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Cold-sensitive and caffeine-supersensitive mutants of the *Schizosaccharomyces pombe dis* genes implicated in sister chromatid separation during mitosis

Hiroyuki Ohkura, Yasuhisa Adachi, Noriyuki Kinoshita, Osami Niwa, Takashi Toda and Mitsuhiro Yanagida

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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We isolated novel classes of *Schizosaccharomyces pombe* cold-sensitive *dis* mutants that block mitotic chromosome separation (nine mapped in the *dis1* gene and one each in the *dis2* and *dis3* genes). Defective phenotype at restrictive temperature is similar among the mutants; the chromosomes condense and anomalously move to the cell ends in the absence of their disjoining so that they are unequally distributed at the two cell ends. Synchronous culture analyses indicate that the cells can enter into mitosis at normal timing but become lethal during mitosis. In comparison with the wild-type mitosis, defects are found in the early spindle structure, the mitotic chromosome structure, the poleward chromosome movement by the spindle elongation and the telophase spindle degradation. The *dis* mutants lose at permissive temperature an artificial minichromosome at higher rates than occur in the wild type. We found that all the *dis* mutants isolated are supersensitive to caffeine at permissive temperature. Furthermore, the mutant cells in the presence of caffeine produce a phenotype similar to that obtained at restrictive temperature. We suggest that the *dis* genes are required for the sister chromatid separation at the time of mitosis and that caffeine might affect the *dis* gene expression. We cloned, in addition to the $dis2^+$ and $dis3^+$ genes, multicopy extragenic suppressor sequences which complement *dis1* and *dis2* mutations. A complex regulatory system may exist for the execution of the dis^+ gene functions.

Key words: mitosis/spindle dynamics/chromosome disjoin/cyclic AMP

Introduction

A major event in mitosis is the disjoining of the chromosomes that comprise each of two sister chromatids after replication (e.g. Inoué, 1981; Murray and Szostak, 1985). Chromosome separation has to be accurate as in DNA replication. Failure to distribute chromosomes equally between two daughter cells would cause aberration or lethality. The error in chromosome separation (either due to loss or non-disjunction) has to be kept to a minimum; otherwise the stable transmission of genetic materials cannot be assured. Chromosome separation may be complex and made up of a number of highly ordered steps. An initial sign of the chromosome separation is the dissolution of the cytoplasmic microtubules. The chromosomes then condense, and the spindle apparatus forms. The chromosomes align in the metaphase plate and, in anaphase A, the sister chromatids are disjoined by kinetochore microtubules. The kinetochores (alternatively called centromeres) are special sites in the chromosomes where the two chromatids are held together after DNA synthesis. They are also the attachment sites for the kinetochore microtubules. In anaphase B, the spindle elongates and pushes the poles apart so that the distance between them increases, resulting in the two sets of separated chromosomes moving to the opposite ends of cell. In the end, the spindle disappears and two new daughter nuclei are formed.

We anticipate that the basic mechanism for mitosis may be similar among different eukaryotes. In the fission yeast *Schizosaccharomyces pombe*, the organism we have been studying as a model for the chromosome separation, the steps described above or analogous ones take place (e.g. Hiraoka *et al.*, 1984; Marks *et al.*, 1986; Tanaka and Kanbe, 1986; Hagan and Hyams, 1987; Uemura *et al.*, 1987; Hirano *et al.*, 1988). *S. pombe* is one of those organisms suitable for cell cycle analysis (Mitchison, 1970). It divides by fission, contains three chromosomes and has distinct cell cycle stages.

We report here the isolation and characterization of *S.pombe* cold-sensitive (cs) mutants which appear to block the sister chromatid separation in mitosis. These novel classes of cs mutants designated *dis* (defective in sister chromatid disjoining) were isolated by the DAPI (4′,6-diamidino-2-phenylindole)-stain screening method and classified into three complementation groups, *dis1*–*dis3*, by tetrad analyses. Their phenotypes at restrictive temperature are similar. In all the *dis* mutants, the chromosomes condense but do not separate, although the undivided chromosomes anomalously move to the cell ends, accompanied by the spindle elongation so that the chromosomes are unequally distributed at the two cell ends. The other interesting characteristic of the *dis* mutants is that they are hypersensitive to caffeine at permissive temperature. The same caffeine concentrations which block the growth of the *dis* mutants have no inhibitory effect on the wild-type *S.pombe*. Furthermore, the caffeine-induced phenotype of the mutant cells is similar to that obtained at restrictive temperature.

Results

Chromosome condensation

To isolate the *S.pombe* mutants defective in nuclear division, the DAPI-stain screening method has been most efficient (Toda *et al.*, 1981; Hirano *et al.*, 1986; Uemura *et al.*, 1987). We constructed a new set of 982 cs mutant strains and screened them for the phenotype of the condensed chromosomes in the arrested cells. [In the previous survey of cs mutants (Toda *et al.*, 1981, 1983), we employed another criterion for the nuclear division arrest phenotype, i.e. the single nucleus in the elongated cell (Nurse *et al.*, 1976).] The cs mutants were individually grown at per-

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Table I. Cold-sensitive mutants that produce condensed chromosomes at 20°C

Genes	Strains	Number of strains
nda2	49, 69, 79, 83, 167, 185, 347, 890	8
nda3	None	0
nuc2	None	0
dis1	80, 105, 203, 288, 341, 615, 804, 909, 945	9
dis2	11	1
dis3	54	1

Among the 982 cs strains examined in the present study, the 19 strains above showed the condensed chromosomes frequently in the cells incubated at 20°C for 10–12 h. They were classified by the complementation test.

missive temperature (33–36°C) and examined for the phenotype of the arrested cells at restrictive temperature (20°C) by fluorescence microscopy (Materials and methods). Nineteen strains showed high frequencies of the condensed chromosomes in the arrested cells (Table I). The strains were classified by the complementation test and also crossed with *nda2*-52, *nda3*-311 and *nuc2*-663 which were previously known to produce the condensed chromosomes at restrictive temperature (Umesono *et al.*, 1983; Hiraoka *et al.*, 1984; Hirano *et al.*, 1988). We found that eight strains were *nda2* mutants (the gene encoding α 1-tubulin; Toda *et al.*, 1984a; Adachi *et al.*, 1986). None of them was *nda3* nor *nuc2*. [The *nda3*$^+$ and *nuc2*$^+$ genes encode β-tubulin (Hiraoka *et al.*, 1984) and an insoluble nuclear protein p67 (Hirano *et al.*, 1988) respectively.] In *nuc2* and *nda3*, the chromosomes condense but do not separate at restrictive temperature.

We pair-wise crossed the remaining 11 strains and found that they can be grouped into three complementation groups, designated *dis1*–*dis3* respectively. There are nine strains for *dis1* and one each for *dis2* and *dis3*. All of these cs mutations are recessive and segregate 2^+:2^-. The phenotype of the condensed chromosome formation co-segregated with the Cs^- phenotype. A fluorescence micrograph of the *dis1*-288 cells incubated at 20°C for 12 h is shown in Figure 1. The growth properties, cell size distribution and viability of the *dis* mutants at restrictive temperature are shown in Table II and Figure 2a and b. The *dis3*-54 is somewhat leaky. None of the *dis* mutants is supersensitive to thiabendazole (TBZ) which is the inhibitor for the tubulin assembly (Toda *et al.*, 1983), whereas most of the cs *nda2* mutants isolated in the present study are supersensitive to TBZ at the permissive temperature (data not shown). The average cell size of the *dis* mutants is slightly longer than that of the wild type at 33°C and increases 10–20% at 20°C (the wild-type cell length decreases at 20°C). The DNA content was measured for the *dis1*, *dis2* and *dis3* mutant cells incubated at 20°C and found to be ~2C per nucleus.

Chromosome localization patterns

By DAPI staining we found that the condensed chromosomes were peculiarly located at one end or were asymmetrically distributed at the two ends of the cells. That is, there appear to be two chromosome localization patterns: the 3:0 pattern with all the chromosomes at one end of the cell (Figure 3, left) and the 2:1 pattern with two chromosomes at one end and the remaining one at the other end (Figure 3, right). The high frequency of the 2:1 chromosome

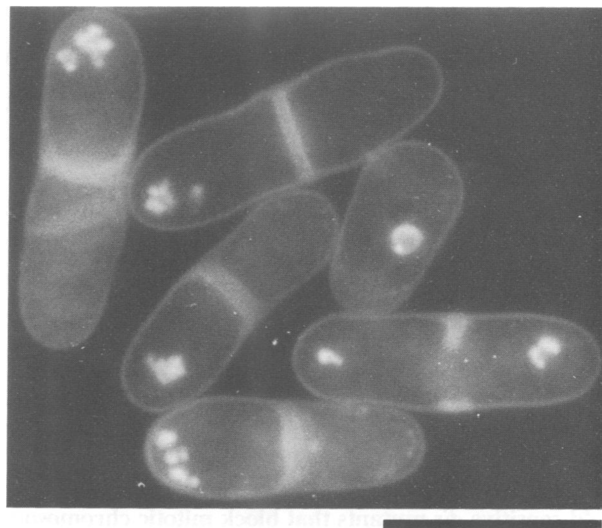

Fig. 1. Fluorescence micrograph of the *S.pombe* cs mutant cells *dis1*-288 incubated at 20°C for 12 h in YPD liquid medium, fixed with 2.5% glutaraldehyde and stained with a DNA-specific fluorescent probe DAPI. The condensed chromosomes are localized at the cell ends. The septum is partly formed. The bar indicates 10 μm.

Table II. Growth properties of *dis* mutants

Strains	Colony size 36°C	Colony size 30°C	Colony size 26°C	Colony size 22°C	TBZ (10 μg/ml)	Cell length (μm) 33°C	Cell length (μm) 20°C
HM123	++	++	++	++	++	11.4 ± 2.2	10.1 ± 2.5
dis1-80	++	+	+	–	++		
dis1-105	++	+	+	–	++		
dis1-203	++	+	±	–	++		
dis1-288	++	++	+	–	++	13.1 ± 3.1	15.8 ± 2.7
dis2-11	++	+	±	–	++	12.4 ± 2.8	13.7 ± 3.0
dis3-54	+	±	±	±	+	12.6 ± 3.1	14.0 ± 3.6

Each strain was back-crossed, and the segregation of Cs^+:Cs^- was examined by tetrads. All of them showed the 2^+:2^- segregation. The length was measured for the cells incubated at 33°C and 20°C for 12 h in liquid YPD. The wild-type colony sizes of HM123 (h^- *leu1*) on YPD plates at different temperatures are indicated as ++. TBZ (thiabendazole) is a tubulin inhibitor. Most of the isolated cs *nda2* mutants are hypersensitive to TBZ and did not grow on YPD plates containing 10 μg/ml TBZ at 33°C. None of the *dis* mutants was hypersensitive to TBZ.

distribution pattern is novel and strikingly different from the *nda2*, *nda3* and *nuc2* mutants in which the condensed chromosomes are situated close to each other because the spindle is absent or unable to elongate (Hiraoka *et al.*, 1984; Hirano *et al.*, 1988). In the *dis* mutants, however, the spindle forms and elongates (described below).

To determine whether the cell polarity which can be distinguished by the Calcofluor stain (Mitchison and Nurse, 1985) has any relation to the chromosome localization pattern, the frequencies of the chromosome localization patterns were determined in regards to the old or the new ends by the double staining with DAPI and Calcofluor. The Calcofluor stain reveals the birth scar located near the new cell ends. The results of the measurements (Table III) showed that the chromosomes have no preference in cell polarity; the condensed chromosomes were located at the new and the old ends in about the same frequency.

We examined whether the nucleolar structure separates

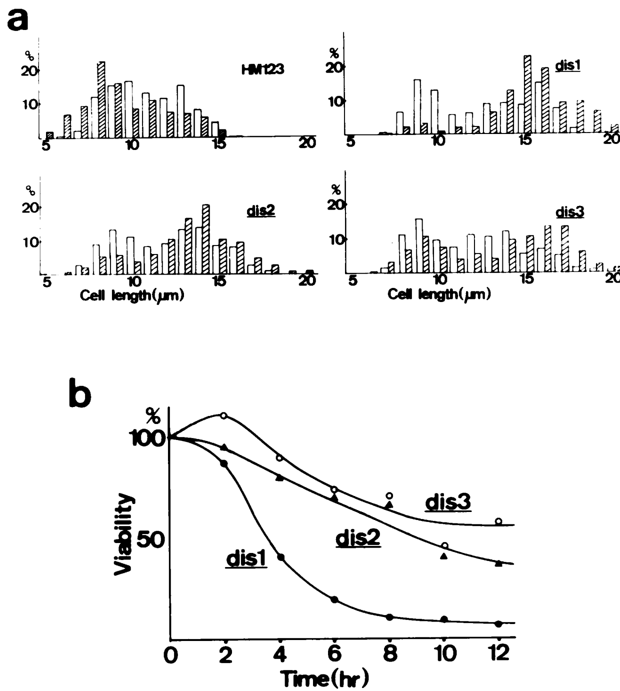

Fig. 2. The cell size and the viability of *dis* mutant cells at permissive and restrictive temperatures. (**a**) The cell size distribution of *dis1*-288, *dis2*-11 and *dis3*-54 grown at permissive temperature (33°C, open column) and arrested at restrictive temperature (20°C for 12 h, hatched column). The results of the wild-type control (HM123) are also shown. (**b**) Viability of HM123, *dis1*-288, *dis2*-11 and *dis3*-54 in the non-synchronous cultures incubated at 20°C for different time lengths and plated on YPD at 33°C.

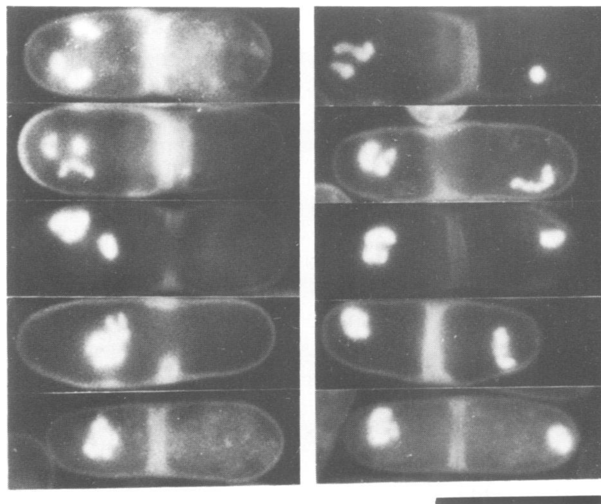

Fig. 3. Chromosome distribution patterns in the arrested *dis* mutants. Cells of *dis1*-288, -203, -80 and *dis2*-11 were incubated at 20°C for 12 h, fixed and stained with DAPI. Two classes of cells showing either all the chromosomes at one end of the cells (3:0 distribution; left) or the 2:1 chromosome distribution (right). The bar indicates 10 μm.

in the *dis* mutant cells. The double stain of the *S.pombe* cells by DAPI and ethidium bromide (Toda *et al.*, 1981; T.Matsumoto, unpublished results) revealed a blue nuclear chromosomal region and an intense orange red nucleolar region. In the wild-type *S.pombe* mitosis, the nucleolus divides into two parts which migrate to the opposite ends of the cells

Table III. Relationship between chromosome localization and cell polarity

Mutants	Pattern of chromosome distribution (%)			
	3:0	0:3	2:1	1:2
dis1	25	23	23	29
dis2	22	20	30	29
dis3	14	12	40	34

The mutant cells were incubated in YPD liquid media at 20°C for 12 h, collected and double stained in DAPI (30 μg/ml) and Calcofluor (0.3 mg/ml). Calcofluor stains the birth scar which is located near the new cell end because a greater part of the cell growth occurs at the old cell end (Mitchison and Nurse, 1985). 3:0, three chromosomes at the new cell end; 0:3, three chromosomes at the old cell end; 2:1, two chromosomes at the new and one at the old end; 1:2, one chromosome at the new end and two at the old end.

Table IV. Nucleolar localization in the *dis* mutants at 20°C

Mutants	Pattern of nucleolar distribution (%)			
	3*:0	2*:1	2:1*	2*:1*
dis1	55	40	4	1
dis2	32	55	11	2
dis3	25	65	9	1

Cells were incubated at 20°C for 12 h and stained by DAPI and ethidium bromide. The nucleolar stain was intensely orange–red (Toda *et al.*, 1981; T.Matsumoto, unpublished). Asterisk indicates the location of the nucleolus (e.g. 2*:1 represents the location of the nucleolus in one cell end containing two chromosomes). Only 1–2% of the cells (2*:1*) showed the separated nucleoli. In the control experiment using the wild-type cells, the nucleolus was separated into two during the nuclear division.

in association with chromosome III which contains the ribosomal RNA gene clusters (Hiraoka *et al.*, 1984; Toda *et al.*, 1984b; Niwa *et al.*, 1986). As shown in Table IV, the nucleolar structure does not divide but locates primarily at one cell end in association with the condensed chromosomes.

It should be noted that the frequencies for the 3:0 (plus 0:3) chromosome patterns in Table III are ~50% for *dis1*, 40% for *dis2* and 25% for *dis3*. If each chromosome should move randomly, the frequency of the 3:0 (plus 0:3) pattern would be 25%. Therefore, it seems that the chromosomes in *dis1* tend to cluster during the movement to the cell ends.

Abnormal chromosome movements

Although we found the condensed chromosomes at the cell ends to be the terminal phenotype of the *dis* mutants, it was essential to determine the course of the chromosome movement from the beginning. To investigate when the chromosomes condense and move to the cell ends at restrictive temperature, synchronous cultures were made and incubated at 20°C as described below. The cells of *dis1*-288 were exponentially grown in YPD liquid culture at 33°C and run in a sucrose gradient centrifugation. The top layer small cells were collected and incubated at 20°C in YPD. An aliquot of the culture was taken after a 60-min interval, immediately fixed with glutaraldehyde and stained with DAPI and/or anti-tubulin antibody for immunofluorescence microscopy. The wild-type cells collected under the same conditions are at the early G_2 stage and concertedly divide after 2 h at 36°C or 5 h at 20°C. If the same *dis1* cells described above are incubated at 36°C (the permissive temperature), they also synchronously divide after 2 h. Therefore, the *dis1*-288 cells

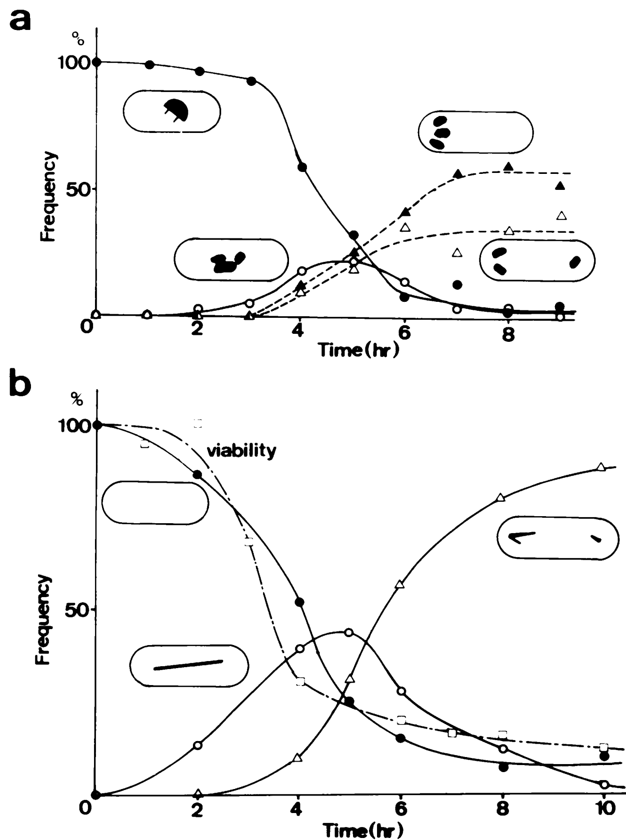

Fig. 4. Chromosome movement and spindle dynamics in the synchronous *dis1* mutant cells incubated at 20°C. The *dis1*-288 mutant cells grown at 33°C were fractionated by sucrose gradient centrifugation, and the small cells were obtained from the top of the cell band. The early G_2 phase cells thus selected were incubated at 20°C in liquid YPD. Cells were collected at 60-min intervals, fixed and stained by DAPI and anti-tubulin. The frequencies of the cells in different stages of the chromosome movements (**a**) and the spindle dynamics (**b**) are shown. (**a**) Chromosome movement. Cells with the normal interphase chromosome domain, ●; cells with the condensed chromosomes in the middle of the cell, ○; cells with all the condensed chromosomes at one cell end, ▲; cells with the condensed chromosomes at two ends, △. (**b**) Spindle structural change. Cells containing the normal interphase chromosome domain and no spindle structure, ●; cells with the spindle in the middle, ○; cells with the residual spindle at the cell ends, △. Viability of the cells is shown by □.

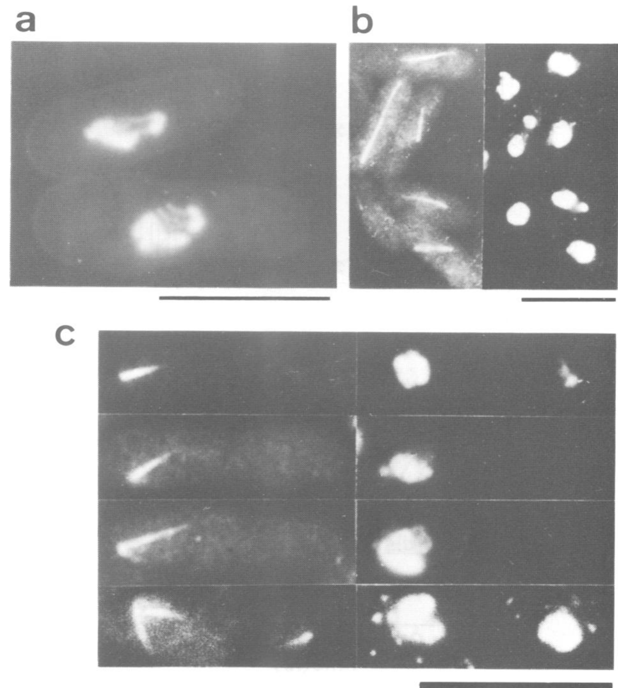

Fig. 5. Fluorescence micrographs of the *dis1* mutant cells in the synchronous culture stained by DAPI or anti-tubulin. (**a**) The condensed mitotic chromosomes of *dis1*-80 seen at 20°C for 4 h. DAPI staining. (**b**) Elongated spindle of *dis1*-288 seen at 20°C for 6 h. Left, anti-tubulin: right, DAPI stain of the same cells. (**c**) The residual spindle structure *dis1*-288 seen at 20°C for 8 h after most of the spindle had disappeared. Left, anti-tubulin; right, DAPI stain of the same cells. The bar indicates 10 μm.

at permissive temperature appear to grow normally, and the initial cell cycle stage of the *dis1* cells collected for the synchronous cultures is at the early G_2 stage as in the wild type.

By DAPI staining, we found a very unusual course of chromosome movements in the synchronous culture of *dis1*-288 at 20°C. For the first 3 h at 20°C the normal interphase chromosomal domain (the hemispherical shape) was seen in most of the cells but then those cells decreased, followed by an increase of the cells with the condensed chromosomes (Figure 4a). During 4–6 h there was a temporal rise of the condensed chromosomes which had not yet moved but were situated close to the center of the cell (a DAPI-stained micrograph is shown in Figure 5a). The U-shaped intermediate form representing the partial separation of the chromosomes in the wild type (Toda *et al.*, 1981) was never observed. After 4 h there was a continuous rise in the number of cells with chromosomes moving to the ends (the maximal frequency was reached at 7 h), and the total cells of which chromosomes moved to the ends reached 90%. Thus inactivation of the *dis1* gene product caused an unequal distribution of the condensed chromosomes. These abnormal mitotic events took place at times approximately the same as the mitosis of the wild-type cells incubated at 20°C. Therefore, the timing of the mitotic events appears to be normal in *dis1*-288 but the chromosomes move asymmetrically, apparently without disjoining.

Immunofluorescence microscopy using anti-tubulin antibody showed that the spindle formed and elongated in the synchronous *dis1* cells at restrictive temperature. In Figure 4b the frequencies of the cells with or without the spindle are plotted versus the time of incubation at 20°C. Initially no spindle structure was seen in the cells [the spindle appears only during mitosis in *S.pombe* (e.g. Hiraoka *et al.*, 1984; Tanaka and Kanbe, 1986)]. Then, the spindle appeared and elongated (Figure 5b). The frequency of the cells with the spindle peaked at 5 h. This timing is normal if compared with the wild-type spindle elongation in the synchronous culture at 20°C. Subsequently, the spindle structure disappeared except the end(s) which had been associated with the chromosomes (Figure 5c). Most of the *dis1* mutant cells after 10 h at 20°C showed such residual spindles.

Viability of the cells in the synchronous cultures was measured by plating (Figure 5b). Its sharp decrease at the onset of mitosis (the midpoint at 4 h) suggests that the *dis1*$^+$ gene product may become essential during mitosis. The viability curve approximately coincides with that of the cells without the spindle.

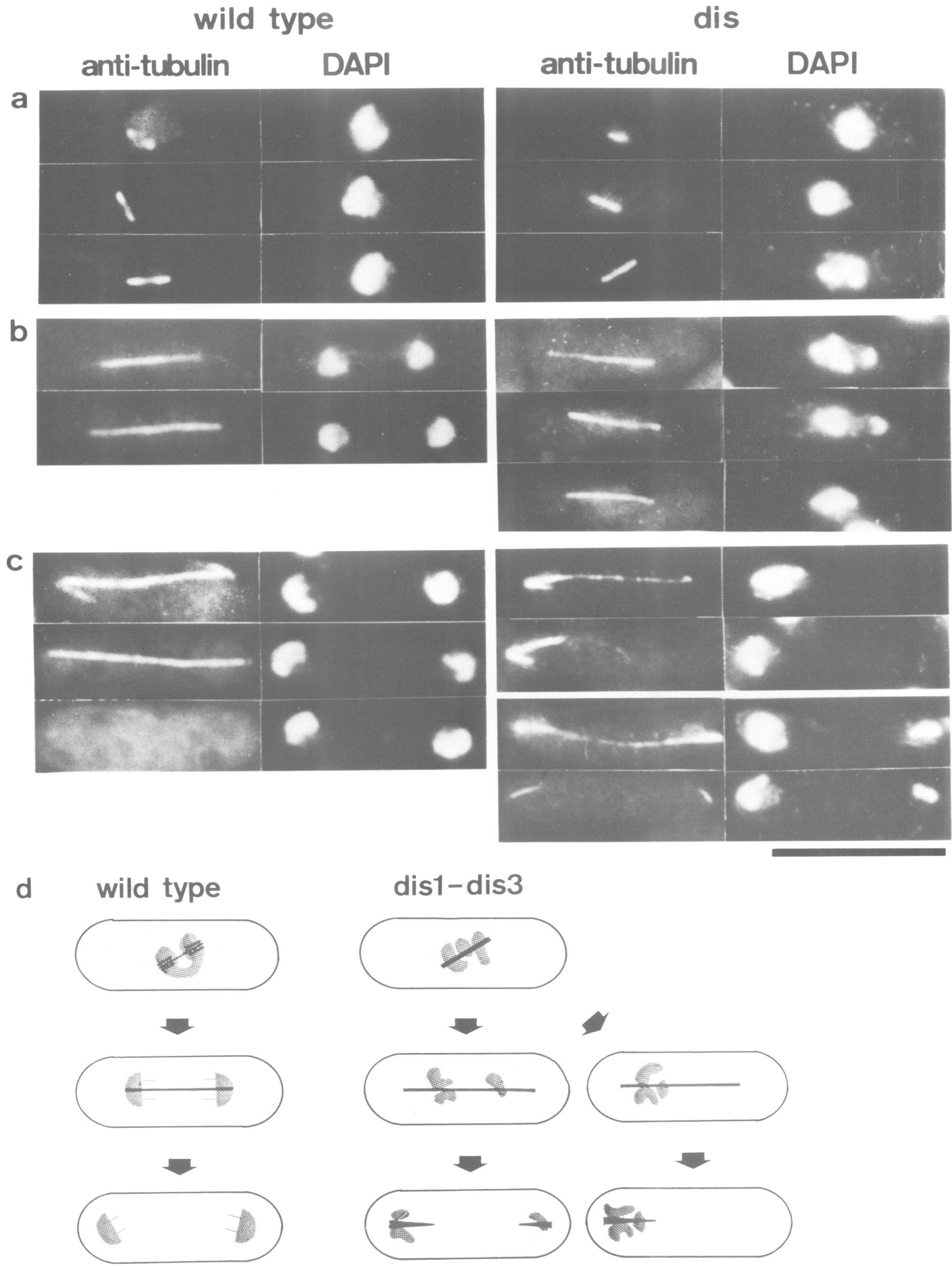

Fig. 6. Comparison between the wild type (left panel) and the *dis1* mutant at 20°C (right panel). See text for explanation. Left, anti-tubulin; right, DAPI stain of the same cells. (**a**) Early spindle and chromosome. (**b**) Chromosome movement by elongating spindle. (**c**) Fully extended and terminal spindles and the separated chromosomes. (**d**) Schematic illustration depicting chromosome and spindle movements. The bar indicates 10 μm.

Comparison with the wild-type nuclear division

Three significant differences are found in the mitotic events between the wild-type and the *dis* mutant cells by DAPI and anti-tubulin staining. Firstly, the early short spindle structure appears to be altered or partly missing in the *dis* mutant cells at 20°C. In the wild-type cells, the short spindle shows a thinner central part with thicker sides toward both ends (Figure 6a, left). The shorter spindles, having a minimal central part, appear to consist of two blobs in immunofluorescence micrographs. In the *dis* mutant cells, however, the short spindles look uniform in thickness (Figure 6a, right). The blob-like spindle ends have never been observed in the *dis* cells.

Secondly, whereas in the wild-type cells the separating chromosomes situate close to the ends of the elongating spindle (Figure 6b, left), the chromosomes in the *dis* mutant cells remain in the middle of the elongating spindle (Figure 6b, right). Thus the mode of the chromosome movement in *dis1* at 20°C must differ from that of the wild-type, although its mechanism is unknown. Thirdly, the spindle disappears in the wild type after completing the anaphase B extension, but in the *dis* mutant cells the ends of the extended spindle remain even after prolonged incubation at 20°C for 12 h. Such residual spindles are always found to be accompanied by the condensed chromosomes; the other ends without the chromosomes disappear. Thus the degradation of the extended spindle is partly impaired in the *dis* mutant cells. These differences are schematically illustrated in Figure 6d, where the rod indicates the spindle.

High-frequency loss of the minichromosome

We found that, at permissive temperature, the *dis* mutants lose an artificial minichromosome Ch16 (Niwa *et al.*, 1986) at rates 20- to 600-fold higher than occur in the wild type (Table V). The loss rates per cell division are 1–30% in the *dis* mutants compared with 0.05% in the wild type. The highest rate of 30% per division in *dis1*-288 is surprising, but this does not apply to normal chromosomes because there is no indication of the high-frequency loss of the normal chromosomes in *dis1*-288 whose viability is high at permissive temperature. However, the failure to construct a homozygous diploid for *dis1*-288 may suggest the instability of the normal chromosomes in diploid; the haploidization might have taken place due to the loss of normal chromosomes from the diploid [aneuploidy is lethal except for the disome for chromosome III in *S.pombe* (Niwa and Yanagida, 1985)].

Hypersensitivity to caffeine

We discovered that *dis* mutants are hypersensitive at permissive temperature to caffeine (trimethylxanthine). As shown in Table VI, the *dis* mutants are unable to grow on YPD plates in the presence of 7.5–10 mM caffeine at 33°C whereas the wild type and other cs mutants such as *nda3* normally grow on YPD plates containing 12.5 mM caffeine. The other *dis1* mutants are also supersensitive to caffeine, but most of cell cycle mutants previously isolated are not (data not shown). It is surprising that all the *dis* mutants independently isolated are supersensitive to caffeine.

Furthermore, when the *dis1*-288 cells were incubated in YPD containing 10 mM caffeine at permissive temperature (33°C) for 8 h, the phenotype similar to that produced at 20°C was found in ~40% of the cells (Figure 7). Although the frequency (40%) by caffeine was lower than that (90%) at 20°C without the drug, it was much higher than that (0%) for the wild type in YPD containing 10 mM caffeine at 33°C or that (2%) for the *dis1*-288 cells in YPD containing no drug at 33°C. Thus caffeine specifically affected the *dis* mutant cells. In the wild type the cells arrested by higher concentrations of caffeine (e.g. 15 mM caffeine) were elongated without the condensed chromosomes, differing from the *dis* mutant cells.

Because it is known that caffeine inhibits cAMP (3′,5′-

Table V. Minichromosome loss in *dis* mutants at permissive temperature

Strains	Loss rate per cell division (%)
Wild-type	0.05
dis1-288	30
dis1-945	5
dis2-11	2
dis3-54	1

The procedures for estimating the loss of the minichromosome Ch16 are described in Materials and methods.

Table VI. Effect of caffeine on the growth of *dis* mutants at permissive temperature

Strains	Growth on plate				
	Caffeine concentrations (mM)				
	5	7.5	10	12.5	15
HM123	++	++	++	+	±
nda3-311	++	++	++	+	±
dis1-80	+	±	–	–	–
dis1-288	+	±	–	–	–
dis2-11	+	–	–	–	–
dis3-54	+	–	–	–	–

Colony size was compared on YPD plates containing different concentrations of the caffeine after 3–4 days at 33°C.

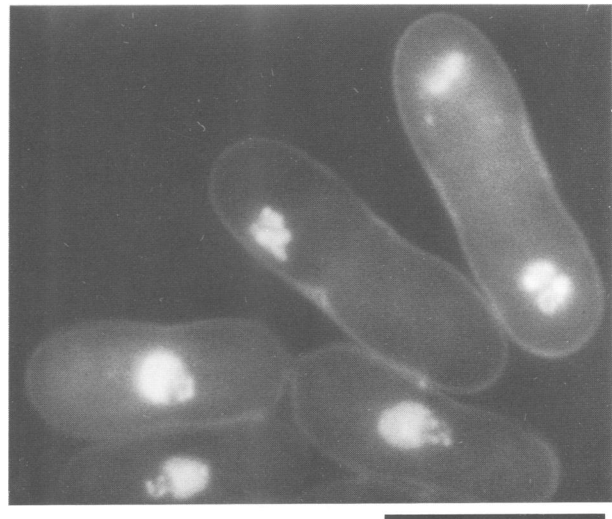

Fig. 7. DAPI-stained fluorescence micrograph of the *dis1*-288 cells incubated at 33°C for 8 h in the presence of 10 mM caffeine. The condensed chromosomes which have moved to the cell ends are seen in ~40% of the mutant cells. The bar indicates 10 μm.

cyclic AMP) phosphodiesterase (Beach *et al.*, 1985), we examined the effect of cAMP on the growth of *dis* mutants. The *dis* mutants appear to be somewhat more sensitive to cAMP than the wild type. Their growth in liquid culture is retarded by 50 mM cAMP, whereas the wild type normally grows even in 100 mM cAMP. There is, however, no significant difference between the *dis* mutants and the wild type in the colony sizes on YPD plates containing 50–100 mM cAMP. Therefore, we consider that further study is required to determine the cAMP sensitivity of *dis* mutants.

Cloning of the genomic DNA sequences by transformation

Genomic DNA sequences of *S.pombe* that complement the cs *dis* mutations were isolated by transformation (Materials and methods). *dis1*-203, *dis2*-11 and *dis3*-54 were used as hosts. Surprisingly, six different genomic DNA sequences (pDS101, 104, 105, 106, 107 and 109) complement *dis1*-203. None of them can complement *dis2* and *dis3* mutants. The clone derived from the $dis1^+$ gene has not yet been identified. We further found that four different genomic sequences (pHR110, pHR120, pHR140, pHR150) complement cs *dis2*-11. Among those, one (pHR120) contains the sequence derived from the $dis2^+$ gene. This was determined by homologous chromosome integration with the marker sequence followed by tetrad analysis. The *dis2* locates in the long arm of chromosome II. pHR120 also complements the caffeine-supersensitive phenotype of *dis2*-11. The transformants show a sensitivity to caffeine identical to the wild type. These four *dis2*-complementing sequences cannot complement *dis1* and *dis3* mutants. We cloned one genomic sequence (pCD112) that complements *dis3*. By homologous integration followed by tetrad analysis, this was found to be derived from the $dis3^+$ gene that locates in the long arm of chromosome II. None of the other *dis* mutants is complemented by pCD112.

The complementation of the *dis1* and *dis2* mutations by various multicopy extragenic suppressors suggests that the $dis1^+$ and $dis2^+$ genes may be the members of regulatory circuits, if the number of cloned sequences reflects the complexity of the systems. These genomic sequences (total 11) are currently being subcloned and characterized for identification of their encoded gene products.

Discussion

We isolated novel classes of *S.pombe* cs mutants that show anomalous chromosome movements during mitosis at restrictive temperature. They are classified into three complementation groups: *dis1*, *dis2* and *dis3*. Their phenotypes at 20°C are similar to each other but clearly differ from those of any previously known cs or ts mutations. The inactivation of the *dis* gene products causes aberrant mitosis. As compared with the wild type, differences are found in the spindle formation, the mitotic chromosome structure, the poleward chromosome movement by the spindle elongation and the telophase spindle degradation. The chromosomes appear to move in the absence of disjoining so that they are distributed unequally (3:0 or 2:1) at the two cell ends.

There was a possibility that each chromosome in the *dis* mutant cells at restrictive temperature consisted of only one duplex DNA. If this were the case, the primary defect should lie in the absence of DNA synthesis rather than in the failure of chromosome disjoining. Evidence against this possibility is as follows. The amounts of DNA measured for the arrested *dis1*, *dis2* and *dis3* cells are 2C, equivalent to the two sets of sister chromatids. There is no indication in the phenotype of *dis* mutants of the defect found in the DNA synthesis mutants of *S.pombe* (Nasmyth and Nurse, 1981). Furthermore, the synchronous *dis1* cells collected from the top layer band in sucrose gradient centrifugation show the normal chromosome separation (after 2 h) at permissive temperature, whereas the same cells, if incubated at restrictive temperature, show the anomalous 3:0 or 2:1 chromosome distribution. Thus, initially the synchronous *dis1* cells at the early G_2 phase must contain the chromosomes consisting of pairs of chromatids. We conclude that the *dis* mutations cause the chromosome non-disjunction in mitosis at restrictive temperature.

What kind of roles do the *dis* genes play in the cell cycle? The highly uniform terminal phenotype of the *dis* mutants incubated at restrictive temperature strongly suggests a specific role in the cell cycle. Analyses of the *dis1* synchronous cultures showed that the cells become lethal during mitosis; the *dis1* gene products may become essential in mitosis. Because the chromosomes condense at the time of mitosis, the condensation process appears not to be impaired. The absence of the U-shaped chromosome domain (Toda *et al.*, 1981; Hiraoka *et al.*, 1984) in the *dis* mutants, however, indicates that these are defective in a step analogous to anaphase A (i.e. the stage in which the sister chromosomes dissociate and move to the opposite poles by the pulling force of the kinetochore microtubules). On the other hand, the spindle elongation that would push the poles apart as in anaphase B appears to take place, although the mode of the poleward chromosome migration and the telophase spindle degradation signficantly differ from that of the wild type. The events observed in the *dis* mutant cells might be an example of uncoordinated mitosis involving the occurrence of the spindle elongation in the absence of chromosome disjoining; a part of anaphase B might independently take place before the completion of anaphase A. We previously described another example of uncoordinated mitosis: the occurrence of spindle dynamics and cytokinesis in the absence of nuclear division in the *S.pombe top2* and *cut* mutants (Hirano *et al.*, 1986; Uemura *et al.*, 1986).

The hypothesis that the *dis* mutants are primarily defective in the mitotic events prior to the spindle elongation may be consistent with the following facts. (i) The viability of the *dis1* cells at restrictive temperature decreases at the onset of mitosis. (ii) The early short spindle in the arrested *dis* mutant cells lacks the blob-like thick ends; kinetochore microtubules might not be assembled or may be functionally impaired. Alternatively, the spindle may be malformed. (iii) The condensed chromosomes in the *dis* mutant cells do not change into the U-shaped form but become three discrete bodies arranged in a line. The chromosomal proteins required for the chromosome separation may be defective in the *dis* mutants. To understand precisely the primary defects in the *dis* mutants, however, it is essential to identify the gene products and their molecular functions.

A striking characteristic of the *dis* mutants is their supersensitivity to caffeine at permissive temperature. We found that certain concentrations of caffeine block the growth of the *dis* mutants but not the wild type. This phenotype can be reversed by transforming the *dis2* mutants with the cloned $dis2^+$ gene. Furthermore, the cytological phenotype of

the *dis1* mutant cells produced by caffeine at permissive temperature is similar to that produced at restrictive temperature in the absence of the drug. Because all the *dis* mutants isolated are supersensitive to caffeine, the effect of the drug cannot be coincidental. The *dis* gene expression might be directly or indirectly controlled by caffeine. The physiological effect of caffeine may be pleiotropic but it is a known inhibitor of cAMP-dependent phosphodiesterase and would increase the intracellular concentration of cAMP. Our preliminary study indicates that the intracellular concentration of cAMP increases in *S.pombe* by addition of caffeine (T.Hirano, unpublished result). Although not conclusive, the *dis* mutants appear to be more sensitive to cAMP than the wild type. Furthermore, multicopy plasmids carrying *S.cerevisiae* phosphodiesterase (PDE) genes (Sass *et al.*, 1986) complement *dis* mutations (T.Toda, unpublished result). These suggest that the rise of the intracellular cAMP concentration might effectively inactivate the mutant *dis* gene products at permissive temperature. Alternatively, the inactivation of the *dis* gene products might cause the rise of the intracellular cAMP concentration. The *dis* gene functions, and concomitantly the sister chromatid separation, might directly or indirectly be under the control of cAMP concentration. Further investigation is required to determine a possible involvement of cAMP in the *dis* gene expression. It is well established that cAMP greatly influences the cell cycle pathway in *S.cerevisiae* especially at the G_1 phase (Matsumoto *et al.*, 1982, 1983). In *S.pombe* the expression of certain meiotic genes is affected by cAMP or caffeine added to the culture medium (Beach *et al.*, 1985; McLeod *et al.*, 1987). In higher eukaryotes the levels of cAMP have been implicated in the control of cell division (Bourne *et al.*, 1975; Pastan *et al.*, 1975).

Little is known about the dis^+ gene products. The similarity in the phenotype of different *dis* mutants suggests that their gene functions may be related. Based on the morphological phenotype, we speculate that the *dis* gene products may be implicated in the formation of the mitotic chromosomes and/or the spindle apparatus. We have cloned 11 genomic sequences which complement the *dis1*, *dis2* or *dis3* mutations and identified the clones derived from the $dis2^+$ and $dis3^+$ genes. The nucleotide sequences of the subclones are currently being investigated. A characteristic of the $dis1^+$ and $dis2^+$ products is that their mutation is complemented by high-copy suppressor sequences. A complex regulatory system with many coordinated genes may exist for the execution of the dis^+ gene functions.

Materials and methods

Strains, media and chemicals

Haploid strains of *S.pombe* were used by the standard genetical procedures described by Gutz *et al.* (1974). The strains *nda2*-52, *nda3*-311 and *nuc2*-663 were previously described (Toda *et al.*, 1983; Yanagida *et al.*, 1986; Hirano *et al.*, 1988). Rich YPD and minimal EMM and sporulation SPA media were used. Thiabendazole (Sigma) stock solution was made in dimethylsulfoxide (20 mg/ml). Caffeine (trimethylxanthine, 100 mM; Wako) was dissolved in distilled water. 3',5'-cAMP was directly dissolved in YPD and used after Millipore filtration.

Isolation of cold-sensitive dis mutants

The procedures were similar to those described for the isolation of a cs *top2* mutant (Uemura *et al.*, 1987). The 982 cs strains were individually grown at 36°C and the exponentially grown cells were transferred to 20°C and incubated for 12 h. The cells were fixed with 2.5% glutaraldehyde, stained with DAPI and observed by epifluorescence microscopy. Strains which showed the cells with the condensed chromosomes (Umesono *et al.*, 1983; Hiraoka *et al.*, 1984) in high frequencies (>30%) were isolated.

DAPI stain and fluorescence microscopy

The procedures described by Toda *et al.* (1981) for DAPI stain and by Mitchison and Nurse (1985) for Calcofluor stain were followed. Calcofluor solution (1 mg/ml) was freshly made for each experiment. For the double stain of DAPI (final concentration 30 μg/ml) and Calcofluor (final concentration 0.3 mg/ml), the cells were fixed, washed with distilled water and stained by a mixed stain solution. For the double stain of DAPI (final concentration 30 μg/ml) and ethidium bromide (final concentration 0.3 mg/ml), procedures similar to those described above were followed.

Indirect immunofluorescence microscopy

The procedures (Hiraoka *et al.*, 1984) were similar to those described by Kilmartin and Adams (1984) and Adams and Pringle (1984). The procedures described by Hagan and Hyams (1988) were also applied. The cell walls were digested with 0.2 mg/ml Zymolyase and partially purified Novo mutanase. Monoclonal antibody against *S.cerevisiae* α-tubulin YL1/2 was used. The second antibody was rhodamine-conjugated rabbit anti-rat IgG antibody.

Synchronous culture

The selection synchrony method (Mitchison and Carter, 1975) was followed. The small early G_2 phase cells were collected from the top layer of 10–40% sucrose gradient centrifugation and incubated in YPD liquid medium. An aliquot of the cultures was taken at intervals, fixed and observed by fluorescence microscopy using anti-tubulin and DAPI.

Chromosome loss in dis mutants

The artificial minichromosome Ch16 (Niwa *et al.*, 1986; Matsumoto *et al.*, 1987) consisting of the centromere III region was introduced into the *dis* mutants by crossing. Small colonies of these mutant cells were made on YPD plates at 33°C for 48 h. All cells from a single colony were collected and resuspended in 1 ml YPD. 0.05 and 0.2 ml of the suspension were plated on YPD and incubated at 33°C for 4 days. Then, the numbers of Ade^- red colonies lacking the minichromosome and Ade^+ white colonies were counted.

Culture of dis mutants in the presence of caffeine or cAMP

The wild type and *dis*-mutant strains were plated on YPD containing different concentrations of caffeine and cAMP, and incubated at 33°C. Colony sizes were examined after 4 days. For liquid cultures, the cells were first grown at 33°C to the concentration of 1×10^7/ml, then diluted 20-fold by YPD, YPD containing 10–100 mM cAMP or YPD containing 50 mM 5'AMP and incubated at 33°C. The cells were examined by fluorescence microscopy and their cell numbers were counted by a phase-contrast microscope after 14 h.

Transformation and gene cloning

The lithium acetate method (Ito *et al.*, 1983) was used for transformation of the *S.pombe* cells. The gene library was made using the pDB248' vector (Beach and Nurse, 1981; Beach *et al.*, 1982) inserted with *Sau*3A1-partial digests of the genomic DNAs. Plasmid DNAs were recovered from transformants by the procedures described by Beach *et al.* (1982). YIp33 was used as an integration vector (Botstein *et al.*, 1979; Toda *et al.*, 1983).

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