Cell Cycle-dependent Specific Positioning and Clustering of Centromeres and Telomeres in Fission Yeast

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Abstract. Fluorescence in situ hybridization (FISH) shows that fission yeast centromeres and telomeres make up specific spatial arrangements in the nucleus. Their positioning and clustering are cell cycle regulated. In G2, centromeres cluster adjacent to the spindle pole body (SPB), while in mitosis, their association with each other and with the SPB is disrupted. Similarly, telomeres cluster at the nuclear periphery in G2 and their associations are disrupted in mitosis. Mitotic centromeres interact with the spindle. They remain undivided until the spindle reaches a critical length, then separate and move towards the poles. This demonstrated, for the first time, that anaphase A occurs in fission yeast. The mode of anaphase A and B is similar to that of higher eukaryotes. In *nda3* and *cut7* mutants defective in tubulin or a kinesin-related motor, cells are blocked in early stages of mitosis due to the absence of the spindle, and centromeres dissociate but remain close to the SPB, whereas in a metaphase-arrested *nuc2* mutant, they reside at the middle of the spindle. FISH is therefore a powerful tool for analyzing mitotic chromosome movement and disjunction using various mutants. Surprisingly, in *top2* defective in DNA topoisomerase II, while most chromatid DNAs remain undivided, sister centromeres are separated. Significance of this finding is discussed. In contrast, most chromatid DNAs are separated but telomeric DNAs are not in *cut1* mutant. In *cut1*, the dependence of SPB duplication on the completion of mitosis is abolished. In *crm1* mutant cells defective in higher-order chromosome organization, the interphase arrangements of centromeres and telomeres are disrupted.

THE organization of eukaryotic nuclei is designed for the storage and expression of the genetic material that consists of a set of linear chromosomes surrounded by the nuclear membrane (Hiraoka et al., 1990). Eukaryotic chromosomal DNAs are highly folded (the 2×10^6-μm-long human genome DNA is stored in a 10-μm-diameter nucleus), even in micro-organisms with small genomes (total yeast DNA is 4,000 μm long and is packed in a 2-μm-diameter nucleus). A number of nuclear proteins may be involved in chromosomal DNA compaction (Gasser and Laemmli, 1987; Earnshaw and Bernat, 1991). Specific DNA sequences may also function in the organizational principles of chromosomal packaging. A question relevant to this problem is whether certain DNA sequences are essential for spatial arrangements of chromosomes in the nucleus. The fission yeast *Schizosaccharomyces pombe* is an ideal organism in which to address this question, since it has a small genome consisting of only three chromosomes and is amenable to fine genetic analysis (Yanagida, 1989; Nurse, 1990).

We recently reported the application of the fluorescence in situ hybridization (FISH)[1] method to this organism (Uzawa and Yanagida, 1992) and suggested its exploitation in the localization of individual DNA sequences within the nucleus. As an initial step towards understanding the principles of nuclear organization in fission yeast, we have investigated the behavior of centromere and telomere sequences in wild-type and mutant cells defective in cell division cycle progression and higher order chromosome structure.

Centromeres and telomeres are DNA sequences which are essential for maintaining and propagating eukaryotic linear chromosomes (Blackburn and Szostak, 1984). Fission yeast centromeres are very large (30–100 kb), consisting of several repetitive sequences and their entire sequences are determined (e.g., Nakaseko et al., 1986; Chikashige et al., 1989; Murakami et al., 1991; Takahashi et al., 1992) so they should be much more easily visualized than nonrepetitive unique sequences. The telomeres and telomere-adjacent sequences also contain repetitive sequences (N. Sugawara, personal communication). Furthermore, knowledge of FISH

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1. *Abbreviations used in this paper*: cs, cold sensitive, FISH, fluorescence in situ hybridization; SPB, spindle pole body; topo II, topoisomerase II; ts, temperature sensitive.

combined with the physical map locations of the probes used (Fan et al., 1988; Chikashige et al., 1989) will be useful in understanding the spatial arrangements of individual chromosomes within the nucleus.

Fission yeast is also an excellent model organism for the analysis of the eukaryotic cell division cycle (Nurse, 1990). A number of cell cycle controlling genes have been identified (Fantes, 1989; Yanagida, 1989), and the dramatic nuclear and cytoplasmic G2/M transitions are well documented (Toda et al., 1981; Hiraoka et al., 1984; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988; Hagan et al., 1990; Kanbe et al., 1989). However, the fission yeast nucleus is so small that kinetochores and kinetochore microtubules, which are essential for chromosome separation in higher eukaryotes, have not been visualized. The actual mechanics of chromosome disjunction in wild-type cells is still poorly understood. Whether sister chromatids are separated in a step analogous to higher eukaryotic anaphase A is an important issue in the assessment of fission yeast as a model system. In anaphase A, the distance between the spindle poles and the centromeres decreases, whereas the distances between the spindle poles increases in anaphase B. A step analogous to anaphase B has been well established in fission yeast (McCully and Robinow, 1971; Hiraoka et al., 1984; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988). If the behavior of the centromeres and the mitotic apparatus can be pursued simultaneously, information essential to the resolution of such questions should be obtained.

In the present paper, we will describe results obtained by using the FISH method in combination with immunofluorescence microscopy to localize centromeres, telomeres, spindles, and spindle pole bodies (SPBs) in fission yeast wild-type and mutant cells. We demonstrate the dramatic alterations in centromere and telomere positioning and association during cell cycle progression. Analyses using mitotic mutants suggested that these changes are genetically controlled. The centromeres and the telomeres appear to be key structural elements in the localization of individual chromosomes in the fission yeast nucleus.

Materials and Methods

Strains and Media

The *S. pombe* haploid wild-type strain used in the present study is *972h*$^-$ (Gutz et al., 1974). The following mutants were used: *h*$^+$ *nda3-KM311 leu1-32* (Hiraoka et al., 1984); *h*$^-$ *nuc2-663 leu1-32* (Hirano et al., 1988); *h*$^-$ *crm1-809 leu1-32* (Adachi and Yanagida, 1989); *h*$^-$ *top2-191 leu1-32* (Uemura and Yanagida, 1984); *h*$^-$ *cut1-206 leu1-32* (Hirano et al., 1986); *h*$^-$ *cut7-24* (Hagan and Yanagida, 1990; this allele was a kind gift of R. Bartlett and P. Nurse, Oxford University, England); *h*$^-$ *cdc25-22* (Nurse et al., 1976); *h*^{90}/*h*^{90} *ade6-M210/ade6-M216* (this study). The wild-type haploid strain *972h*$^-$ and a diploid strain were grown to the cell density of 3×10^6 or 1×10^7 cells/ml at 33°C in rich YPD medium (1% yeast extract, 2% polypeptone, 2% glucose). Temperature-sensitive mutant strains were grown in YPD to 2×10^6 or 5×10^6 cells/ml at the permissive temperature, 26°C, and then shifted to the restrictive temperature of 36°C, for 1-2 (*top2* and *cut1*; Uemura and Yanagida, 1986; Uzawa et al., 1990), 3 (*cut7*; Hagan and Yanagida, 1990) or 4 (*nuc2*; Hirano et al., 1988) h. *cdc25* was grown in minimal EMM2 (Nurse, 1975) to 3×10^6 cells/ml at the permissive temperature, 26°C, and shifted to the restrictive temperature of 36°C, for 4 h (Hagan and Hyams, 1988). Cold-sensitive mutants were grown in YPD to 2×10^6 cells/ml at the permissive temperature of 33°C, and shifted to the restrictive temperature, 20°C, for 8 and 12 h (*nda3*; Hiraoka et al., 1984) or 12 h (*crm1*; Adachi and Yanagida, 1989). Cells were collected and used for the preparation of specimens for FISH and immunofluorescence microscopy.

Preparation of Digoxigenin-labeled Probe DNA

pRS140 (Chikashige et al., 1989), YIp10.4 (Toda et al., 1984) and cos212 (this study) were used to visualize the centromere, rDNA and the telomere, respectively. The DNA fragments used to prepare the probes were digested by a mixture of AluI, DdeI, HaeIII, RsaI, and Sau3AI to yield an average fragment size of ~300 bp. These fragments were labeled by digoxigenin-dUTP using the random priming labeling kit (Boehringer Mannheim Corp., Germany). Nonreacted nucleotides were removed by a Sephadex G50 spun-column. Once made the probes were kept at 4°C for 6 mo without affecting their utility.

Immunofluorescence Microscopy

Cells to be used for immunofluorescence microscopy were fixed with aldehyde and stained with antibodies as previously described (Hagan and Hyams, 1988). Briefly, cells suspended in YPD were fixed in 3.8% paraformaldehyde and 0.2% glutaraldehyde for 1 h, and treated with Novozyme 234 (0.1 mg/ml, Novo) and Zymolyase 100T (0.6 mg/ml, Seikagaku) at 37°C for 90-120 min. They were suspended in 1% Triton X-100, washed three times with 1 mg/ml sodium borohydride and then incubated with primary antibodies. The mAb TAT1 (Woods et al., 1989; a gift of Dr. K. Gull, University of Manchester, UK) was used for microtubule staining with a goat anti-mouse Texas red-conjugated polyclonal sera (EY Lab) as the secondary antibody. For SPB staining, rabbit polyclonal antibodies were raised to sad1 fusion protein (Hagen, I., and M. Yanagida, manuscript in preparation) and sheep anti-rabbit FITC-conjugated sera (Cappe, Durham, NC) were used as the secondary antibody. Immuno-labeled cells were re-fixed by 3% paraformaldehyde for 20 min, washed three times in PEM (100 mM Pipes, 1 mM EGTA, 1 mM MgSO4, pH 6.9) and treated with RNase A (0.2 mg/ml) for 2 h at 36°C followed by hybridization with the digoxigenin-labeled DNA probes for FISH.

Fluorescence In Situ Hybridization

The procedures for FISH were as described in Uzawa and Yanagida (1992) with modifications. For alkali-denaturation of nuclear DNA, 1×10^7 to 2×10^7 cells were suspended in 100 μl 0.1 M NaOH and kept at room temperature for 2 min, followed by brief centrifugation. Cells were resuspended in 100 μl heat-treated (65°C for 10 min) hybridization buffer (50% formamide, 2× SSC, 10% dextran sulphate, 5× Denhardt's solution, 0.5 mg/ml salmon sperm DNA) containing 0.1-1.0 ng/μl digoxigenin-labeled probe and incubated for hybridization at 36°C for 14-16 h. Cells were washed three times in 2× SSC for 30 min each at room temperature, resuspended in PBS-BAG (PBS, 1% BSA) (Sigma Chemicals, St. Louis, MO), 0.1% sodium azide, 0.5% cold water fish skin gelatin (Sigma Chemicals) and kept at room temperature for 30 min. Cells were resuspended in 100-200 μl PBS-BAG containing anti-digoxigenin-rhodamine or -fluorescein, Fab fragments (2.0 μg/ml) (Boehringer Mannheim Corp.) and incubated for 12 h followed by three washes in PBS-BAG. Chromosomal DNA was stained with 0.2 mg/ml DAPI and cells were then washed with PBS containing 0.1% sodium azide. Cells were air dried on poly-L-lysine-coated coverslips and inverted onto mounting medium (90% glycerol, 1 mg/ml *p*-phenylenediamine). Specimens for fluorescence microscopy were observed by a Zeiss Axiophot (Carl Zeiss, Inc., Oberkochen, Germany) with a 100W light source (HBO 100W/2). Photographs were taken on Kodak T-Max 400 or Ektachrome 400 (Eastman Kodak Co., Rochester, NY).

Isolation of Cosmids Containing Telomere-adjacent DNAs

To obtain the cosmids containing the telomere adjacent DNAs a fission yeast genomic cosmid library was screened by colony hybridization using the 1.0 kb NsiI-HindIII fragment of pNSU21 (a kind gift of N. Sugawara and J. W. Szostak, Massachusetts General Hospital, Boston, MA). This fragment lies 6.5 kb away from the telomere (N. Sugawara and J. W. Szostak, personal communication). The fragment was labeled by digoxigenin-dUTP (Boehringer Mannheim Corp.) and the hybridization signals were detected by Southern Light™ Test Kit (Tropix Inc., Bedford, MA). 9 independent cosmids (cos212, cos232, cos566, cos750, cos773, cos1004, cos1300, cos1749, and cos1767) were obtained and in this study cos212 was used as a telomere-adjacent probe. NotI-digested genomic DNA was run in PFG electrophoresis (Fan et al., 1988) and probed with cos212. The fragments containing the telomeres of chromosomes I and II were hybridized. It is not known whether the sequence homologous to cos212 is present in the ends of chromosome III.

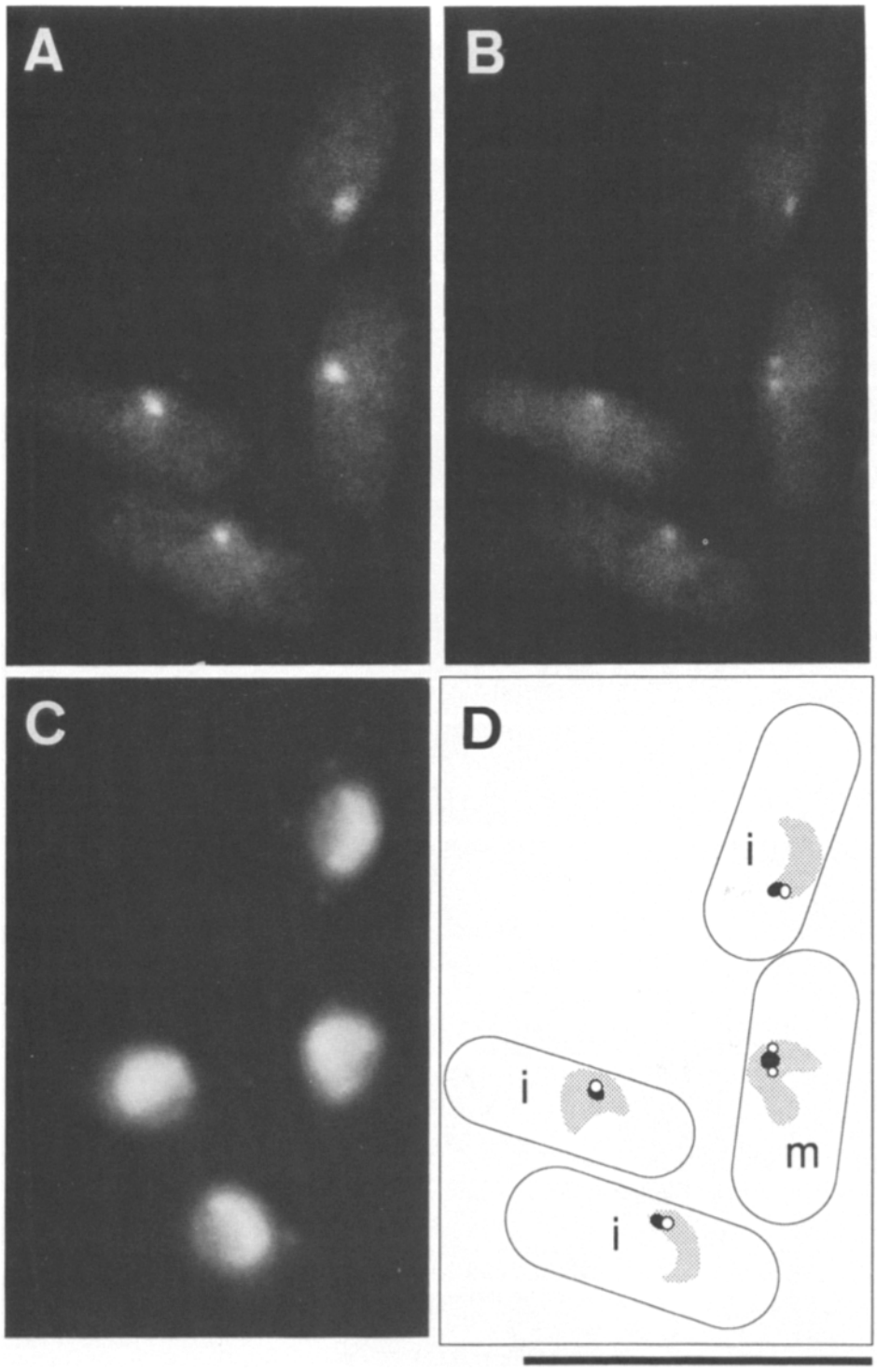
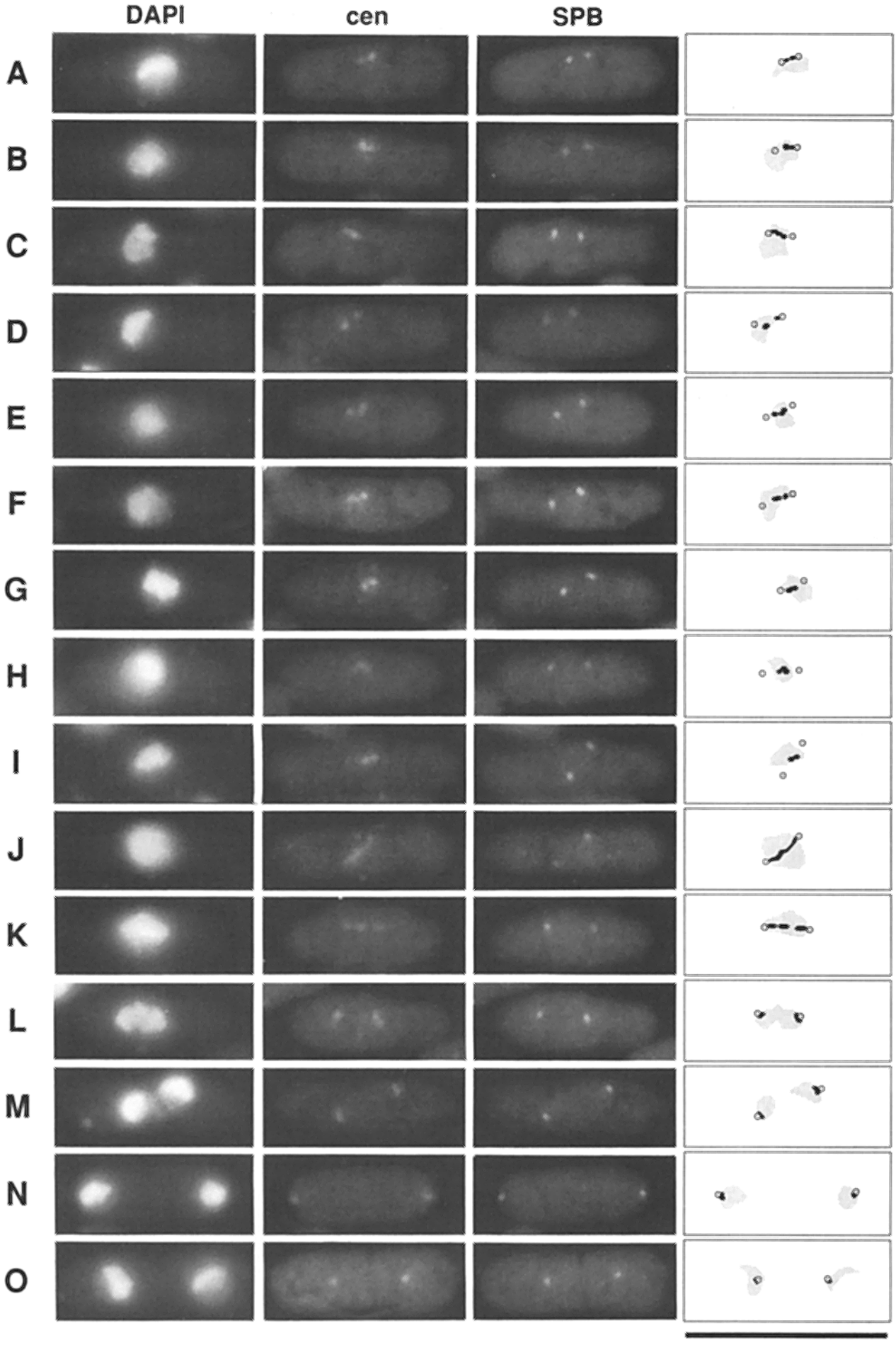


Figure 1. FISH of the centromeres combined with immunofluorescence microscopy of the SPB in fission yeast. The centromeres (*A*) were visualized by FISH in combination with immunofluorescence microscopy of the SPB (*B*) in haploid wild type fission yeast cells. Whole chromatin DNA (*C*) was stained by DAPI. A cartoon of the superimposed images is shown in *D*. (*A*) Chromosomal areas of the cells were denatured and hybridized with the digoxigenin-labeled centromere DNA probe which, followed by reaction with anti-digoxigenin antibodies conjugated with rhodamine. (*B*) The same cells stained by immunofluorescence microscopy using anti-sad1 antibodies which revealed the SPB. The second antibodies used were FITC-conjugated sheep anti-rabbit antibodies. (*C*) The chromosomal domain of the same cells were stained with a DNA-specific fluorescent probe, DAPI. (*D*) A cartoon depicting the centromere DNA (*filled area*), the SPB (*open circles*), and the chromatin region (*hatched area*). The interphase and mitotic cells are indicated by *i* and *m*, respectively. Bar, 10 μm.

Results

Centromeres in G2 Are Found Adjacent to the SPB

Fission yeast centromeres and the SPB or microtubules were simultaneously observed in the same cells by using the FISH method in combination with immunofluorescence microscopy. Cells were processed for immunofluorescence microscopy first and then by the procedures for FISH. The antibodies used are directed against sad1, an SPB protein (anti-sad1; Hagan, I., and M. Yanagida, manuscript in preparation), and *Trypanosoma* α-tubulin (TAT1; Woods et al., 1989). The DNA hybridization probe pRS140 contained a common repeat, *otr*, present only in the centromeres (Chikashige et al., 1989; Takahashi et al., 1992). The probe DNA was labeled with digoxigenin-dUTP by using random priming, and the hybridized probe DNA was detected with anti-digoxigenin


DAPI
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antibodies conjugated with rhodamine or fluorescein (see Materials and Methods). From this point on centromere localization with this probe will be referred to as cenFISH. In the present paper, micrographs obtained by FISH combined with anti-sadl are shown, because the SPBs are better preserved than microtubules under hybridization condition. Consistent results are obtained by FISH combined with TAT1.

In Fig. 1, cenFISH (*A*), SPB (*B*), and DAPI whole chromatin (*C*) staining are shown for the same haploid wild-type cells along with a cartoon (*D*) schematizing the visualized structures (*filled areas*, centromeres; *open circles*, SPBs; *hatched areas*, whole chromatin DNA). G2 (indicated by *i*) and mitotic (indicated by *m*) phase cells contain single and double SPBs, respectively. The mitotic spindle runs between the two anti-sadl stained SPBs (Hagan, I., and M. Yanagida, manuscript in preparation). G1 phase is negligible in exponentially growing wild-type cells. S phase begins shortly after nuclear division and is completed before cytokinesis, thus G2 cells are defined with a single nucleus containing a single SPB.

G2 centromeres are invariably present at the periphery of chromosomal domain (Uzawa and Yanagida, 1992). In the present study, we found that G2 centromeres locate adjacent to the SPB (Fig. 1). Among 230 G2 cells examined, all the hybridization signals except one were found in the vicinity of SPB. Furthermore, separated three individual centromeres were rarely seen in G2, usually being observed as a single intense cluster. The centromeres appear to have a strong affinity for the SPB in G2 phase.

Centromeres Interact with the Mitotic Apparatus

We found that, during mitosis, centromeres display stage specific alterations in their position relative to the SPB. A series of mitotic cells are shown in Fig. 2 (from left to right; whole DNA, cenFISH, SPB, and a superimposed scheme). Upon the entry into mitosis, centromeres dissociate from the SPB, and then distribute along the spindle. The clustered centromere structure is disrupted in mitosis; two or three hybridization signals probably representing individual centromeres were observed in early mitotic cells. The DAPI staining of the nuclei in Fig. 2, *A–K*, does not show any sign of nuclear chromosome separation, but they all contain two SPBs between which a relatively short spindle should run. Most individual centromeres (*filled area* in the cartoon) lie along a line connecting the SPBs, however, some (Fig. 2, *D* and *G*) clearly do not, suggesting that such chromosomes are yet to be captured by the microtubules or that the kinetochore microtubules might radiate away from the spindle's main axis. Through-focusing observation indicated that, in most mitotic cells shown in Fig. 2, the SPBs and the centromeres exist in the same focal plane; no other SPB or centromeres were found in other planes.

Irregular positioning along the spindle in Fig. 2, *A–F* possibly reflects a stage similar to prometaphase movement which might occur in fission yeast. Upon initial centromere attachment to microtubules, centromeres may undergo a series of oscillatory movements.

Sister Centromeres Separate after Metaphase

In Fig. 2, *H* and *I*, centromeres gather in the middle of the short spindle, while DAPI-stained chromatin of these cells showed no sign of division. They are reminiscent of mitotic metaphase cells. Sister centromere disjunction should occur after this metaphase-like stage. The images representing sister centromere separation have been assigned as Fig. 2, *J* and *K*. Note that although the sister centromeres are separating, the overall chromatin structure, as judged by DAPI staining, is not separated in these cells.

In contrast, Fig. 2, *L–O* show chromatin during or after nuclear division. The sister centromeres of these nuclei appear to be fully separated and located near the SPB. The distance between the SPBs for the cells in Fig. 2, *L–N* exceeds that of those in Fig. 2, *A–K*, indicating that the cells are in anaphase B or telophase. In these cells, the centromeres are located at the ends of the mitotic spindle. In cell O the nuclei have moved to the middle of the daughter cells after spindle degradation and cytokinesis is being initiated. In short, fission yeast centromeres gather in the middle of the short spindle, and separate before the full spindle extension, indicating the presence of anaphase A movement in fission yeast.

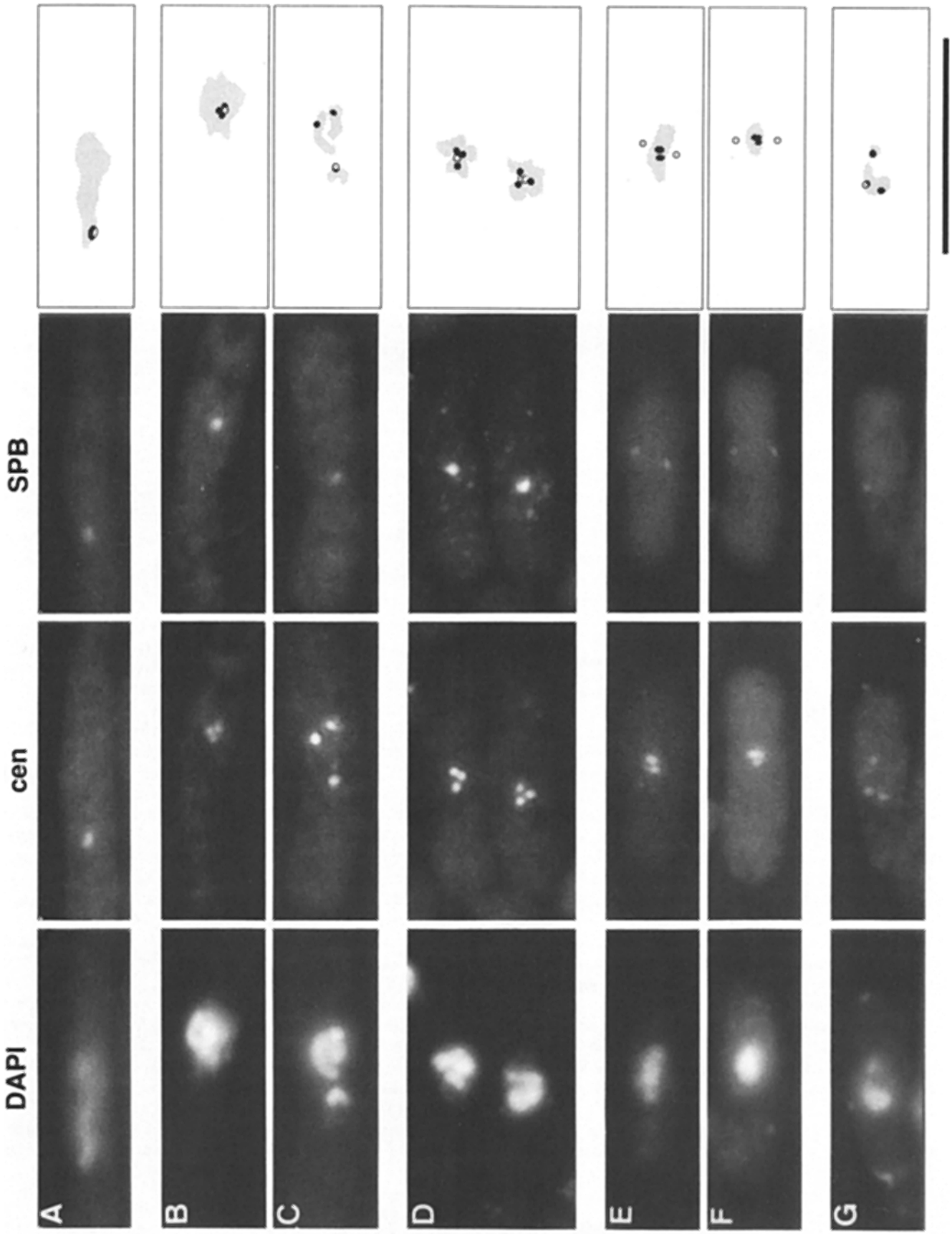
Centromere Localization in Mitotic Mutants

We examined centromere localization in mutant cells arrested at different stages of mitosis by cenFISH and SPB staining. In the temperature-sensitive (ts) *cdc25-22* cells blocked in G2 due to the absence of mitotic inducer (Nurse et al., 1976; Nurse, 1990), the centromeres form a cluster and locate closely to the SPB (Fig. 3 *A*). Note that the nuclear chromatin shown by DAPI stain is much extended in *cdc25* cells but the centromeres still remain clustered. In *cdc25*, microtubules do not form the spindle but are localized in the cytoplasmic array (Hagan and Hyams, 1988).

The cold-sensitive (cs) *nda3-311* mutant contains a defect in *S. pombe*'s single β-tubulin so that at the restrictive temperature (20°C) cells are synchronously arrested in early mitosis due to their inability to form the spindle (Hiraoka et al., 1984). Chromosomes are condensed, the SPB remains single and cells contain an elevated high histone H1 kinase activity (Hiraoka et al., 1984; Kanbe et al., 1990; Moreno et al., 1989; Kinoshita et al., 1991). In Fig. 3, *B* and *C*, two *nda3-311* cells are shown (*B*, 8 h and *C*, 12 h at 20°C). The centromeres were localized in the vicinity of SPB but their mutual association was weakened (Fig. 3 *B*) as the signal was split into three. Prolonged incubation at 20°C (Fig. 3 *C*) results in centromere and chromosome scattering (see DAPI stain). Interestingly, even after scattering, one of the three centromeres always associates with the single SPB.

ts mutants *cut7* are unable to form a normal mitotic spindle due to defects in a kinesin-related mitotic motor (Hagan

Figure 2. Localization of centromeres relative the SPB in wild-type mitosis. Exponentially growing wild-type cells were fixed and treated for immunofluorescence microscopy followed by cenFISH as described in Fig. 1. A series of mitotic cells containing the two SPBs were selected and shown. From left to right: whole chromatin DNA stained by DAPI, cenFISH, anti-sadl antibodies immunofluorescence, a cartoon of the superimposed images. Bar, 10 μm.


DAPI
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crm1

and Yanagida, 1990, 1992). At the restrictive temperature (36°C), mutants are blocked at a stage after *nda3-311* but before *nuc2-663* (see below); chromosomes condense and the SPB duplicates but the normal spindle is not seen, probably due to the lack of interdigitation of the half spindles. Fig. 3 *D* shows cenFISH staining of *cut7-24* mutant cells. Three distinct centromere signals were arranged in a triangular fashion around the SPBs. The centromeres took up a position where they could interact with both SPB and spindle microtubules.

In the ts *nuc2-663* mutant, a 67-kD protein, containing the TPR repeat motif, is defective (reviewed in Goebl and Yanagida, 1991). At the restrictive temperature, mutant cells arrest at a mid-mitotic stage (Hirano et al., 1988, 1990). Two *nuc2* cells in Fig. 3, *E* and *F* show cenFISH signals in the middle of condensed chromosomes and at the center of the spindle line running between two SPBs. The spindle axial view showed three triangular centromere positioning (data not shown). Such cenFISH staining further supports the interpretation of a metaphase-like arrest in *nuc2* mutant cells.

Disruption of Centromere Clustering in Mitosis

Quantitative data of cenFISH experiments (Fig. 4 *A*) supported the above conclusions. The number of the centromere signals in the nucleus was counted in >200 cells of wild-type and mutant cells. G2 phase cells were identified by their single nucleus and SPB, while M phase cells by their duplicated nuclei and SPBs. >80% of wild-type G2 cells showed a single centromeric signal, whereas 64% of wild-type M phase cells contained two or three centromere signals. More signals (up to six) were observed in the dividing nucleus (Fig. 3, *J* and *K*), but they were omitted, because the frequency was low.

Results obtained by mutants are also shown in Fig. 4 *A*. ts *cdc25-22* mutant growing at 26°C and arrested at 36°C for 4 h had one clustered cenFISH signal in 91 and 66% cells, respectively. In *nda3-311* incubated at 20°C for 12 h and *nuc2-633* at 36°C for 4 h, three signals were frequently (87 and 51%, respectively) seen. These results strongly indicated that the centromere clustering in G2 is disrupted upon the entry into mitosis. In wild-type control cells, the FISH signals were identical at different temperatures (20, 33, and 36°C).

Centromere Clustering Requires Functional crm1$^+$ *Gene Product*

We found that centromere clustering is disrupted in a cs *crm1-809* mutant. As shown in Fig. 3 *G*, two or three cenFISH signals were observed in *crm1-809* at 20°C for 12 h, while SPB remained single. A high proportion of *crm1* mutant cells, which contained cytoplasmic microtubules and no spindle at the restrictive temperature and are thus in interphase (data not shown), had two or three cenFISH signals (Fig. 4 *A*). This suggests that interphase centromere clustering requires normal *crm1*$^+$ gene function. The *crm1*$^+$ gene encodes a highly conserved 110-kD protein essential for maintaining the higher order chromosome structure and interacting with an AP-1 like transcription factor papl (Adachi and Yanagida, 1989; Toda et al., 1991, 1992). The *crm1*$^+$ gene product locates within the nucleus and the nuclear periphery.

Telomeres Also Cluster in G2 and Disperse in M Phase

Fission yeast has ~300 bp of telomeric 5′-$C_{1\text{-}6}G_{0\text{-}1}T_{0\text{-}1}GTA_{1\text{-}2}$-3′ repeats at its chromosomal ends (Matsumoto et al., 1987; Allshire et al., 1988; Zakian, 1989). However, such a small probe is too short to visualize telomeric DNA by FISH. The telomere adjacent region of the chromosomes usually contains a complex mixture of repetitive sequences called telomere-associated sequence (Zakian, 1989). For example, budding yeast chromosomes have 250–650 bp telomeric DNA and ~30 kb telomere-associated DNA. Since fission yeast has also telomere-associated sequences (Sugawara, N., and J. W. Szostak, personal communication), we used a cosmid (cos212) containing 30 kb of the telomere-adjacent DNA as a telomere specific FISH probe (see Materials and Methods). This cosmid was obtained from a fission yeast genomic library by colony hybridization using a telomere adjacent DNA fragment as the probe.

To visualize the chromosome ends in fission yeast, FISH was performed using cos212 as the probe. This sequence is adjacent to the extreme telomeric ends of chromosomes I and II (see below and Materials and Methods). We presumed that there are at least four hybridizable chromosomal ends present in the genome of *S. pombe*. From here on FISH telomere localization using cos212 will be referred to as telFISH. In Fig. 5, DAPI-stain, telFISH and the doubly exposed color micrographs of wild-type diploid cells are shown. Telomere signals appear to locate at the nuclear periphery.

Haploid wild type 972 cells grown exponentially at 33°C were fixed and treated for telFISH and SPB staining. As shown in Fig. 6, *A–H*, depending upon the cell cycle stage, one to up to 7–8 signals were found in wild-type nuclei (from left to right; DAPI, telFISH, SPB, and a cartoon). In Fig. 6, *A–C*, G2 phase cells containing a single SPB are shown with one or two telFISH signals situated at chromatin region periphery. In these selected cells, the SPB and telomere signals are in the same focal plane, and by focusing through the whole cells, no other SPB or telomere signals were found. Telomeres were apparently positioned randomly with respect to the SPB, but were invariably close to the edge of the chromatin region.

The number of telomere signals was one or two in 88% G2 phase cells (Fig. 4 *B*). Only 3% G2 cells showed four telomere signals. In G2-arrested ts *cdc25* cells at 36°C for

Figure 3. Centromere location in mutants. Five mutants *cdc25-22* (*A*, 36°C, 4h), *nda3-311* (*B*, 20°C, 8 h; and *C*, 20°C, 12 h), *cut7-24* (D, 36°C, 3 h), *nuc2-663* (*E* and *F*, 36°C, 4 h) and *crm1-809* (*G*, 20°C, 12 h) were incubated at the restrictive temperatures for the times indicated in parenthesis. Cells were fixed and treated for anti-sad1 stain (indicated by *SPB*) followed by cenFISH (indicated by *cen*). The chromatin region was stained by DAPI. In cartoons, the centromeres and SPBs are illustrated by the filled circles and the open circles, respectively. The chromatin regions are hatched. Bar, 10 μm.

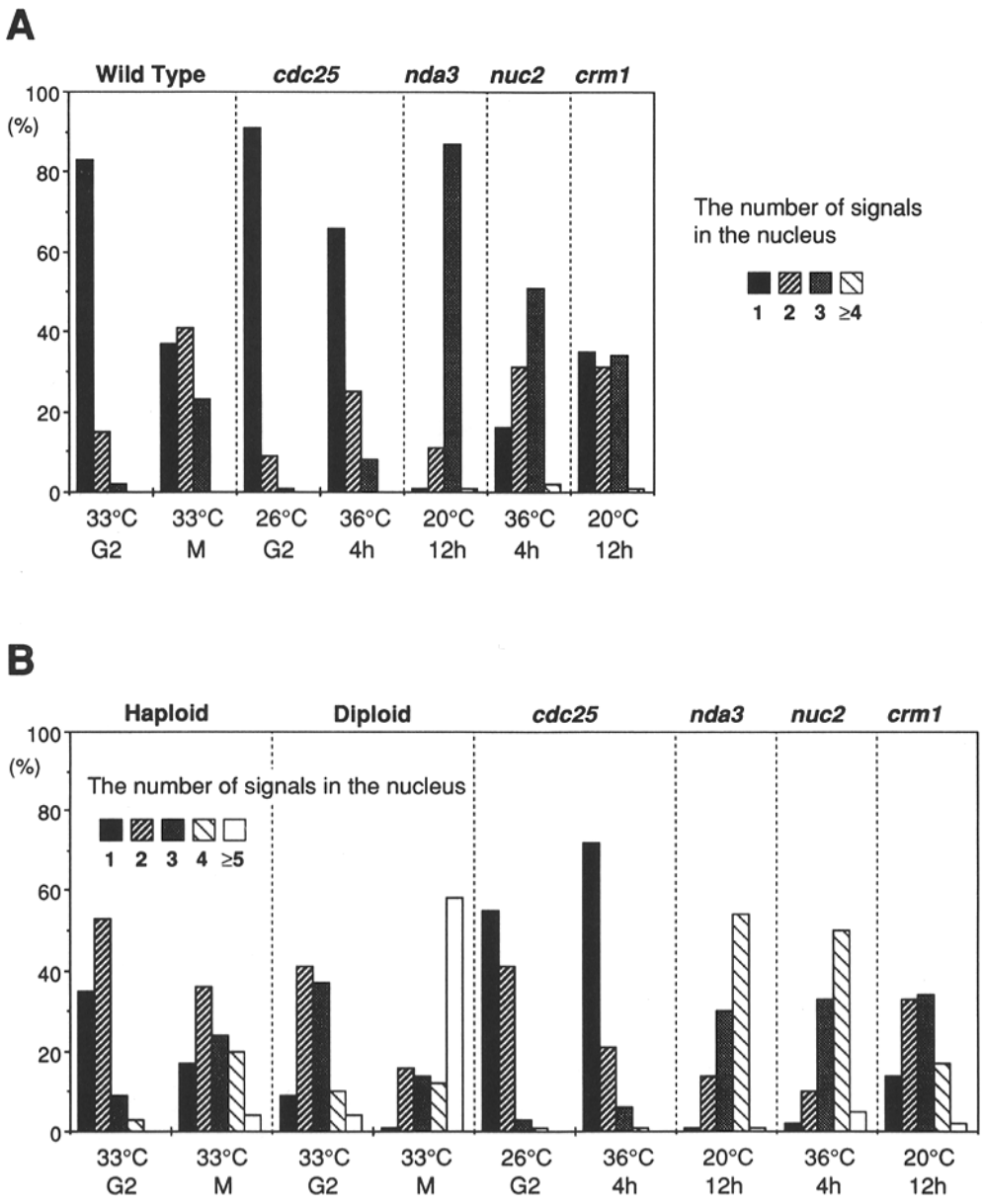

Figure 4. Centromere and telomere clusters are disrupted in mitosis. (*A*) The number of centromere signals per cell was counted by cenFISH. Mutant cells, grown at the permissive temperature, were transferred to the restrictive temperature for the appropriate time. Cells were fixed and treated for immunofluorescence microscopy using anti-sad1 antibodies followed by cenFISH. Whole nuclear chromatin was stained by DAPI. For each strain, > 100–300 cells were counted. The G2 cells contained a single nucleus and SPB, while mitotic cells contained two SPBs. The frequency (%) of the centromere signals in wild-type G2 and M phases are indicated by G2 and M, respectively. cdc25, *cdc25* mutant grown at 26°C or blocked at 36°C for 4 h. nda3 and nuc2, cs *nda3* and ts *nuc2* cells blocked at 20°C for 12 h and 36°C for 4 h, respectively. crm1, *crm1* mutant incubated at the restrictive temperature for 12 h. (*B*) The number of telomere signals per cell was counted by telFISH. Strains and experimental conditions used are similar to those described above. A diploid h^{90}/h^{90} *ade6-210/ade6-216* were used. The number of telomeric signals in each nuclear chromatin region was counted.

4 h, 73% contained one telomere signal. Therefore, four telFISH signals were rarely observed in G2 phase cells, suggesting that chromosome ends might be associated. Further association of the telomeres may cause the single telomeric signal.

In mitotic cells, however, 4–8 telFISH signals were found in the nucleus (Fig. 6, *D–H*). Mid-mitotic cells (Fig. 6 *E*) produced four clearly visible signals at the edge of the chromatin region. In earlier mitotic stages (Fig. 6 *D*), cells often still have only two signals. For dividing nuclei (Fig. 6 *F*), up to eight signals were seen. Interestingly, four signals were still seen in the telophase nuclei (Fig. 6 *G*) and even in those just prior to cytokinesis (Fig. 6 *H*). Note that S phase in wild-type cells takes place after telophase and before cytokinesis so that disruption of telomeric association may continue through S phase.

Quantitative estimation of the telomeric signals in wild-type mitotic cells, and mitotically arrested *nuc2* and *nda3* mutants indicated that on average three and four signals were found (Fig. 4 *B*). Four signals were found in 50 and 54% of arrested *nuc2* and *nda3* cells, respectively. In diploid wild type cells, G2 cells revealed 2–3 telomeric signals, while in mitosis, 58% of cells exhibited more than five signals. Consistent with the results of cenFISH, the number of telomeric signals increased in *crm1* mutant cells at the restrictive temperature.

To confirm that the probe used for telFISH actually bound to the ends of chromosomes, the mitotically condensed chromosomes of *nda3-311* cells were stained. Hybridization took place only at the ends of the two large chromosomes I and II (Fig. 6 *I*). The small chromosome III did not show any hybridization signal, suggesting that the telomere adjacent

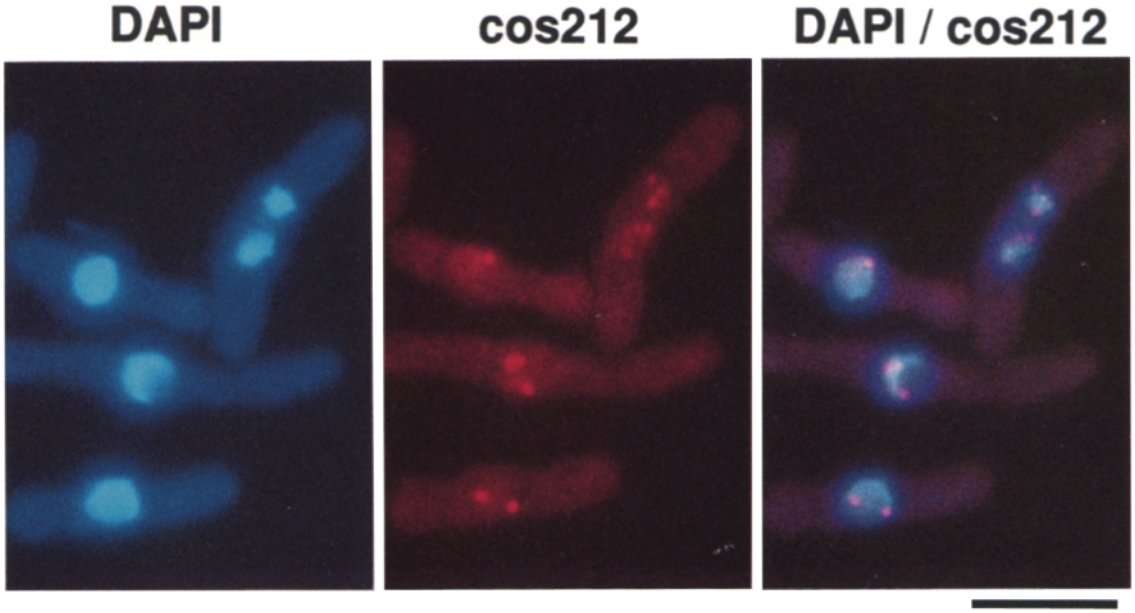

Figure 5. Telomere localization in wild-type diploid cells. Color fluorescence micrographs of DAPI stain (*blue*), telFISH using cos212 as the probe (*red*) and the double exposure of a wild-type diploid cell. The telFISH signals were observed at the nuclear periphery in the G2 phase cells. Bar, 10 μm.

sequence hybridizable to cos212 sequence might be missing in chromosome III.

Centromeres Are Separated but Most Chromatid DNAs Are Not in DNA Topoisomerase II Mutant

DNA topoisomerase II (topo II) is essential for viability and plays an important role in mitotic chromosome condensation and separation (reviewed by Yanagida and Sternglanz, 1990). It is heat inactivated in fission yeast ts *top2* mutants (Uemura and Yanagida, 1984, 1986; Uemura et al., 1987). When ts *top2* mutant cells enter mitosis, the chromosomes do not fully condense and the spindle is transiently formed. Cytokinesis takes place and the undivided nucleus is cut in two, causing lethality. This terminal phenotype is called the cut phenotype (cell untimely torn; Hirano et al., 1986).

top2 mutant FISH staining is shown in Fig. 7. Mutants ts *top2* cells, incubated at 36°C for 1–2 h, frequently revealed mitotic cells in which DAPI-stained chromatin showed two thin protrusions extending from either side of the main chromatin mass. These correspond to the portions of chromatin DNA pulled by the transiently observed spindle (Uemura and Yanagida, 1986). The centromere FISH signals in such cells were distributed along the spindle and moved toward the cell ends. Several examples of cells exhibiting this frequent phenotype are shown in Fig. 7, *A* and *B*. Note that the intensities of cenFISH signals are approximately equal in two sides, strongly suggesting that the centromeres are separated in *top2* cells. If only the centromere clustering was disrupted and each undivided centromere was pulled by the spindle in *top2* cells, then the asymmetric distributions (2:1 or 3:0) of the cenFISH signals should be observed. However, no cell contained the FISH signal at only one end, and most cells displayed equally separating centromere signals.

Judging from DAPI staining, most other chromosomal DNAs were not separated in the absence of topo II function. Results of FISH using the rDNA and the telomeric probes are also consistent with the DAPI images; they remained in the undivided nuclear chromatin (data not shown). The results presented here provide evidence that centromere DNA movement is dependent upon microtubule-mediated force.

An important question with regards to the role of topo II in chromosome segregation is whether the centromeres might be prematurely separated in early stages of mitosis before anaphase in the absence of topo II function. If topo II molecules have a positive role in holding the sister centromeres together and the spindle kinetochore microtubules in the metaphase exert a separating pulling force toward the opposite ends of cell, as in higher eukaryotes, the sister centromeres in *top2* mutants might fail to associate and thus prematurely appear as separate dots before anaphase. As shown in Fig. 7 *C*, centromere DNA behavior in certain top2 mutant cells at the restrictive temperature is highly reminiscent of that seen in early and mid-mitotic wild-type cells. Sister centromeres are not separated and lie between the two SPBs until the spindle attains its metaphase length. These metaphase-like centromere arrangements were frequently observed as transient structures in *top2* cells containing a short spindle, suggesting that the centromere positions relative to the spindle were normal in *top2* until mid-mitosis. Hence, cenFISH signal movements in *top2* appeared to occur at the time of anaphase.

Centromere and Telomere Movements in cut1 *Mutant*

We determined whether centromeres and telomeres were normally separated in ts *cut1* mutant cells at the restrictive temperature. ts *cut1* mutant cells exhibit the archery-bow shaped chromatin which is pulled toward the ends of cell by

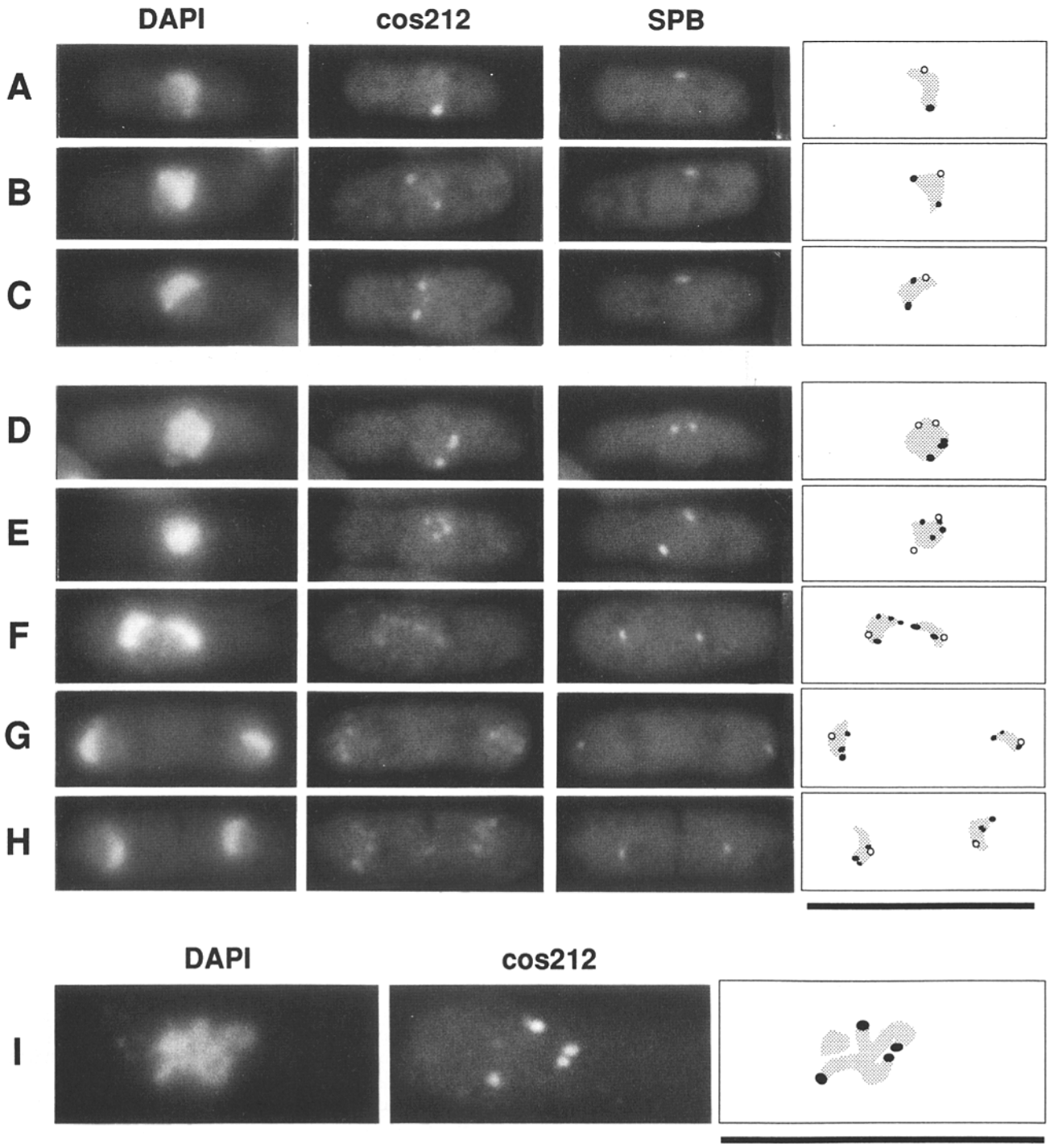

Figure 6. Telomere localization in haploid wild-type and *nda3-311* cells. (*A–H*) Haploid wild-type cells were treated for immunofluorescence microscopy using anti-sad1 antibodies followed by telFISH. DAPI staining revealed the whole chromatin. In G2 cells which contained a nucleus and one SPB signal (*A–C*), there was either one or two telomere signals. In mitotic cells, which contained a single chromatin region with the two SPBs (*D–E*), from two to four telomere signals were seen. In mitotic cells, which contained dividing or divided chromatin regions with the two SPBs (*F–H*), the number of the telomere signals per cell was approximately eight. (*I*) a *nda3-311* cell incubated at 20°C for 12 h. Four telomeric signals correspond to the ends of two large chromosomes I and II. See Results. Bar, 10 μm.

the spindle which forms and then disappears after spindle extension (Hirano et al., 1986; Uzawa et al., 1990). It was of interest to know which part of the chromosomes remain undivided in *cut1* mutants as a large fraction of the chromosomes seem to be separated. The *cut1*$^+$ gene encodes an essential 210-kD protein (Uzawa et al., 1990). FISH staining of archery-bow chromatin is shown in Fig. 8. While a significant portion of chromatin remained unseparated, the centromeres were separated apparently normally (Fig. 8 *A*). Furthermore FISH staining using an rDNA repeat unit probe

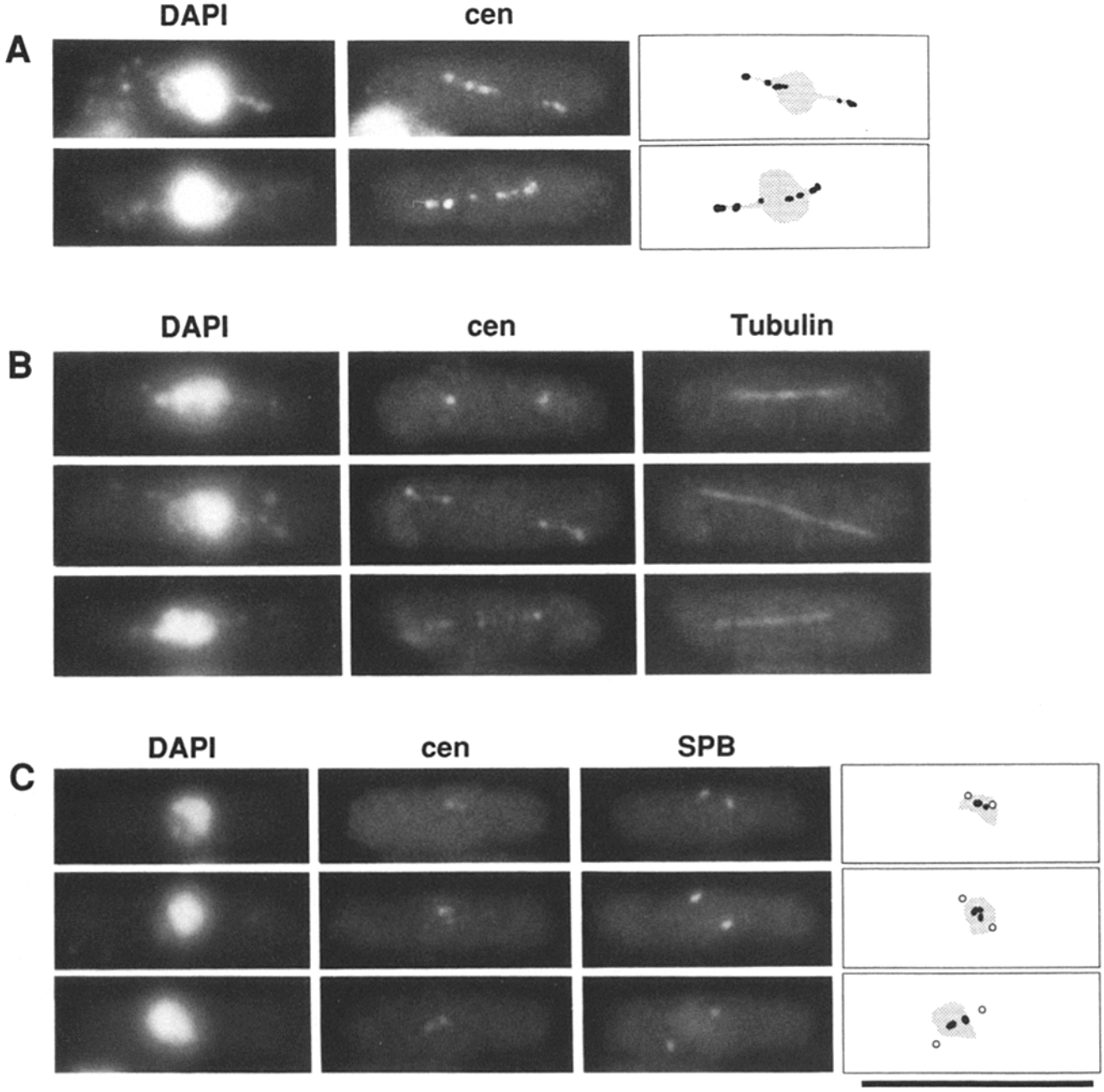

Figure 7. Centromere and telomere behavior in *top2* mutant. ts *top2* cells, grown at the permissive temperature, were transferred to the restrictive temperature for 1–2 h, fixed and treated for immunofluorescence microscopy of cenFISH (indicated by cen). In the *A* and *B*, mitotic cells with the nuclear chromatin pulled by the spindle (Uemura et al., 1986) are shown. The centromeres are separated and moved toward the spindle poles. (*A*) DAPI (*left*) revealed the nuclear chromatin region. cenFISH (*middle*) of the same cells. (*B*) anti-tubulin stain (TAT1 antibody) exhibiting the mitotic spindle is shown. DAPI stain, cenFISH of the same cells are also shown. (*C*) In earlier mitotic stages with shortly separated SPBs, the centromeres were transiently situated in the middle between the two SPBs. Bar, 10 μm.

indicated that nucleolar rDNA repeats were also normally segregated (data not shown). An intriguing result was obtained by telFISH (Fig. 8 *B*). TelFISH signals invariably remained in the undivided region of the chromosomes. This is in contrast to the results of *top2* mutants. In *cut1* mutants, most chromatid DNAs are often divided, but telomere-adjacent DNAs are always included in the undivided region. The number of detectable telomeres was four to eight, suggesting that telomere fusion and clustering may be disrupted and they may be even partly separated in *cut1* mutant at the restrictive temperature.

Discussion

Location of Interphase Centromeres and Telomeres

In the present study fission yeast centromeres were simultaneously localized with the SPBs in wild-type and

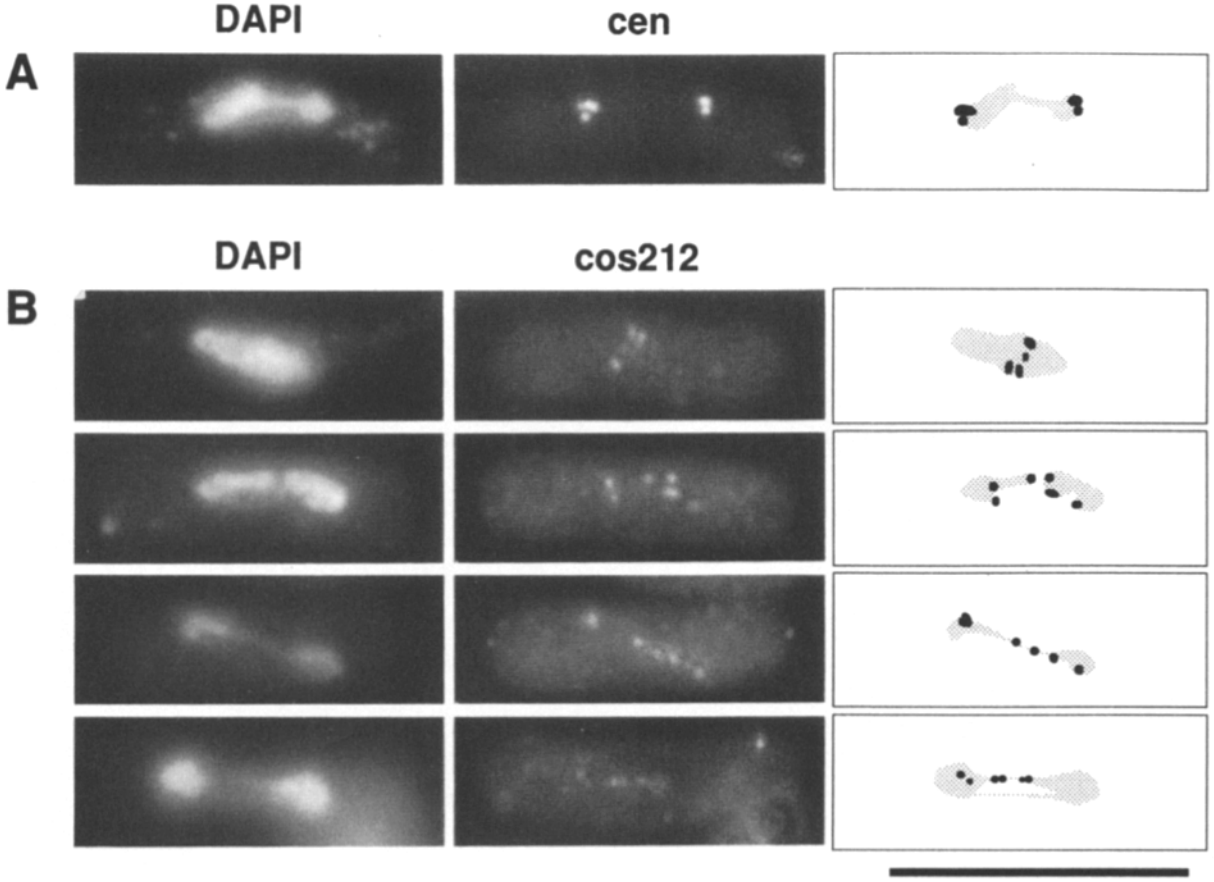

Figure 8. Centromere and telomere behavior in *cut1* mutant. ts *cut1* cells grown at the permissive temperature were transferred to the restrictive temperature for 1–2 h and treated for FISH using the probes of centromere (*A*) and telomere (*B*). Mitotic cells with the DAPI stained chromatin pulled by the spindles are shown. The centromere signals are separated and have moved toward the ends of the cell, whereas the telomere signals remain in the central chromatin region. Bar, 10 μm.

mitotically-defective mutant cells by a combination of the FISH and immunofluorescence microscopy. Both ends of two chromosomes I and II were also visualized using a probe adjacent to the telomeres. In the initial study (Uzawa and Yanagida, 1992), only one centromere, containing the largest number of repeats (*cen3*) was reproducibly visualized. The inability to see the other centromeres was probably due to the short size of the probe used. Combined anti-tubulin and FISH staining revealed the interaction of the centromeres with the spindle (Uzawa and Yanagida, 1992; Takahashi et al., 1992), however this study establishes the interphase centromere localization for the first time by using antibodies against an SPB component. We definitively demonstrated that the centromeres located near the SPB throughout cell cycle except in mitosis (schematically shown in Fig. 9).

Interphase centromere clustering has been previously documented in organisms such as in *Drosophila* (Mathog et al., 1984; Hochstrasser et al., 1986; Foe and Alberts, 1985) and others (Hilliker and Appels, 1989), however this is the first case establishing linkage between interphase centromeres and the SPB (equivalent to centrosome). Fission yeast telomeres, at least a part of them, are localized in the nuclear periphery, but their location relative to the SPB was apparently not fixed. *Drosophila* telomeres are attached to the nuclear envelope (Hochstrasser et al., 1986; Hiraoka et al., 1990). There may be a striking similarity in centromere and telomere localization mechanisms between fruit fly and fission yeast. Rabl (1885) argued that the interphase chromosomes occupy a "telophase configuration" with all centromeres and all telomeres at opposite poles of the nucleus. Arrangements of interphase centromeres and telomeres supporting Rabl configuation were found in organisms including insects (Mathog et al., 1984; Foe and Alberts, 1985; Hiraoka et al., 1990), mammals (Sperling and Luedtke, 1981; Cremer et al., 1982) and plants (Hilliker and Appels, 1989), but were not found in other organisms including humans (Manuelidis and Borden, 1988; Bartholdi, 1991; Billia and Boni, 1991). In the fungus *Saprolegnia ferax*, the kinetochores are arranged near the centriole, but no apparent interphase stage has so far been documented for growing hyphae *S. ferax* (Heath, 1980) so that this situation may be quite different from *S. pombe*. At present, a general statement about eukaryotic chromosome location in the interphase nucleus can not be made.

Positioning the clustering of the fission yeast centromeres and telomeres in the interphase may have functional implications. There may exist genes responsible for the spatial organization of chromosomes in the interphase, and mutations affecting the arrangement will be obtained and analyzed.

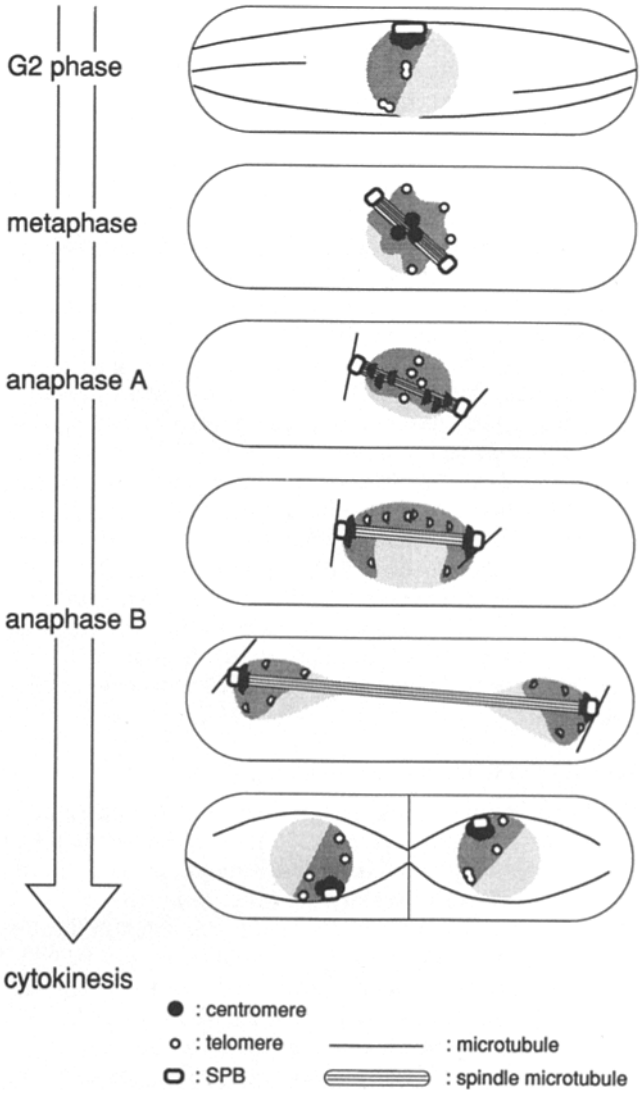

Figure 9. Positional changes and association/dissociation of centromeres and telomeres through the fission yeast cell cycle. Centromeres (indicated by the *filled circles*) and telomeres (*small open circles*) alter their positions during cell cycle as schematized. The SPB and chromatin region are indicated, by the large rectangle and the hatched area, respectively. Cytoplasmic microtubule arrays are schematized by the curved lines in cells at the top (G2 phase) and bottom (postanaphase before cytokinesis). Spindle microtubules are indicated by parallel lines. The short line anchored to SPB represents the astral microtubules seen in anaphase. The centromeres cluster and associate with the SPB in interphase but dissociate and interact with the spindle in mitotic metaphase and anaphase A. The telomeres of chromosomes I and II (see Results) are clustered and located near the nuclear envelope in G2, while they are dissociated in mitosis and postanaphase before cytokinesis.

crml might be an example of such mutants although it also grossly affects general higher order chromatin structure so that it is unlikely that the *crml*$^+$ gene product specifically affects centromere and telomere positioning.

~20-, 50-, and 90-kb centromere repetitive DNAs (designated *otr*, Takahashi et al., 1992) in chromosomes I, II, and III, respectively, can hybridize to the probe used. These sequences are essential for centromere functions in both mitosis and meiosis (Niwa et al., 1989; Hahnenberger, 1989 and 1991; Matsumoto et al., 1990). Additional 15-kb *imr* and *cnt* sequences, present in the central region as low copy repetitive sequences (Takahashi et al., 1992), do not hybridize to the probe, but their locations are assumed to be similar to those of the *otr* sequences which surround them. In G2 cells, centromere DNAs are apparently associated and observed as a single large dot. In mitotic cells, on the other hand, three dissociated signals were seen, and their sizes in the FISH images are ~0.3 μm, that is, at the resolution limit for light microscopy so that the actual sizes might be smaller than those observed. One signal each was found for mitotic chromosomes; the sister centromeres might be too close to be resolved by light microscopy. There may be DNA sequences responsible for association of the centromeres with the SPB and they may be a part of the *otr* or the central *imr* and *cnt*. High resolution approaches such as immune-electron microscopy using centromere DNA probes should identify the nearest sequence to the SPB.

Two-States for the Control of Centromere Location in the Cell Division Cycle

A major finding of the present report is that the centromere locations alternate between two states in cell cycle, namely, SPB position in G2 and spindle position in M phase. A control mechanism possibly exists to switch off SPB localization upon entry into mitosis. It is unlikely that microtubules are involved in association of the centromeres with the SPB, because no microtubules have been found near the SPB in G2 nuclei prepared by a variety of fixation procedures for thin section electron microscopy (McCully and Robinow, 1971; Tanaka and Kanbe, 1986), although cytoplasmic microtubules run parallel to the G2 SPB (Tanaka and Kanbe, 1986; Kanbe et al., 1989). Furthermore, the centromere clustering remained intact by the cold treatment of cells (2°C for 25 min) although the cytoplasmic microtubule arrays were disrupted by the same treatment (our unpublished results). In addition, the centromere was normally clustered in *nda3-311* mutant cells briefly (2 h) incubated at 20°C. In these cells, cytoplasmic microtubules were completely disrupted. However, the possibility that the minute quantity of cold-resistant or functional *nda3-311* mutant tubulins was present as a component of SPB can not be rejected. Anti-γ-tubulin antibodies stain the SPB throughout cell cycle (Horio et al., 1992).

In fission yeast, the SPB is inactive as a microtubule organizing center (MTOC) in vitro in G2 phase but active in M phase (Masuda et al., 1992). The *cut7* motor protein, essential for spindle formation, associates only with the mitotic SPB (Hagan and Yanagida, 1992). Electron microscopic studies (McCully and Robinow, 1971; Tanaka and Kanbe, 1986) show that SPB morphology changes as cells enter M phase. Therefore a parallel relationship may exist between mitotic SPB activation, the dissociation of the centromeres from the SPB, the association of cut7 and morphological rearrangements.

Centromere-microtubule interaction appears to occur only when the mitotic spindle forms; in fission yeast the spindle is present only in mitosis which comprises only one fifth of the cell cycle. This is very different from the situation

in budding yeast where the spindle microtubules are present throughout the cell cycle (Byers, 1981). Among fission yeast mitotic mutants examined, centromeres were situated in the middle of the spindle in *nuc2* (Hirano et al., 1988), consistent with previous proposals that this represents a metaphase plate.

Steps Analogous to Anaphase A Occur in Fission Yeast

An important conclusion of the present study is that a step analogous to anaphase A exists in fission yeast. The evidence supporting this conclusion was that centromeres in fission yeast wild-type cells were not separated until the spindle reached a critical length, and that they were completely separated before full spindle extension. Individual centromeres were initially distributed along short spindles, and then clumped in the middle of the spindle, as the chromosomes make a structure similar to the metaphase plate. Subsequently the centromeres separate before, or coincidental with, anaphase B spindle elongation. All the centromeres were found near the SPBs in late anaphase. Thus the distance for centromeres to move toward the poles in putative anaphase A is roughly 1 μm. Assuming a rate of chromosome movement is 0.5 μm per min (Hiraoka et al., 1984), it would take 2 min which is consistent with the rarity of anaphase A images. These results are important because they predict the presence of both a kinetochore and a minus end mitotic motor protein in fission yeast.

Centromere distributions possibly related to the prometaphase chromosome shuffling movement well described in higher eukaryotic cells (McIntosh and Hering, 1991; Rieder, 1991) were observed in wild-type early mitotic cells. Centromere signals were arrayed in an apparently random and irregular manner along the spindles that were shorter than metaphase spindles. The existence of such premetaphase-like movements in yeast can only be established by future study involving direct visualization in an unfixed cell system.

Sister Centromere Separation Does Not Require Topo II

In *top2* mutants, while most other chromatid DNAs remained undivided, the centromere DNAs were separated by the spindle, indicating that topo II is not essential for sister centromere separation. This suggested that the topological nature of the centromere DNAs might differ from other parts of the chromatids. For example, the sister centromeres may not be intertwined, but most other parts are. This is an intriguing possibility, considering that the centromeres are the sites where the two chromatids are tightly held together from prophase to metaphase. Alternatively, the sister centromeres may be intertwined, but the pulling force exerted by the microtubules bound to the centromeres is sufficient to force separation, leading to the accumulation of intertwined chromatid portions at the edges of separated chromatids. Another possibility raised was that the sister centromeres are not intertwined but topo II molecules associate with the centromeres and might play a role in holding the sister centromeres together but, if topo II was inactivated, they might be prematurely separated by the force of the metaphase kinetochore microtubules. The possibility of premature separation was unlikely, however, because the normal metaphase-plate centromere clustering was observed before the abortive anaphase in *top2* mutant cells at the restrictive temperature. Thus the sister centromeres may be held together by components other than topo II. In short, topo II is essential for separation of sister chromatids but not for sister centromeres.

In contrast to the results with *top2* mutants, most chromatid DNAs are separated in *cut1* mutants, but *cut1* cells still fail to complete nuclear division. We demonstrated that telomeres remained in the undivided central chromatin region; *cut1* mutations might be implicated directly in the telomere separation or indirectly in the separation of a nuclear component which is associated with the telomeres. Fission yeast telomeres may be bound to the nuclear envelope as shown in the present study or nuclear matrix like human telomeres (de Lange, 1992). Consistently, cut1 protein is an insoluble nuclear component (Uzawa et al., 1990). Temperature-sensitive *cut1* produces the large nucleus containing a number of SPBs and polyploidy chromosomal DNAs if septation is blocked (Uzawa et al., 1990; Creanor and Mitchison, 1990). The dependency of SPB duplication and DNA replication on the completion of mitosis is abolished in *cut1*. Combination of the present finding with the previous ones suggests that SPB and chromosome duplications can continue to occur without a final stage of chromosome separation in *cut1*.

Location of Chromosomal Termini

Only one or two signals were observed in G2 phase cells using a telomere adjacent hybridization probe, while up to eight signals were found in dividing mitotic nuclei, indicating that the telomeric regions are fused and clustered in G2 phase, but that telomere interaction is disrupted in mitosis (Fig. 9). Interestingly, four signals were frequently found in telophase nuclei before cytokinesis so that telomere dispersal appears to continue in the S phase in wild-type cells.

The finding that telomeres are located at the nuclear periphery during G2 phase may indicate their anchoring into the nuclear membranes. Their positioning relative to the SPB is apparently random, suggesting that telomere positioning is not fixed, possibly moving along with movements of the nuclear envelope. Micrococcal nuclease digestion indicated that fission yeast telomeres consist of nonnucleosomal chromatin (Chikashige et al., 1989). Telomere interactions and location to the nuclear periphery is also found in many other eukaryotes (Wagenaar, 1969; Agard and Sedat, 1983; Hochstrasser et al., 1986; Chung et al., 1990; Rawlins et al., 1991; Klein et al., 1992). While this is the first report for the occurrence of disruption of the telomere association in yeast mitosis, such a phenomenon has been predicted as telomere association has to be disrupted before sister chromatid separation.

The results presented in this study are a step in our effort towards understanding the spatial organization of chromosomes in eukaryotic nuclei using fission yeast as a model organism. Fission yeast centromeres and telomeres appear to play important roles in the spatial arrangement of chromosomes in the nucleus. The availability of a large number of mutants offers an opportunity to expand our understanding of the genetic control of nuclear organization.

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