterial strain BL21(DE3) harboring pFA280 as described by Studier and Moffatt (1986), and partially purified according to the method described by Watt et al. (1985), with the following modifications. Insoluble pellets of the extracts treated with 5 M urea were solubilized by extraction with 8 M urea and 0.05% SDS on ice for 30 min. A 100-ml culture of E. coli yielded ~400 µg of partially purified fused protein.

Antiserum Preparation

The partially purified bacterial crml+ polypeptides were further purified by SDS-PAGE followed by electroelution of the protein from gel as described by McDonald et al. (1986). The eluted protein was concentrated to an appropriate volume and dialyzed against PBS. A rabbit was injected with 200 µg of purified polypeptides mixed with complete Freund's adjuvant at ~50 sites on the back. Subsequently, 100 µg of the protein in incomplete Freund's adjuvant was injected 14 and 42 d after primary immunization. Blood collection was performed 21, 28, and 50 d after primary immunization, and whole blood was collected after 57 d.

Affinity Purification of Antibodies

For affinity purification of antibodies, the procedure described by Smith and Fisher (1984) was followed. About 200 µg of partially purified φ10–crml+ fusion protein from E. coli was subjected to SDS-PAGE (2-mm-thick gel) (Laemmli, 1970) and electroblotted to a nitrocellulose filter (pore size, 0.45 µm). A part of the filter was stained with 0.1% Amido black in 20% methanol and 10% acetic acid, destained with PBS, and the corresponding part of the filter where the fusion protein was blotted was excised. After blocking with 5% skim milk in PBS for 1 h, the filter was incubated in anti–crml+ antiserum diluted 10-20-fold with 5% skim milk at 4°C overnight. The filter was washed five times with PBS and then eluted by 30-s washes with 1 ml of 5 mM glycine-HCl, pH 2.3, 500 mM NaCl, and 100 µg/ml of BSA; eluates were immediately neutralized by the addition of 1/10 vol of 0.5 M Na2PO4 and dialyzed against PBS.

Immunoblot Analysis

Immunoblot analyses were performed by transferring the protein electrophoretically to nitrocellulose after SDS-PAGE (Burnette, 1981). 5% skim milk in PBS was used for blocking nitrocellulose filters, and the dilution of antibodies and 121I-protein A.

Indirect Immunofluorescence Microscopy

The procedures (Hirao et al., 1984) used were similar to those described by Kilmarin and Adams (1984) and Adams and Pringle (1984). The procedure described by Clark and Abelson (1987) in which cell walls were digested before fixation was also applied. Culture was fixed with 3.3% formaldehyde for 30 min at room temperature and washed four times with 1.2 M sorbitol, 20 mM KPO4 (pH 6.5) (S buffer). Cells were suspended in 1/5 to 1/10 vol of S buffer and the cell walls were digested with 0.3 mg/ml zymolyase and partially purified Novo mutanase at 33°C for 60–90 min. Cells were washed once with S buffer and were permeabilized by treatment with S buffer containing detergent (1% or 0.5% Triton X-100, 0.05% SDS). After washing with S buffer, cells were incubated with 1 mg/ml BSA/PBS containing the first antibodies at 26°C for overnight. Cells were washed three times with 1 mg/ml BSA/PBS followed by incubation with secondary antibodies for ~8 h. Cells were then washed again with 1 mg/ml BSA/PBS three times and were mounted on polylysine-coated glass slides with p-phenylenediamine/90% glycerol containing 0.1% DAPI.

Results

Isolation of Cs Mutants with Altered Chromosome Domains

We isolated 982 cs mutants by replica plating from 200,000 mutagenized colonies of a haploid S. pombe strain HM123 (see Materials and Methods). They grew at a permissive temperature (36°C) but did not at a restrictive temperature (20–22°C). Each strain was cultivated exponentially in liquid YPD-rich medium at 36°C, and then transferred to 20°C for 12 h (equivalent to a two generation period for the wild-type cells). Nuclei were stained with the DNA-specific fluorescent probe DAPI and observed under an epifluorescence microscope. In the wild-type cells, the interphase nucleus show a smoothly stained hemispherical chromosomal domain (Fig. 1, a and e), which was seen in most stages of the S. pombe cell cycle and briefly changed its shape during mitosis (Toda et al., 1981).

Three cs strains (cs10, 119, and 809) attracted our attention by their characteristic phenotype of the DAPI-stained chromosomal domains. They were normal at 36°C but strikingly altered at 20°C (Fig. 1, b, c, and d, respectively). Apparently fibrous or rodlike condensed chromosome segments were visible (Fig. 1, f and g), and the overall shape of the chromosome domain was often deformed (Fig. 1 c). The nuclear phenotype of these three mutants was highly uniform and similar. More than 90% of the cells of each mutant strain incubated at 20°C for 6 h contained the fibrous chromosome domains. A time-course change in crml–809 is shown in Fig. 2. A normal hemispherical chromosome domain was initially seen at 36°C (0 h), and then by transferring the culture to 20°C, relatively thin fibrous segments became visible in the chromosome domain after 4 h, followed by thickening of the fibers into rods that were irregularly folded after 14 h. The nuclear morphological phenotypes of the three strains at 20°C were rather similar, but the double mutant cs10 showed the rodlike condensed chromosomes more frequently.

Genetic Analyses

Each of the three cs strains was crossed with the wild-type, and in all the tetrads examined, Cs+/Cs− segregated into 2:2 for the strains 119 and 809, indicating that a single mutation caused their Cs− phenotype. The nuclear phenotype cosegregated with the cs. For the remaining strain 10, however, Cs+/Cs− segregated 4:0, 3:1, or 2:2. As described below, double mutations cause the Cs− phenotype of the strain 10.

The cs alleles in 119 and 809 belonged to the same complementation group. This was found by crossing them and examining resulting spores for the cs phenotype. Only 20 Cs+ spores were obtained among 10⁶ spores. In S. pombe, the frequency of intragenic recombination is <500 recombination per 10⁶ spores (Nurse and Thuriaux, 1980). We designated the genetic locus for 119 and 809 as crml. The cs-lethal phenotype of crml–809 is recessive because the heterozygous diploid made by crossing with meil (Egel, 1973; Kohli et al., 1977) is Cs+.
Figure 1. DAPI-stained fluorescence micrographs of the *S. pombe* wild-type (a and e) and *crml* mutant cells (b–d, f, and g). Exponentially growing cells at 33°C were transferred to 20°C for 12 h in YPD liquid medium, fixed with 2.5% glutaraldehyde and stained with DAPI. (a and e) Wild-type (HM23) cells grown at 20°C. The shape of nuclear chromatin regions is hemispherical. (b) Arrested cells of the double mutant (csl0). (c) Arrested cells of *crml-119*. (d, f, and g) Arrested cells of *crml-809*. Bars, 10 μm.
We mapped the chromosomal locus of crml–809 in the right arm of chromosome I. By the haploidization method (Kohli et al., 1977), crml was allocated in this chromosome (data not shown). A subsequent series of tetrad analyses showed a close linkage between crml and his6 (and also nda4) in the right arm of chromosome I. The map distances between crml and his6 and between crml and nda4 are 2.5 and 1.1 cM, respectively (Table II).

Genetic analyses of strain 10 indicated that it contained a Cs⁺ nonlethal mutation in crml which produced the Cs⁻ phenotype when combined with another nonlethal mutation in a different locus designated crm2 (data not shown).
Accumulation of p25.

Phenotypes at Permissive Temperature

Accumulation of p25. SDS-PAGE of the mutant cell extracts showed that the amount of one particular protein greatly increased in the *crm* mutant cells (Fig. 4). An intense Coomassie blue-stained band (designated p25) was present at the position of 25,000 mol wt in all the mutant extracts of *crm-*809, 1-809, and the double mutant cs10 both at 36°C and 20°C, but not in the wild type. The amount of other peptides might also have changed but the increase of p25 was most prominent.

Tetrad analysis indicated that the accumulation of p25 cosegregated with *crm*; Cs*+* segregants always contained an excess amount of p25 in the extracts (data not shown). For tetrads of the double mutant cs10 crossed with the wild-type, the 2+:2− segregation of the p25 accumulation was also observed. The Cs*+* segregants with the accumulated p25 were presumed to be *crm*.

The Arrested Phenotype in *crm* Mutants

The average cell length of *crm-*809 was ~30% longer at 20°C than at 36°C (Fig. 3a), and the frequency of the single-nucleated cells increased from 79% at 36°C to 95% at 20°C. These results suggested that a considerable fraction of the *crm-*809 cells elongated in the absence of nuclear division at 20°C. On the other hand, the cell length of the cs10 was 10% longer at 20°C than at 36°C, and the frequency of single-nucleated cells at 20°C was approximately the same as that at 36°C. Therefore, the arrest of cs10 did not seem to be specific to a cell cycle stage.

The total amounts of DNA, RNA, and protein in 1 vol of the wild-type and mutant cultures at 20°C are shown in Fig. 3b. In *crm-*809 at 20°C, the increase rates of protein, RNA, and DNA in the cultures all decreased to ~50% of those of the wild type. The cell number (Fig. 3b, open triangles) increased 1.7-fold and remained constant. In the double mutant cs10, however, the increase rates were severely reduced. The cell number increase stopped immediately after the shift to 20°C.

In spite of these arrested phenotypes, cells of *crm-*809 remained viable at 20°C, nearly 100% for 9 h and 80% for 15 h. Viability of the cs10, however, was only 15% after 15 h at 20°C.

Table II. Tetrad Analyses for the Linkage Between *crm*, *his*, and *nda4* on Chromosome I

| Cross          | PD | NPD | TT | cM  |
|----------------|----|-----|----|-----|
| *nda4* x *crm*-809 | 44 | 0   | 1  | 1.1 |
| *his6* x *crm*-809 | 96 | 0   | 5  | 2.5 |
| [LEU2] x *his6*   | 41 | 0   | 3  | 3.4 |
| [LEU2] x *crm*-809 | 91 | 0   | 0  | <0.54 |

PD, parental diphy; NPD, nonparental diphy; TT, tetra type. The map distance (cM) was calculated according to Perkins' formula (Kohli et al., 1977). [LEU2] indicates a strain in which the cloned *nda4* gene was integrated on the chromosome with the *S. cerevisiae* LEU2 marker gene.

Cs*+* *crm* segregants derived from strain 10 grew with apparently normal nuclear structure but showed the phenotype (the accumulation of p25, see below) characteristic of the *crm* mutants. Thus, to our surprise, all three cs mutants independently isolated by the gross nuclear phenotype turned out to be derived from mutations in a novel, identical locus, *crm*.

Resistance to a Protein Kinase Inhibitor. We examined a number of drugs to learn if any would affect the growth of *crm* mutants at 36°C, and found that both the single and double *crm* mutants are resistant to the drug staurosporine that is known to inhibit protein kinases, especially kinase C (Tamaoki et al., 1986). Table III shows that, while the wild-type *S. pombe* cells grew poorly on YPD plates containing 0.5 μg/ml staurosporine (no growth in 1 μg/ml) (Toda, T., and M. Yanagida, manuscript in preparation), all three *crm* mutants grew normally on YPD plates containing 1.0 μg/ml of the drug. This resistance of *crm-*809 was lost when the strain was transformed with the cloned *crm*+ gene (Table III). (Cloning of the *crm*+ gene is described in the next section.) The *crm* mutants were also resistant to another kinase inhibitor, K-252 (data not shown).

Hypersensitivity to Ca2+. As kinase C activity is known to be related to the concentration of Ca2+ (Takai et al., 1979), effect of Ca2+ on growth of the *crm* mutants was tested. (The *S. pombe* cells, like *S. cerevisiae* [Ohya et al., 1986], became sensitive to only very high concentrations of Ca2+.)
Ca\(^{2+}\) in the culture medium, probably because of the poor penetration. Table IV shows that crml mutant was more sensitive to Ca\(^{2+}\) than the wild type; crml–809 did not grow even in 100 mM Ca\(^{2+}\) in the YPD liquid culture, whereas the wild-type cells grew in 300 mM of Ca\(^{2+}\). Other divalent cations Mg\(^{2+}\), Zn\(^{2+}\), and Cu\(^{2+}\) did not have such effect. The sensitivity of crml–809 to trifluoperazine (an inhibitor of calmodulin) was the same as the wild type (data not shown).

**Table III. Effect of Staurosporine on crml Mutants**

| Strain   | 0    | 0.5  | 1.0  |
|----------|------|------|------|
| HM123    | +    | –    | –    |
| crml–809 | +    | +    | –    |
| crml–119 | +    | +    | +    |
| crml–crm2 (cs10) | +    | +    | +    |
| HM123/pDB248 | +    | –    | –    |
| HM123/pYA262 | +    | –    | –    |
| crml–809/pDB248 | +    | +    | +    |
| crml–809/pYA262 | +    | +    | +    |

The wild-type (HM123; h– leu), crml strains (h– leu crml–809 or crml–119) and double-mutant cs10 were streaked on YPD plates containing 0, 0.5, or 1.0 μg/ml of staurosporine. (+) Normal growth at 33°C after incubation for 3 d. The wild-type (HM123; h– leu) and a crml mutant (h– leu crml–809) were transformed with the vector pDB248 or pYA262 (containing the crml\(^{+}\) gene) on pDB248. The transformants (indicated by pDB248 or pYA262) were streaked on EMM2 minimal plates containing 0, 0.5, or 1.0 μg/ml of staurosporine and incubated at 33°C. On the EMM2 plates, all the strains tested were more sensitive to staurosporine than on YPD plates.

**Cloning of crml\(^{+}\) gene**

An S. pombe genomic DNA library in the shuttle vector pDB248 (Beach and Nurse, 1981) was used to transform cs crml–809 leu1, however, we failed to obtain any Cs\(^{+}\) transformant (the reason is not clear). Therefore, we attempted to “walk” to the crml locus from the nda4 locus which is tightly linked to crml. The genetic distance between crml and nda4 is 1.1 cm (Table II). This would correspond to a physical distance of <10 kb (Nakaseko et al., 1986). The nda4\(^{+}\) gene had been previously cloned and integrated on the chromosome with the S. cerevisiae LEU2 gene and pBR322 sequence (Yanagida et al., 1986). To obtain the genomic DNA sequences adjacent to the nda4\(^{+}\) gene, we isolated the genomic DNA from a transformant integrated with a plasmid carrying the nda4\(^{+}\) gene. DNA was partially digested with Hind III, self ligated, and used for transformation of E. coli to clone the pBR322 sequence which was co-integrated with the nda4\(^{+}\) gene.

Some of the plasmids (rl–r6; Fig. 5 a) recovered from the E. coli transformants were used to transform the S. pombe crml mutants. Among the six plasmids examined, two (pCRM1–r3 and pCRM1–r5) could complement the crml mutations. pCRM1–r5 with a 13.5-kb-long genomic DNA insert complemented the cs phenotype of crml–119, crml–809, and also the double mutant cs10. Subcloning of the crml\(^{+}\) gene was done by testing various clones for the ability to complement the crml mutations. Results indicated that the 4.1-kb Sph I–Bgl II fragment was sufficient for complementation (Fig. 5 a). This fragment was ~10 kb apart from the nda4 gene and 5 kb apart from the integrated LEU2 gene.

The smallest subclone which could complement crml was 4.1 kb long (pCRM1–284). Northern blotting probed with the 2.5-kb Hind III coding fragment (see below) showed a ~4-kb-long, weak band (data not shown). Genomic Southern hybridization probed with the 2.5-kb Hind III fragment showed a single set of bands for S. pombe DNA digested with Hind III or Eco RI, which was consistent with the cloned crml\(^{+}\) gene, indicating that the crml\(^{+}\) gene is unique in the genome (Fig. 5 b, lanes 1, 2, and 3). A set of bands strongly hybridized to the S. pombe crml\(^{+}\) probe was also present in the S. cerevisiae genomic DNA (Fig. 5 b, lanes 4, 5, and 6), suggesting that an equivalent for the crml\(^{+}\) gene may exist in S. cerevisiae.

The one-step, gene-disruption method (Rothstein, 1983) was applied by integrating the 2.5-kb Hind III crml\(^{+}\) DNA fragment ligated with the S. cerevisiae LEU2 gene onto the chromosome. In the tetrads dissected, only two spores were

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**Figure 4.** SDS-PAGE of S. pombe crml extracts shows the accumulation of a specific protein, p25. Extracts were prepared from cells grown at 36°C or arrested at 20°C. For the wild type, cells were grown at both temperatures. Cells were broken by glass beads with shaking, and polypeptides in the extracts were run in 9–18% gradient SDS-PAGE and stained with Coomassie blue. W.T., the wild-grown at 36°C or arrested at 20°C. For the wild type, cells were transformed with pDB248 carrying the crml\(^{+}\) gene; 10/pDB222, the cs10 cells transformed with multicopy plasmid pDB248 carrying the crml\(^{+}\) gene; 10/pCRM1, cs10 transformed with pDB248 carrying the crml\(^{+}\) gene; crml/pCRM1, crml–809 cells transformed with pDB248 carrying the crml\(^{+}\) gene. Intensity of the p25 band (arrowhead) is high in crml mutant cells either at 36 or 20°C. In the transformants, the amount of p25 is as low as the wild-type cells. Numbers at left are the molecular mass (expressed in kD) determined by standard markers.

**Table IV. Effect of Ca\(^{2+}\) on the Growth of crml Mutant**

| Strain   | 0   | 100 | 200 | 400 |
|----------|-----|-----|-----|-----|
|          | mM  | mM  | mM  | mM  |
| HM123    | 59  | 51  | 27  | 2.4 |
| crml–809 | 35  | 1.5 | 1.2 | 0.8 |

The cell concentrations were initially adjusted to 0.5 × 10\(^6\) per ml in the liquid YPD containing 0–400 mM CaCl\(_2\) and were measured after 24 h at 33°C. HM123 (h– leu1) is a wild-type strain.

* Cell numbers shown are all × 10\(^5\) cells/ml.
Figure 5. Cloning of the crml+ gene. (a) The crml+ gene was cloned by chromosome walking, knowing that the crml and nda4 loci are tightly linked. Genomic DNA was isolated from a transformant integrated with YIp33 containing the nda4+ gene, the S. cerevisiae LEU2 gene, and pBR322, and transformed to E. coli. Restriction maps of the six clones obtained (r1–r6) are shown. Hind III sites are represented by vertical lines. Distances between restriction sites are given in kilobase pairs. Subclones derived from r3 are also shown. The ability of these clones to complement the cs lethality of S. pombe crml is shown (+). (–) No complementation. The white arrow indicates the coding region of crml+ (see text). (b) Genomic DNAs of S. pombe (lanes 1-3) and S. cerevisiae (lanes 4-6) were cut with Hind III (lanes 1 and 4), Eco RI (lanes 2 and 5), or Hpa I (lanes 3 and 6), run in agarose, blotted, and probed with the nick-translated 2.5-kb Hind III fragment of the crml+ gene. (Note that Hind III digest of S. pombe DNA in lane 1 is partial.) Hybridization was done at 50°C in 5x SSPE/0.3% SDS with 100 µg/ml denatured salmon sperm DNA as carrier. The length of hybridized bands are given in kilobase pairs.

viable and they were all Leu–. The crml+ gene, therefore, seems to be essential.

A putative crml2 gene was cloned by transformation of the double mutant strain cs10 (crml–crml2) with an S. pombe genomic DNA library in the shuttle vector pDB248. Two Leu+ Cs+ transformants were obtained. Plasmids recovered from them rescued the cs lethality of strain 10 but did not complement the crml single mutants. The two plasmids contained a common 4.2-kb Hind III fragment. Integral genetic mapping of the 4.2-kb Hind III fragment followed by tetrad analysis showed that this fragment was apparently derived from the crml2 locus. (When a Leu+/Cs+ integrant was crossed with the Cs– double mutant csl0 and the resulting 700 Leu+ spores were examined, none of them was Cs–.)

Sequence Determination and Predicted Amino Acid Sequence

We determined the nucleotide sequence of the ~3.7-kb-long region of the cloned crml+ gene. There is a 3,231-bp-long open reading frame (the arrow in Fig. 5 a and Fig. 6). This open reading frame encodes a 1,077 amino acid, hypothetical protein. The calculated molecular mass is 124 kD. It is an acidic protein (pI, 5.6). The hydropathy plot (Kyte and Doolittle, 1982) shown in Fig. 6 indicates that the NH2-terminal 210 residues are relatively hydrophilic, while the 210–320 and the 690–1,077 regions contain several hydrophobic stretches. Computer analyses of available protein data bases (Swiss protein data base and the National Biochemical Research Foundation) have shown no strong sequence homology to any known protein.

Identification of the crml+ Gene Product by Immunoblotting

To identify the crml+ gene product in S. pombe extracts, we raised an antiserum against the crml+ polypeptide which was overexpressed in E. coli. The 2.5-kb Bgl II fragment encoding the carboxy-terminal 723 amino acids of the crml+ protein was cloned into a Bam HI site of pET-3a (pAR3038), an expression vector constructed by Rosenberg et al. (1987). The resulting plasmid pYA280 contained the promoter of T7 bacteriophage gene φ10 and its first 12 residues fused to the COOH domain of crml+ gene. The fused gene produced a 735–amino acid residue polypeptide (83.6 kD) in E. coli (see Materials and Methods). SDS-PAGE of the extracts of E. coli, which was induced for the fused protein production by pYA280, showed an intense polypeptide band with the expected molecular weight (data not shown). The polypeptide was purified (described in Materials and Methods) and in-
jected into a rabbit. Polyclonal antibodies were affinity-purified with the crml+ polypeptide and used for immunoblotting and immunofluorescence microscopy.

By immunoblotting of the wild-type S. pombe extract, we detected a 115-kD protein (designated pl15) that reacted with anti-crm/+ antibodies (Fig. 7 a, lane 1). Preimmune serum of the same rabbit did not show any band. Pl15 is likely to be the crml+ gene product because it has nearly the same molecular mass as that predicted from the nucleotide sequence. An approximately twofold increase of pl15 was seen in the cells transformed with pCRMI (Fig. 7 a, lanes 2 and 3). The pl15 band was also present in the crml mutant extracts (Fig. 7 a, lanes 4 and 5).

Using the affinity-purified anti-crm/+ antibodies, we tested for cross-reacting proteins in other eukaryotes. The antibodies recognized a polypeptide with a similar molecular mass in the budding yeast S. cerevisiae and also in HeLa cell extracts (Fig. 7 b).

Subcellular Fractionation of pl15

To determine the subcellular localization of pl15, we fractionated the crml+ mutant cell homogenates by Percoll gradient centrifugation (Hirano et al., 1988). The cells grown at 36°C or arrested at 20°C were lysed by zymolyase digestion followed by homogenization, and were overlaid on a 10-30% linear Percoll gradient. After centrifugation at 8,500 rpm in a type SW27 rotor (Beckman Instruments Inc., Fullerton, CA) for 15 min, fractions were collected. Nuclei made a band in the middle of the gradient. Soluble cytosol components, mitochondria, and other small organelles remained at the top of the gradient. Cell walls and nondisrupted cells were pelleted at the bottom. Each fraction was run in SDS-PAGE and immunoblotted using anti-crm/+ antibodies to examine the presence of pl15. A larger part of pl15 was present in the top fractions of the gradient, while a smaller part was in the nuclear fractions (Fig. 8). No significant difference was found between the homogenates made at 36 and 20°C. Similar results were obtained for the homogenates of the wild-type cells (data not shown). Evidence will be discussed below that crml+ protein in the top fractions might have leaked out from the nuclei or been released from the fragile nuclear peripheral region.

Roughly half the amount of the pl15 in the top fractions and all the pl15 in the nuclear fractions could be pelleted in the absence of Percoll by centrifuging at 8,000 g for 10 min in 20 mM KPO4, pH 6.8, 1.2 M sorbitol (data not shown). About 50% of this insoluble pl15 could be solubilized by 1.0 M NaCl and 2% Triton X-100. Thus, pl15 appears to have at least three different forms, soluble, 2% Triton soluble, and Triton insoluble.

Immunofluorescence Microscopy by Anti-crm/+ Antibodies

Immunofluorescence micrographs of S. pombe wild-type cells stained with affinity-purified anti-crm/+ antibodies are shown in Fig. 9, a and c, together with the corresponding DAPI-stained micrographs (Fig. 9, b and d). No immunofluorescence was seen when preimmune serum was used (data not shown). When the cells were first fixed and subjected to zymolyase digestion (Fig. 9 a), intense immunofluorescence by anti-crm/+ antibodies was seen in the nucleus. This intense nuclear staining was not uniform but seen as patches (inset, Fig. 9 a). Cytoplasmic immunofluorescence was weak. When cells were first treated by zymolyase before fixation, anti-crm/+ antibodies particularly strongly stained the nuclear periphery (Fig. 9, c and d). Under these conditions, cell fixation was not complete so that the DAPI-stained areas were greatly shrunken. The perinuclear staining was not continuous (inset, Fig. 9 c). The crml–809 mutant cells ar-

![Figure 7](https://example.com/figure7.png)  
Fig. 7. Immunoblotting to detect the crml+ gene product using anti-crm/+ antiserum.  
(a) Detection of the crml+ gene product in S. pombe extracts. S. pombe extracts were run in SDS–polyacrylamide gel and immunoblotted with affinity-purified anti-crm/+ antibodies. The amount of proteins for each lane was adjusted to be equal. An autoradiograph of the blot probed with 125I-protein A is shown. Lane 1, extract of wild-type (HM123) cells transformed with multicopy vector pDB248 without insert; lane 2, extract of wild-type (HM123) cells transformed with pCRMI–r5; lane 3, extract of wild-type (HM123) cells transformed with pFA284, the smallest subclone of the crml+ gene in pDB248; lane 4, extract of crml–809 grown at the permissive temperature, 33°C; and lane 5, extract of crml–809 arrested at 20°C. The radioactivity of the bands in lanes 2 and 3 were approximately twofold higher than that of the band in lane 1. Molecular masses determined by standards are shown. (b) Immunoblotting of extracts of S. cerevisiae and HeLa cells with affinity-purified anti-crm/+ antibodies. (Lanes 1, 2, and 3) S. pombe wild-type extracts; (lane 4) S. cerevisiae wild-type extract; (lane 5) HeLa cell extract. The blot was probed with anti-rabbit IgG antiserum conjugated to horseradish peroxidase.

![Figure 8](https://example.com/figure8.png)  
Fig. 8. Cellular fractionation of crml+ product by Percoll gradient centrifugation. S. pombe crml–809 cells grown at 36°C or arrested at 20°C were digested with zymolyase followed by homogenization. Extracts were centrifuged on a 10–30% linear Percoll gradient. Fractions were subjected to SDS-PAGE and immunoblotted using anti-crm/+ antibodies (crml 36°C) crml–809 cells grown at 36°C; (crml 20°C) crml–809 cells were grown exponentially at 36°C, then transferred to 20°C, and incubated for 12 h.
Figure 9. Indirect immunofluorescence microscopy of *S. pombe* using affinity-purified anti-*crm1*+ antibodies (a, c, and e) and DAPI stain (b, d, and f). (a and b) The wild-type cells were first fixed with formaldehyde followed by zymolyase digestion. Nuclei were preferentially stained with the antibodies. (c and d) The wild-type cells were first digested with zymolyase and then fixed. (e and f) The *crm1−809* mutant cells arrested at 20°C for 12 h. The cells were first fixed and then digested with zymolyase. Bar, 10 μm.
Discussion

We isolated a novel class of *S. pombe* cs mutants, *crlm*, by examining the DAPI-stained nuclei for the cells of 982 cs mutant cultures individually grown at a permissive temperature and then transferred to a restrictive one. Their phenotype, recognized by fluorescence microscopy, was a striking alteration in higher order chromosome structures at restrictive temperature. By DAPI staining, the fibrous or thread-like, condensed, chromosome segments were seen in the mutant nuclei at restrictive temperature, and by prolonged incubation, deformation of the entire chromosome domain occurred with thickening of the fibers into rodlike chromosome structures. In contrast, the normal nuclei at permissive temperature were normal looking and smoothly stained by DAPI without any nuclear deformation. Gene cloning showed that the *crlm* gene encodes a 1,077-residue polypeptide. Southern hybridization and immunoblotting suggested that the *crlm* gene and product may be conserved in evolutionarily distant organisms such as *S. cerevisiae* (the gene equivalent to *crlm* has recently been cloned from *S. cerevisiae*; Yamano, H., and M. Yanagida, unpublished results).

The altered chromosomes produced in *crlm* mutants at 20°C are not abnormal dead structures because the arrested cells were viable at 20°C for 10 h and grew again when the culture was moved back to 36°C. The nuclear phenotype observed, however, should not be necessarily interpreted to mean that the physiological role of the *crlm* gene product is related to the maintenance of higher order chromosome structure. For instance, a cytoplasmic defect might produce a substance affecting the nuclear function so that the chromosome structure is indirectly altered. A nuclear defect in scavenging toxic substances such as free radicals formed in the nucleus also might affect the chromosome superstructure. Results obtained by analyses of the *crlm* mutants, however, appear to be consistent with a view that the *crlm* gene product plays a positive role in the maintenance of chromosome architecture or at least a certain function in the nuclear structure. The nuclear phenotype of *crlm* mutants is not allele specific; three independently isolated mutants produced the same phenotype. Furthermore, all of the mutants showed marked reduction in macromolecular synthesis at the restrictive temperature.

The strongest evidence we have so far for the active participation of the *crlm* gene in the nuclear function is perhaps the localization of the *crlm* gene product within the nucleus and at the nuclear periphery. This was obtained by indirect immunofluorescence microscopy using affinity-purified antibodies against a *crlm* fusion polypeptide. When the cells were first fixed with formaldehyde and then digested by zymolyase, immunofluorescence was intense in the nucleus. It was weak in the cytoplasm.

On the other hand, when the cells were first digested with zymolyase followed by fixation, most immunofluorescence was apparently localized at the nuclear periphery. Spheroplasting before formaldehyde fixation apparently caused the removal of the *crlm* protein from the inner nucleus during preparation of immunofluorescent specimens. This may be consistent with the results of cellular fractionation experiments which show that the *crlm* protein is partly soluble. In Percoll gradient centrifugation, such soluble *crlm* protein might have leaked out from the nuclei. It should also be mentioned that the yeast nuclear envelope is fragile; thin section electron micrographs of isolated nuclei often showed broken nuclear envelopes (Hiraoka, Y., and M. Yanagida, unpublished result). Indeed, longer homogenization of extracts causes the decrease of *crlm* protein in the nuclear fractions (data not shown).

The function of *crlm* protein is little understood at the molecular level. The fact that anti-*crlm* antibodies stained the nucleus of *S. cerevisiae* suggests that *crlm* protein might have a nuclear function generally preserved in eukaryotes. The alteration of chromosome structures at the level of chromatin might be responsible for the reduction of macromolecular synthesis. One particular protein, p25, was accumulated in all the *crlm* mutants at permissive temperature. We purified p25 and raised anti-p25 serum, and immunoblotting confirmed the large increase of p25 in the mutant cells (Adachi, Y., unpublished result). Partial amino acid sequence of p25 was determined in collaboration with Drs. S. Kuramitsu and H. Kagamiyama of Osaka Medical College (Osaka, Japan) so that we were able to clone the gene for it by hybridization using oligonucleotide deduced from the amino acid sequence as the probe (Adachi, Y., and M. Yanagida, manuscript in preparation). Northern hybridization indicated that the level of p25 mRNA is greatly increased in the *crlm* mutant cells. Thus, all the mutations in the *crlm* genes enhanced the transcription for the gene for p25. Nothing is known about the function of p25 which would facilitate an understanding of the role of the *crlm* gene product. The large increase of p25 apparently does not reduce the growth of *crlm* mutants. We speculate that the accumulation of p25 rescues the *crlm* mutations at permissive temperature. To test this hypothesis, disruption of the p25 gene is necessary.

We suspect that p115^mut^ might be one of those nuclear components that modify the chromosome structure or regulate the nuclear environment required for maintaining the higher order chromosome structure. To investigate such a possibility, it is essential to examine the existence and the role of p115^mut^ in the nuclei of higher eukaryotes. In mammalian cells, various nuclear components including the nuclear membrane, lamina, and the pores are firmly established. We are currently undertaking to isolate the mammalian-equivalent gene for *crlm* and to identify its product and function.

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