A Low Copy Number Central Sequence with Strict Symmetry and Unusual Chromatin Structure in Fission Yeast Centromere

Kohta Takahashi, Shin Murakami, Yuji Chikashige,
Hironori Funabiki, Osami Niwa, and Mitsuhiro Yanagida

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

Submitted April 27, 1992; Accepted June 8, 1992

Fission yeast centromeres vary in size but are organized in a similar fashion. Each consists of two distinct domains, namely, the ~15-kilobase (kb) central region (*cnt* + *imr*), containing chromosome-specific low copy number sequences, and 20- to 100-kb outer surrounding sequences (*otr*) with highly repetitive motifs common to all centromeres. The central region consists of an inner asymmetric sequence flanked by inverted repeats that exhibit strict identity with each other. Nucleotide changes in the left repeat are always accompanied with the same changes in the right. The chromatin structure of the central region is unusual. A nucleosomal nuclease digestion pattern formed on unstable plasmids but not on stable chromosome. DNase I hypersensitive sites correlate with the location of tRNA genes in the central region. Autonomously replicating sequences are also present in the central region. The behavior of truncated minichromosomes suggested that the central region is essential, but not sufficient, to confer transmission stability. A portion of the outer repetitive region is also required. A larger outer region is necessary to ensure correct meiotic behavior. Fluorescence in situ hybridization identified individual *cens*. In the interphase, they cluster near the nuclear periphery. The central sequence (*cnt* + *imr*) may play a role in positioning individual chromosomes within the nucleus, whereas the outer regions (*otr*) may interact with each other to form the higher-order complex structure.

INTRODUCTION

Eukaryotic chromosomes have distinct functional domains to ensure their stable maintenance and propagation, namely, replication origins, centromeres, and telomeres (e.g., Murray and Szostak, 1983, 1985; Blackburn and Szostak, 1984; Newlon, 1988). The centromere DNAs interact with proteins to form a complex structure called the kinetochore, the region responsible for association with microtubules and motor proteins that segregate the divided sister chromatids toward the spindle poles in mitosis (e.g., Mitchison, 1988). The kinetochores of vertebrate chromosomes are highly complex (e.g., Earnshaw, 1991; Earnshaw and Bernat, 1991). Mammalian centromere DNAs contain a large number of tandemly arranged repetitive sequences. The alphoid satellite DNA contains a 17-base pair (bp) repeat consensus that associates with a protein called CENP-B (e.g., Masumoto *et al.*, 1989; Wevrick *et al.* 1990; Willard,

1990). Other kinetochore proteins have been recognized by human autoimmune sera (Moroi *et al.*, 1980; Bernat *et al.*, 1991; Compton *et al.*, 1991; Yen *et al.*, 1991).

Functional centromeric DNAs have been isolated and characterized in two organisms, namely, the budding yeast *Saccharomyces cerevisiae* (Clarke and Carbon, 1985; Murray and Szostak, 1985; Newlon, 1988; Cotterel *et al.*, 1989), which has 16 chromosomes, and the fission yeast *Schizosaccharomyces pombe* (Clarke *et al.*, 1986; Nakaseko, *et al.*, 1986, 1987; Niwa *et al.*, 1986, 1989; Fishel *et al.*, 1988; Chikashige *et al.*, 1989; Hahnenberger *et al.*, 1989, 1991; Clarke and Baum, 1990; Matsumoto *et al.*, 1990; Murakami *et al.*, 1991), which has three. In budding yeast, a short stretch of DNA (~125 bp) containing a consensus sequences confer centromere function. Nucleotide substitutions in the consensus sequences in native and artificial chromosomes impair mitotic and meiotic chromosome behavior.

In contrast, fission yeast centromeres are large. The 35- to 110-kb long DNA, 300–900 times larger than budding yeast centromeres, is implicated in determining centromere function. Meiotic recombination within these regions is minimal (Nakaseko *et al.*, 1986; Chikashige *et al.*, 1989; Niwa *et al.*, 1989). Artificial linear or circular chromosomes (>30 kb) containing these sequences segregate normally in both mitosis and meiosis (Niwa *et al.*, 1986, 1989; Hahnenberger *et al.*, 1989, 1991; Clarke and Baum, 1990; Matsumoto *et al.*, 1990). Shorter (<10 kb) DNA fragments derived from *cen* regions, however, fail to confer centromere function (Nakaseko *et al.*, 1986).

The organization of the fission yeast centromeres (designated *cen*) is represented schematically in Figure 1(a). The outer repetitive (designated *otr*) domains consist of repeating elements common to all *cen*s. *otr* nucleotide sequences were reported previously; the dg element is indicated by filled arrows (Nakaseko *et al.*, 1986; similar to K, Clarke *et al.*, 1986), whereas the dh element is indicated by hatched arrows with and without the open box (Nakaseko *et al.*, 1987; Chikashige *et al.*, 1989; similar to L in Fishel *et al.*, 1988). dg and dh were defined by sequence determination, whereas K and L by restriction enzyme digestion analysis.

There are 2, 3, and 13 repeat motifs in *otr1*, *otr2*, and *otr3*, respectively (Chikashige *et al.*, 1989; Murakami *et al.*, 1991). They are invertedly arranged toward the center of each *cen*. Unexpectedly, the shortest chromosome III (Fan *et al.*, 1988) has the longest *otr* region. The repeat motif in each *otr* is composed of dg and dh sequences that have centromere specific fixed sizes and orientations (Figure 1(a)). For example, dg and dh are aligned in a tail-to-tail fashion in *otr1*, whereas they are head-to-tail in both *otr2* and *otr3* (Chikashige *et al.*, 1989; Murakami *et al.*, 1991). Most of the dh repeats in *otr3* are shorter than those in *otr1* and *otr2* (Murakami *et al.*, 1991). The boundary between dg and dh was defined by sequence comparisons (Nakaseko *et al.*, 1987; Chikashige *et al.*, 1989; Takahashi, Murakami, and Kinoshita, unpublished data).

We wanted to determine whether the central region is essential for centromere function. To address this question, we first determined its structure. Although previously studied by restriction mapping (Chikashige *et al.*, 1989; Hahnenberger *et al.*, 1989, 1991; Clarke and Baum, 1990; Murakami *et al.*, 1991; Polizzi and Clarke, 1991), little nucleotide sequence information of the central region has been available (Chikashige *et al.*, 1989; Kuhn *et al.*, 1991; Murakami *et al.*, 1991; Takahashi *et al.*, 1991). Previous data showed that the central region seemed to consist of two sequence classes, namely, *inmost* repeat (designated *imr*) and *center* (designated *cnt*) sequences. Restriction mapping suggested that each *cnt* is flanked by the inverted *imrs* (large open arrows in Figure 1(a)).

In the present study, we will show that the central region (*cnt* + *imr*) consists of low copy number sequences (part of which are unique). We determined the nucleotide sequence of *imrs* and *cnts* of all *cen*s and found a surprising symmetrical conservation in the *imrs*. This novel structural feature may be important for centromere function. The behavior of partially deleted minichromosomes showed that the central region (*cnt* + *imr*'s) is essential but not sufficient for *cen* function. Both the outer and central regions are required for proper mitotic chromosome disjunction. Fluorescence in situ hybridization (FISH) recently has been applied to fission yeast and used to locate the centromeres, telomeres, and nucleolar DNAs in interphase and mitotic cells (Uzawa and Yanagida, 1992). We show that the individual centromere DNAs can be identified and that they are clustered in the interphase nucleus. In the light of these structural, genetic, and cytological results, we propose a model for the functional organization of fission yeast centromeres and discuss its implication for higher eukaryotic centromeres.

MATERIALS AND METHODS

Strains and Media

Haploid-wild type *S. pombe* strain 972 *h*⁻ was used as the standard strain. *S. pombe* variant strains 267 (UCD-FST1; isolated in Australia) and 270 (CBS5680; isolated in Poland) were described in Zimmer *et al.*, (1987) and Levin *et al.* (1990). We also used five other *S. pombe* wild-type strains (EF4, 0208, 0209, CSIR Y-830, NCYC132) (Zimmer *et al.*, 1987) for genomic Southern hybridization probed with pRS138, pKT110, pKT104, and pSS163 (see Figure 1(b)). Intense hybridization bands were obtained, suggesting that these natural variants also contain centromeres similar to those reported here. The strain used for nuclear isolation and nuclease digestion experiments was TM003 (*h*⁻ *leu1 end1*). This strain lacks a major endonuclease (Uemura and Yanagida, 1984) and is suitable for preparation of intact chromatin. *S. pombe* media and culture conditions were previously described (Chikashige *et al.*, 1989).

Plasmids and Cosmids

The following plasmids were used. pKT100 contained a 3.5-kilobase (kb) *EcoRV*-*EcoRI* fragment from *cen1*. Subcloning of pKT100 by *HindIII* and *Sph I* digestions yielded a series of plasmids pKT101–pKT105. pRS138 and pYC101, respectively, contained 1.4-kb *EcoRI* and 5.2-kb *cen1* *EcoRI* fragments. pKT110, 120, 130, and 140 were obtained by subcloning of pYC101 with *HindIII* and *EcoRI* digestions. pYC111 and pYC113 and pYC116, respectively, contained 3.0-, 4.0-, and 5.4-kb *cen2* *HindIII* fragments. pTM5 (5.0 kb *HindIII*), pMU24 (0.3 kb *HindIII*), pMU30 (0.6 kb *HindIII*), pMU10 (3.5 kb *HindIII*-*EcoRI*), pMU35 (1.8 kb *EcoRI*-*Cla I*), and pMU22 (0.4 kb *Cla I*) contained the *cen3* fragments. The vector plasmids used for pRS138 and pYC116 were pUC18 or pUC19 (Yanisch-Perron *et al.*, 1985), whereas pYC12 (Chikashige *et al.*, 1989) was used for pMU10. Bluescript KS(+) (Stratagene, La Jolla, CA) was the vector for the other plasmids, such as pYC14 and pYC11, that contain the *S. pombe* *sup3-5* and the *S. cerevisiae* *LEU2* genes, respectively, as markers. Cosmids Z5 and cos35-3 were previously described (Chikashige *et al.*, 1989; Murakami *et al.*, 1991). Cosmids W1 and Y19 were isolated in the present study by screening an *S. pombe* cosmid library using centromeric sequences pSS107 (Nakaseko *et al.*, 1986) and pCA5 (Chikashige *et al.*, 1989), respectively, as probes.

Primers, Genomic DNA, Hybridization, and Sequence Determination

The following primers were synthesized for cloning using the polymerase chain reaction (PCR) method. They were designated P1–P8.

- P1, 5'-CGATTTCTCAGAATAAGCTT-3';
- P2, TAATAAACAGAGTTGAATTC;
- P3, TCTAGAATAACTGTTGTATACTA;
- P4, AGGAATTCAAGGCGATTGAAATCC;
- P5, CGAAGCTTATACTAAATAAGACC;
- P6, AAAAGCTTTCGATAATTTGAGCTT;
- P7, CCAAGCTTTGACCGACTTCTACA;
- P8, GGCTCGAGACTTTGAGGATGTTGT.

P1–P3 were the sequences used for cloning the boundary regions between the *imr* and *cnt* sequences in *cen1*. P4–P6 were used for cloning the ends of the tm sequences present in the *cnt* regions of *cen1* and *cen3*. P7 and P8 were used for cloning the 5' upstream sequences of the *top2*⁺ gene (Uemura *et al.*, 1986; Shiozaki and Yanagida, 1991). Genomic DNAs were obtained from *S. pombe* strains 972, 267, and 270 by the potassium acetate method (Rothstein, 1985). Southern hybridization was performed using standard procedures (Maniatis *et al.*, 1982) with modifications (Nakaseko *et al.*, 1986). Hybridization was done in 6× SSC, 5% dextran sulfate, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml salmon sperm DNA at 65°C.

The dideoxy method (Sanger *et al.*, 1977) was used for nucleotide sequencing with unidirectional progressive deletion (Yanisch-Perron *et al.*, 1985). DNA fragments obtained by the PCR method were treated with Klenow fragment for 30 min, cleaved with the restriction enzyme for the primer sequence, and run in agarose gel electrophoresis. The agarose block was cut from the band with the expected fragment size and cloned into Bluescript for sequence determination.

Construction of Minichromosomes

Circular minichromosomes CM2104 and CM3112 were previously made by the gap repair method (Matsumoto *et al.*, 1990). To apply the gap repair method for other centromere regions, pEX40 and pEX60, which were similar to pEX20 (Matsumoto *et al.*, 1990), were made. The insert in pEX40 contained the budding yeast *LEU2* gene flanked with the inverted *Bam*HI–*Hind*III fragments of *dhI*, and by using pEX40 for gap repair, CM4130 was obtained. pEX60 contained the *Cla*I–*Hpa*I in the left *imr*IL that was ligated with *Hpa*I–*Bam*HI in the right *otr*1R. CM6137 was obtained by using pEX50. For transformation of *S. pombe*, pEX40 and pEX60 were linearized with *Bam*HI and *Hpa*I, respectively.

Circular minichromosomes RMCdel23, RMCdel37, and RMCdel1 containing deletions in *imr1* were made by exonuclease III–partial digestion of the *Cla*I side of *Cla*I–*Bgl*II fragment, followed by ligation with 11.3-kb *Bgl*II–*Bam*HI fragment. This procedure was necessary because the large inverted DNA segments are hardly replicated in *Escherichia coli*. The ligated DNAs with the marker gene *sup3-5* in Bluescript KS(+) were directly used for transformation of *S. pombe*, and stable *Ade*⁺ transformants were obtained for RMCdel23 and RMCdel37. For RMCdel1, no stable transformant of *S. pombe* was obtained. However, the *in vitro* ligated DNA could replicate in *E. coli* so that the stability of RMCdel1 could be examined in *S. pombe*. pKT23 and pKT21 containing 11.3 kb *Bgl*II–*Bam*HI and 8.8 kb *Eco*RI–*Bam*HI, respectively, were made using the Bluescript KS(+) with *sup3-5* as the vector and used for transformation of *S. pombe*. The host strain used for transformation was HM422 (*h*[–] *leu1* *lys1* *ade6-704*). *Ade*⁺ transformants were obtained (Matsumoto *et al.*, 1990). Transformants that appeared to carry minichromosomes were analyzed by pulsed field gel (PFGE) electrophoresis, and minichromosomes with the expected sizes were detected. Minichromosomes were examined by *Not*

I digestion followed by the partial digestion mapping technique (Chikashige *et al.*, 1989) to determine their structure.

For construction of DC3600 and DC3640, one step gene replacement (Rothstein, 1983) was used for the stable minichromosome CM3112 using linearized *cen3* DNA fragments (*Pst*I–*Nhe*I in *imr3R* for DC3600 and *Sph*I–*Pst*I spanning *cnt3* and a proximal part of *imr3R* for DC3640) interrupted with *ura4*⁺ as the marker. The linearized DNAs were used for transformation of an *S. pombe* host strain (*h*[–] *leu1* *ade6-704* *ura4*) containing CM3112. *Ura*⁺ and *Ade*⁺ transformants were obtained. Cosegregation of *Ura*⁺ *Ade*⁺ markers was tested by streaking transformants on plates. Mitotic minichromosome stability was determined according to Matsumoto *et al.*, (1990).

Micrococcal Nuclease Digestion

The procedures described previously (Chikashige *et al.*, 1989) were used with modifications. Exponentially growing *S. pombe* cells were harvested and treated with Zymolyase-100T (0.2 mg/ml without EDTA; Seikagaku Kogyo, Japan) at 36°C for 20 min. Spheroplasts were resuspended in the SCP buffer (20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [PIPES] at pH 6.3, 0.1 mM CaCl₂, 1 M sorbitol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and mixed with 40 volumes of the lysis buffer (20 mM PIPES, 0.1 mM CaCl₂, 9% Ficoll, and 1 mM PMSF), followed by gentle shaking. Lysed spheroplasts were centrifuged at 10 000 rpm for 5 min, and the pellets containing the nuclei were resuspended in SCP buffer at a cell concentration equivalent to 1–2 × 10⁶/ml. Cells were prewarmed at 36°C for 2 min and then treated with micrococcal nuclease (MNase) (500 unit/ml; Worthington Biochem, Freehold, NJ) at 36°C for 1–20 min. The reaction was stopped by adding EDTA (to a final concentration of 50 mM). The suspension was centrifuged at 12 000 rpm for 20 min. The pellets contained unbroken cells and large aggregates of mitochondria but did not contain the nuclei that had been digested with nuclease, judging from the results of Southern hybridization of DNAs from the pellets. The supernatant containing the nuclease-digested nuclei was mixed with proteinase buffer (final concentration, 50 mM tris(hydroxymethyl)aminomethane-HCl, 0.3 M NaCl, 1% SDS, 50 mM EDTA and 0.1 mg/ml Proteinase K at pH 7.5) and incubated at 50°C for 4 h. DNA was extracted by phenol, precipitated with ethanol, and contaminating RNA was digested with RNase. Electrophoresis was carried out in 1.5% agarose.

DNase I Digestion

The procedures similar to those described for MNase digestion were used. The SMP buffer (20 mM PIPES at pH 6.3, containing 5 mM MgCl₂, 1 M sorbitol, and 1 mM PMSF) was substituted for SCP buffer. The lysis buffer was 20 mM PIPES, 5 mM MgCl₂, 9% Ficoll 400, and 1 mM PMSF.

FISH and Immunofluorescence Microscopy

The digoxigenin-labeled centromeric DNA probe (pRS140; Chikashige *et al.*, 1989) was used for FISH. The procedures previously described (Uzawa and Yanagida, 1992) were used with modifications (H. Funabiki and M. Yanagida, unpublished data). Fluorescence microscopy using anti-tubulin antibody TAT1 (Woods *et al.*, 1989) was performed as described (Uzawa and Yanagida, 1992).

RESULTS

Isolation of Inner Centromere DNAs

We isolated cosmid clones covering the central regions of *S. pombe* centromeres by chromosome walking (Chikashige *et al.*, 1989; Murakami *et al.*, 1991; this study, MATERIALS AND METHODS). Representative clones are shown in Figure 1(b) (Z5, Y19 for *cen1*, W1 for *cen2*,

centromere function (see below). No sequences encoding a putative protein have been found in this region. Sequences similar to the budding yeast centromere consensus sequences CDEI, II, and III are not present in the central regions.

The asymmetric *cnt1* and *cnt3* sequences contained a 3.3-kb long highly homologous segment (designated tm1 and tm3, respectively; indicated by the hatched boxes in Figure 1(b)). The tm-like sequence, however, is not present in *cnt2*, as determined by the nucleotide sequence of *cnt2* and Southern hybridization patterns of cosmids containing *cnt2*. Note that a small portion of tm3 is duplicated and located with inverted symmetry at the end of *imr3L*. A 20-bp AT-rich palindrome TTTTACAATATTGTAAAAA is present toward the right end of tm.

Vertical lines in Figure 1(a) (and the thin arrowheads in Figure 1(b)) show the localization of the tRNA genes (the respective amino acids are indicated by the single letter code) (Kuhn *et al.*, 1991; Takahashi *et al.*, 1991). They are found within the central regions and outside the outer repeats but never within the *otr* repeats. No particular tRNA gene recurred in all the *cen* areas, but several were common to two of the three *cen*s. The role these tRNA genes are playing remains obscure. The previously reported nk sequences (Nakaseko *et al.*, 1986) were found to encode Ile-tRNA (Takahashi *et al.*, 1991). They were completely identical and known to be present in around 10 copies in the entire genome. Many are located in noncentromere areas (Nakaseko *et al.*, 1986; Kinoshita, Nakaseko, and Yanagida, unpublished data).

Structure of the Central Regions of *cen1*, *cen2*, and *cen3*

Figure 2 shows an ~11-kb long nucleotide sequence of the central *cen1* region. The end of an outer repetitive element *otr1L* (boxed, the direction of a repeat unit dG indicated by the arrow) is followed by the left inverted repeat (*imr1L*, ~5.6 kb), a center sequence (boxed; *cnt1*,

~4.1 kb), and a part of the right inverted unit (*imr1R*, ~1.3 kb). The tRNA-encoding sequences are also boxed with the direction of putative transcription and the name of amino acids (Ala, Glu, and Ile) indicated in each case. d1, d23, and d37 are the sites for deletion mutants of minichromosomes (see Table 1). The sequences with the lines are compared below. The tm1 sequence is defined by the arrows with the broken lines. A part of the *cnt1* sequence after the vertical line is duplicated in *cen3*. Sequencing data for the central regions of *cen1*, *cen2*, and *cen3* were deposited in EMBL/GenBank under accession numbers X66738 through X66746. Nucleotide sequence organization in the central regions of *cen2* and *cen3* were similar to *cen1* as shown in Figure 1(b).

To determine whether central sequences were repetitive or unique, Southern hybridization was done under stringent conditions, using probes derived from different parts of the central *cen1* region. The results are shown in Figure 3 (the probes are indicated by the open boxes. E and H represent *EcoRI* and *HindIII*, respectively). The probe used in (a) contains the highly repetitive sequence dG1 so that intensely hybridizing bands (Nakaseko *et al.*, 1986) were obtained within a short exposure time. The probes (b) and (g), containing tRNA-encoding sequences (indicated by the arrowheads), revealed weak bands that are reiterated roughly 10 times. However, the probes (c)–(f) in *imr1* and (h)–(i) in *cnt1* displayed only one and two hybridizing bands, respectively, even after a long exposure time; the two hybridization bands in (h)–(i) represent tm1 and tm3. The sizes of the restriction fragments were consistent with the prediction that only the two sequences (*imr1L/imr1R* or *cnt1/cnt3*), respectively, were detected with these probes.

To estimate the degree of sequence homology between *imr1L* and *imr1R*, their sequences in the wild-type 972 genomic DNA were compared, as shown in Figure 4(a) (identical nucleotides are indicated by the asterisks). Surprisingly, the sequences were 100% identical at least in the 1259 bp long area determined (only a portion of the determined sequences is shown). The

Table 1. Sequence comparison among three *S. pombe* strains

Sequence	Strains	Length (bp)	Difference from strain 972				Change (%)
			Substitution	Insertion	Deletion	Sum	
<i>imr1L</i>	267	721	12	3	0	15	2.1
<i>imr1L</i>	270	719	10	1	0	11	1.5
<i>cnt1</i>	267	278	4	0	0	4	1.4
<i>cnt1</i>	270	640	10	0	2	12	1.9
5'- <i>top2</i>	270	485	5	1	3	9	1.8

DNA clones were obtained by the PCR method from the two wild-type strains 267 and 270, using the primer sequences of the standard wild-type 972 (see MATERIALS AND METHODS). The DNA sequences cloned from 267 and 270 were determined and compared with those from 972. The sequence for *top2*⁺ was derived from the 5'-upstream region of the *top2*⁺ gene (pUCTOP2). Nucleotides altered from those in the strain 972 were scored. Change, the number percentage of the altered nucleotides.

[illegible]

Figure 3 Genomic Southern hybridization probed with fragments in the central *cen1* region. *S. pombe* genomic DNA was isolated, digested with *EcoRI* or *HindIII* nucleases, run in agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with ^{32}P -labeled probes (a)–(i) derived from the central *cen1* (their locations are indicated by the open boxes). Probe (a) was derived from the outer region *otr1*, whereas the others were derived from the central *cen1*. B, *Bam*HI; S, *Sph* I; H, *Hind*III; E, *Eco*RI.

identity abruptly ends at the point of transition into *cnt1* (indicated by the bent arrow). The same situation was found in the *imrs* of *cen2* and *cen3*; the sequenced regions of *imr2L* and *imr2R* and the *imr3L* and *imr3R* were completely identical. As with *cen1*, the loss of homology on the transition to *cnt2* is abrupt; however, that into *cnt3* is gradual (c). The *imr* sequences are in the same chromosomes but 4–7 kb apart.

Comparing the sequenced region of *tm3* (Figure 1(b)) with *tm1* showed they are highly similar (Figure 4(d)). This high degree of conservation is unexpected because *tm1* and *tm3* are found in different chromosomes. It is of interest to determine what kind of constraint exists for maintaining such homology. Note that the decrease of homology between *tm1* and *tm3* was gradual at the left end but abrupt at the right end.

Symmetrically Conservative Changes in imr1 in S. pombe Variant Strains

The question arises as to whether the 100% identity between *imrL* and *imrR* in each *cen* is essential for centromere function. Are any mutations in *imr* tolerated for normal chromosome segregation? As an initial at-

tempt to answer this question, we examined whether the nucleotide sequences of *imrs* derived from fission yeast natural variants were identical to that of a standard laboratory strain 972. We isolated ~500 bp sequences corresponding to the inmost regions of *imr1L* and *imr1R* by the PCR method from the two strains 267 and 270 isolated in Australia and Poland, respectively (see MATERIALS AND METHODS). Genomic Southern hybridization using *otr* and *imr* as probes indicated that the centromeres are highly conserved in these natural variants. Two unique primers from *cnt1* and one repetitive one from *imr1* (see MATERIALS AND METHODS) were made according to the sequence of the standard laboratory strain 972. Sequences of the PCR clones isolated from strains 267 and 270 with these primers are shown in Figure 4(a) (only those nucleotides differing from 972 are shown). For the strains 267 and 270, respectively, six (267) and five (270) substitutions plus two (267) and one (270) insertions were found in the *imr1L* (the upper sequence). The same changes also occurred at the corresponding positions in *imr1R*. Nucleotide changes also occurred within the central *cnt1* region. These results indicate that mutations are tolerated in the *imr* regions but only in a concerted fashion.

Figure 2 The nucleotide sequence of the central *cen1* region. An ~11-kb-long nucleotide sequence spanning the central region of *cen1* is shown. From the top, the boxed sequences are the end of *otr1* (a part of *dgI*) (Nakaseko *et al.*, 1986), three tRNA genes (Ala, Glu, and Ile) (Takahashi *et al.*, 1991), the *cnt1* (large box), and the inverted Ile tRNA gene. The sequences within the lines are compared in Figure 4. The *tm* sequence is defined by the broken arrows in the large box. The short vertical line within in the box indicates the boundary for the duplicated in *cen3*.

Figure 5 Micrococcal nuclease digestion of the central *cen1* region. (a) and (b) *S. pombe* nuclei were isolated and subjected to MNase digestion for differing durations (0 ~ 20 min). DNAs were extracted, run in agarose gel electrophoresis, and hybridized with the ^{32}P -labeled *cen1* probes C, D, F, G, and J (indicated by the boxes). The resulting Southern hybridization patterns are shown in (b). Ethidium bromide (EtBr) staining patterns are shown on the left. The concentration of nuclease used was 500 units/ml. The time for incubation was 0, 1, 2, 5, and 20 min for lanes 1–5, respectively. The probes used were C, pYC148 (*HindIII*-*EcoRI*); E, pKT108 (*Sph* I-*HindIII*); F, pKT105 (*HindIII*-*EcoRI*); G, pYC151 (*HindIII*-*EcoRI*); and J, pYC130 (*Bgl* II-*HindIII*). Only probe C, derived from *otr1*, displayed the ladder-like nucleosomal pattern. (c) Nuclei were isolated from cells transformed with multicopy plasmid pYC310 or pYC306 covering the region of *imr1* or *cnt1*, respectively (indicated in (a)), were used for MNase digestion. Southern hybridization was carried out using the probes D and J. Ethidium bromide staining patterns are also shown on the left. Because the copy number of plasmid was high (~50), the observed ladder-like patterns were presumed to be mostly due to the chromatin on the plasmid, contributions by genomic DNA should be negligible. The nuclease was used at 250 units/ml.

To determine whether the observed rate of change in *imr* was comparable with that in other areas of the chromosome, we isolated portions of *cnt1* and the 5' upstream sequence of DNA topoisomerase II gene (designated *top2*⁺) (Uemura *et al.*, 1986; Shiozaki and Yanagida, 1991) from strains 267 and 270 by the PCR method. Comparison of the nucleotide sequences of

these fragments from these three strains showed that the average rates of changes in *imr1* were 1.5 ~ 2.5%, which was similar to the 1.4 ~ 1.9% obtained for *cnt1* and *top2*⁺ (Table 1). This suggests that the rate of change in the sequence of *imr1* may be as frequent as in the other areas of the *S. pombe* genome. Hence, we concluded that the sequence symmetry between *imr1L* and

a part of pYC116 containing the end of *imr2R*). (c) Two sequences containing the proximal ends of *imr3L* and *imr3R* are shown (above, a part of pTM5 containing the end of *imr3L*; below, a part of pMU30 containing the end of *imr3R*). The transitional loss of sequence identity in *cen3* was not sharp as in *cen1* and *cen2*. (d) Two sequences containing both ends of *tm1* and *tm3* (above, a part of pYC101; below, a part of pTM5 and pMU30). The right end of *tm* sequence identity ends abruptly, whereas at the left end the identity gradually disappears. Although the two sequences shown here are 100% identical, sequencing of the internal region of *tm3* is not complete so that it is not known whether the internal regions are also 100% identical.

imr1R is strictly maintained despite a normal frequency of mutations occurring within the *imr1* regions.

Chromatin in *imr* and *cnt* is Nonnucleosomal

We have shown previously by MNase digestion of isolated nuclei followed by Southern hybridization that the outer repetitive *otr* regions contain regular nucleosomal chromatin (Chikashige *et al.*, 1989). This experiment was extended to the central *cen* regions using the probes from *imr1* and *cnt1* (the probes D, F, G, and J in Figure 5(a)). When hybridized with these probes, the digestion patterns of central *cen* DNA were broad and smeared (Figure 5(b)). This was in marked contrast to the patterns obtained with the outer repetitive region (probe C) and ethidium bromide staining (left end) that gave typical nucleosomal ladders. These results are similar to those of Polizzi and Clarke (1991). The rate of nuclease digestion was similar for *otr*, *imr*, and *cnt*.

To obtain more information about the nonnucleosomal nature of the chromatin in the central region, the following experiment was done. We examined the MNase digestion patterns of chromatin on plasmids pYC310 and pYC306 that carried the probes D and J (Figure 5(a)). These isolated sequences do not support centromere function when combined with an autonomously replicating sequence (ARS); instead, the resulting plasmids behave as unstable multicopy plasmids. As shown in Figure 5(c), nucleosome ladder-like patterns were found on the *imr1* and *cnt1* sequences when contained on these plasmids. Therefore, the central *cen* DNA sequences may be able to form regular nucleosome structure, but the ability is suppressed by the environment in normal chromosomes.

DNase I Hypersensitive Sites within the Central *cen* Regions

Isolated nuclei were partially digested by a dilution series of DNase I in a manner analogous to that used with MNase (see Materials and Methods). Then DNAs were subsequently extracted, digested with restriction enzymes, separated by agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with centromeric DNA probes to identify and determine the DNase I hypersensitive sites. *imr1* probes D and F (Figure 6) were used in combination with the restriction enzymes *EcoRI* and *Sph I* (indicated by E and S). By increasing the DNase I concentration (lanes 1–5 and 8–12), two bands at 2.3 and 2.7 kb increased in intensity for probe F (left), whereas a 2.1-kb band appeared for probe D (right). Interestingly, the location of these DNase I hypersensitive sites (indicated by small arrowheads) exactly correlates with that of the tRNA-like genes in *imr1* (top). No other region in the *imr1* exhibited DNase I hypersensitivity.

Similar analysis of the entire central region of *cen3* using PFG electrophoresis identified DNase I hypersen-

Figure 6 DNase I hypersensitive sites in the central regions. *S. pombe* nuclei were isolated and digested with DNase I (Worthington Biochem; 0 ~ 4 µg/ml at 36°C for 5 min; lanes 1–5 and 8–12) or *Nco I* (lane 6), *Sph I* (lane 7), or *EcoRI* (lane 8). DNAs were extracted, completely digested with *EcoRI* (lanes 1–7) or *Sph I* (lanes 8–13), run in agarose gel electrophoresis, and hybridized with ³²P-labeled probes D (lanes 1–7) or F (lanes 8–13). The restriction sites within *imr1L* are as indicated; E, *EcoRI*; S, *Sph I*; N, *Nco I*. The sizes of the respective restriction fragments are indicated beneath the sites. The small arrowheads with numbers indicate DNase I hypersensitive sites and the size of fragments generated. The location of the tRNA genes are indicated by the large arrowheads. The concentrations of nuclease used were 0 (lanes 1 and 8), 0.5 (lanes 2 and 9), 1 (lanes 3 and 10), 2 (lanes 4 and 11), and 4 µg/ml (lanes 5 and 12). For a reference hybridization band, total genomic DNA was digested with *Nco I*/*EcoRI* (lane 6) and *Sph I*/*EcoRI* (lanes 7 and 13); the restriction fragments with the sizes expected from the map were obtained.

sitive sites in close association with the tRNA-encoding sequences but not in the outer repetitive regions. In contrast, micrococcal nuclease digestion did not show clear hypersensitive sites in the central *cen* regions.

Location of ARS Sequences within the Central Regions

To identify centromeric regions possessing ARS activity and thus being likely to act as origins of DNA replication, a series of DNA fragments derived from the entire *cen1* (Figure 7, top) were inserted into the vector pYC11 that contains the budding yeast *LEU2* marker gene but no ARS to enable propagation in *S. pombe*. The resulting plasmids were transformed into *S. pombe*, and the fre-

quencies of transformation were measured (Table 2). Plasmid 26 contained the authentic *S. pombe* ARS (ARS1) (Maundrell *et al.*, 1988). Judging from the frequency of transformation, putative ARS activity is abundant throughout the central region, one being apparently present in each of *otr1*, *imr1*, and *cnt1*. Consistently, *S. pombe* ARS consensus sequences were found at A, B, and C (Figure 7, see legend). The transformation frequency of 310Sc17 and 306Sc15 was particularly high. To confirm that these plasmids with ARS activity were propagated in a monomeric form, Southern hybridization membranes of transformant DNAs were probed with vector sequences (Figure 7, bottom). P indicates the purified monomeric plasmid DNA from *E. coli*, whereas T represents plasmids recovered from *S. pombe* transformants. Plasmids pYC310, pYC310Sc17, pYC312, pYC306, and pYC306Sc15 were indeed monomeric (indicated by arrows). In this transformation experiment, the control plasmid 26 was propagated as a putative dimer rather than a monomer (see legend).

Central Regions are Essential for Proper Chromosome Segregation

We constructed a series of circular minichromosomes (Figure 8(a)) that contained deletions of various parts

of *cen1* (see MATERIALS AND METHODS). Briefly, CM2104, CM4130, and CM6137, which contain the complete central region with a portion of the outer region, were constructed by the gap-filling method using the vectors pEX20 (Matsumoto *et al.*, 1990), pEX40, and pEX60 made in the present study. These behaved as relatively stable minichromosomes (indicated by ++ or +; see also Table 3). The repair of plasmids gapped at different sites within centromere regions has generated a number of stable circular minichromosomes (Matsumoto *et al.*, 1990). We have failed to obtain minichromosomes missing one of the two *imrs* by gap-filling method. This is consistent with the fact that such a structure made in *E. coli* was very unstable in *S. pombe*. The fidelity of gap repair was high, because the minichromosomes obtained in both the present and previous studies produced the expected Southern hybridization restriction digest patterns.

RMCdel23, RMCdel37, and RMCdel1, deleting a part of *imr1L* but containing one complete *dgI* repeat in *otr1R*, were constructed by in vitro ligation of two DNA fragments and introduced into *S. pombe* cells (see MATERIALS AND METHODS). Deletions in the *imr1L* were obtained by exonuclease III digestion, and the sites of deletions being verified by nucleotide sequencing (in-

Figure 7 ARS sequences in the central *cen1* region. A series of DNA fragments derived from central *cen1* region were inserted into pYC11, a derivative of the vector Bluescript, containing the *S. cerevisiae* *LEU2* as the marker. The resulting plasmids were used for transformation of host *S. pombe* strain *h⁻ leu1*. A high frequency transformation (>100 per μ g DNA) was obtained for a number of clones (indicated by the hatched boxes). The transformation frequency with vector alone was <0.1 per μ g DNA. Only four plasmids produced low frequencies of transformation. The central region is quite unusual, because an ARS fragment is present at an average of every ~20 kb in the *S. pombe* genome. A, B, and C represent the nucleotide sequences homologous with the *S. pombe* ARS consensus sequence (5'-T/ARTT-TATTTAA/T-3') (Maundrell *et al.*, 1988); A, 5'-TATTTATTTAA-3'; B, 5'-TTAAATAAACA-3'; C, 5'-TATTTATTTAT-3'. Plasmid DNAs recovered from transformant cells were run in agarose gel electrophoresis and hybridized with the vector sequence (indicated by T; as a control, purified supercoiled plasmids from *E. coli* were run as indicated by P for each plasmid). For plasmid 310, 312, 306, 310Sc17, and 306Sc15, the monomeric supercoil plasmids were present in the transformant cells. However, other plasmids with a high frequency transformation existed as the presumed dimeric form. In this series of experiments, the control plasmid 26 containing ARS was also present as the presumed dimer in transformants.

Table 2. High-frequency transformation by subfragments of *imr1* and *cnt1*

Plasmids	Number of transformants per μg DNA			
	Exp1	Exp2	Exp3	Exp4
26 (ARS1)	350	293	222	191
301	191	236	108	
307	0	0	0	
313	11	7	6	
308	194	42	12	92
315	131	151	14	53
303	258	220	186	
314	0	0	2	1
304	59	9	12	
310	482	133	246	
312	237	156	92	
306	472	649	176	
310Sc17	208	576	140	515
306Sc15			174	474

Chromosomal location of the inserts for each plasmid is shown in Figure 7. Most plasmids except 304, 307, 313, and 314 produced high frequency of transformation (>100 per μg DNA). Plasmid 26 contains ARS1 (Maundrell *et al.*, 1988).

icated in Figure 2). The mitotic stability of RMCdel37 and RMCdel1, which only differed by the deletion of a 1.8-kb-long sequence from *imr1L* in RMCdel1, was strikingly different (Table 3). pKT23 and 21 were made by directly subcloning the fragments into the vector pYC14.

The stability of these circular minichromosomes was assessed by colony color (Hieter *et al.*, 1985; Koshland

et al., 1985; Niwa *et al.*, 1989) and tetrad analysis (Table 3). RMCdel1, which lacks a large portion of *imr1L*, was highly unstable: its stability was comparable with constructs containing larger deletions or the control ARS plasmid. However, RMCdel37, which contained a proximal half of *imr1L*, exhibited a strikingly improved stability, indistinguishable from that of RMCdel23 or CM6137, which contain most or all of *imr1L*. This suggested that the entire *imr* may not be necessary to confer stability but that the proximal sequence of *imr* or a certain length of exact inverted homology is important for centromere function. In meiosis, the ratio of first division segregation/second division segregation was 10/25 for RMCdel37 and 17/65 for RMCdel23, suggesting that despite abundant precocious sister chromatid separation in meiosis I, they behaved as chromosomes.

Two minichromosomes DC3600 and DC3640 containing internal deletions in *cen3* (Figure 8(b)) were made by one step gene replacement in the stable minichromosome CM3112 (Matsumoto *et al.*, 1990); the deleted portions are indicated by a broken line and the 1.8-kb *ura4⁺* marker gene by a hatched box. DC3600, lacking a central to distal portion of the *imr3R*, was stable (Table 3), consistent with the above results. On the other hand, DC3640, containing a 4.1-kb deletion proximal to *imr3* and *cnt3*, was highly unstable. Structure of DC3600 and DC3640 was verified by partial digestion and Southern hybridization (Matsumoto *et al.*, 1990).

Fluorescence In Situ Hybridization Visualization of Individual *cents*

We have shown previously that the FISH method can be successfully applied to localize fission yeast chro-

Figure 8 Construction of minichromosomes deleting various parts of central regions in *cen1* and *cen3*. (a) The gap repair method (Matsumoto *et al.*, 1990) was used for construction of the circular minichromosomes CM4130, CM2104, and CM6137. They were maintained in *S. pombe*. RMC circular minichromosomes were made by in vitro ligation of the left and right arms prepared in *E. coli* (see MATERIALS AND METHODS). pKT23 and pKT21 were subcloned from cosmids. Their mitotic stability (MT) is represented by ++ (highly stable), + (stable), and - (unstable). Stable minichromosomes are represented by solid thick bars. Quantitative data on mitotic stability and meiotic segregation pattern for these circular DNAs are shown in Table 3. The following abbreviations are used to denote restriction sites; B, *Bam*HI; Bg, *Bgl* II; C, *Cla* I; E, *Eco*RI; H, *Hind*III; Nh, *Nhe* I; P, *Pst* I; S, *Sph* I. (b) Internal deletions of the central *cen3* were made by the one step gene displacement on the circular minichromosome CM3112 (Matsumoto *et al.*, 1990). The disrupted sequence was displaced by the *S. pombe ura4⁺* gene (indicated by hatched boxes). DC3600 was stable, whereas DC3640 was unstable.

Table 3. Mitotic stability and meiotic segregation of circular minichromosomes harboring various deletions

Cen	Minichromosomes	Length (kb)	Cells with minichromosomes ^a (%)	Loss per division ^b (%)	Ade ⁺ :Ade ⁻					Tetrad ratios	
					0:4	1:3	2:2	3:1	4:0	FDS	SDS
<i>cen1</i>	CM4130	30.0	95.1	1.7	14	8	73	1	0	34/73	39/73
	CM2104	20.5	70.5	12.6	33	6	40	6	1	7/40	33/40
	CM6137	23.5	84.9	8.6	42	18	82	9	0	17/82	65/82
	RMCdel23	22.0	86.6	8.4	30	4	35	2	0	10/35	25/35
	RMCdel37	20.5	86.9	10.0	14	3	18	2	1	4/18	14/18
	RMCdel1	19.0	14.3	ND				ND			
	pKT23	15.0	13.3	ND				ND			
	pKT21	12.5	17.5	ND				ND			
<i>cen3</i>	CM3112	36.0	95.6	0.8	18	3	55	0	0	48/55	7/55
	DC3600	35.0	98.3	2.0				ND			
	DC3640	32.0	7.0	ND				ND			

Deletions in the minichromosomes are depicted in Figure 8. The length of centromeric DNA in the minichromosome is indicated in kilobases. ND, not determined because the minichromosomes were too unstable to accurately determine these values; FDS, first division segregation; SDS, second division segregation.

^a Percent of cells carrying the minichromosome in a selective medium (see Matsumoto *et al.*, 1990).

^b Rate of minichromosome loss per division calculated by the method described in Matsumoto *et al.* (1990).

mosomal DNAs (Uzawa and Yanagida, 1992). The centromere DNA was visualized with a probe from the outer repeats and was found to partition to the nuclear periphery of interphase cells and to the middle of the spindle in the mitotic cells. However, only *cen3* was reproducibly visualized using this approach, perhaps due to the fact that it has the longest outer repeats.

By the use of longer probes, we can now observe individual *cens*. Three condensed chromosomes are seen in *nda3-311* mutant cells (Hiraoka *et al.*, 1984), and, as shown in Figure 9a, each *cen* is seen as a dot on the chromosome (upper, DAPI stain for chromosomal DNA; lower, FISH for *otr* repeat). The smallest chromosome III showed the most intense dot, consistent with *cen3* organization. Note that the size of *otr3* is ~100 kb; if fully extended, this would correspond to ~33 μ m. However, it is compacted into ~0.3 μ m in condensed chromosomes, approximately equivalent to 100-fold compaction. The *S. pombe* condensed chromosomes failed to show the two dots of replicated *cens* observed when using FISH alphoid probes for mammalian mitotic chromosomes. The *S. pombe* *cens* might be too small to be resolved into two.

In the interphase nucleus, the *cens* were found to cluster and, as previously reported (Uzawa and Yanagida, 1992), to partition to nuclear periphery (Figure 9b; upper, DAPI; lower, FISH). Two hundred interphase cells showing in situ hybridization signals were examined, and 94% of the cells clearly showed the clustered centromeres. Three closely situated centromeres were resolved in approximately one-half of them, and the rest of them showed a single clustered centromeres with intense hybridization signals. By triple staining (DAPI, FISH, and anti-tubulin) (Uzawa and Yanagida, 1992)

interphase cells, these *cens* were found to be always located near to cytoplasmic microtubules that run close to the nuclear periphery (Figure 9c; top, DAPI; middle, FISH; bottom, anti-tubulin). Thus, the interphase *cens* might be closely associated with the spindle pole body (SPB), because their location resembles that the SPB (Figure 9(d)) (Tanaka and Kanbe, 1986; Hagan and Hyams, 1988; Horio *et al.*, 1991). In contrast, mitotic cells show the centromeres locating at the middle of short spindle (Figure 9(d)) (Uzawa and Yanagida, 1992).

DISCUSSION

In the present study, we determined ~30 kb of the nucleotide sequences of the central domains of the fission yeast centromeres *cen1*, *cen2*, and *cen3*. Sequences for the outer repetitive areas were reported previously (Nakaseko *et al.*, 1986, 1987; Chikashige *et al.*, 1989) as was restriction site information (Chikashige *et al.*, 1989; Clarke and Baum, 1990; Hahnenberger *et al.*, 1991; Murakami *et al.*, 1991). Hence, the total of ~60 kb of nucleotide sequences so far determined has uncovered the complex sequence structures present within the centromeres. Sequencing of *cen1* was complete except for the duplicated regions. However, a part of *cen2* remains to be determined (Chikashige *et al.*, 1989; Clarke and Baum, 1990; Hahnenberger *et al.*, 1991). A striking finding presented in this report is that the central inverted repeats, which are separated by a 4, 7, or 5 kb central sequence, are strictly identical. This strict symmetry was found for all *cens*. Such symmetry may be maintained by a kind of gene conversion. Variations occur at corresponding positions in *imrL* and *imrR*

Figure 9 Nuclear location of centromeres. (a) An *nda3-311* cell (a mutant in the β -tubulin gene) (Hiraoka *et al.*, 1984) was stained with DAPI (a fluorescent probe for DNA, above) or probed with centromere sequence contains *dg* and *dh* by FISH (below). Three dots were seen. Bar, 10 μ m. (b) A haploid wild-type 972 interphase cell stained with DAPI (above) and the centromeres revealed by the probe *dg* (below). The three dots, located near the nuclear periphery, were overlapped. (c) A wild-type diploid interphase cell triple stained with DAPI (top), FISH probed with the repeat *dg* (middle), and the anti-tubulin antibody TAT1 (Woods *et al.*, 1989) (bottom). Bar, 10 μ m. The clustered dots were close to the cytoplasmic microtubules that run close to the SPB. (d) A schematic drawings of FISH in interphase (above) and metaphase (below) cells with the centromeric repetitive sequence as a probe. Each *cen* visualized by FISH and the SPBs are indicated black dots and the open circles, respectively. The nuclear chromosomal domains are represented by the hatched areas. Interphase microtubules and a mitotic spindle are also shown. (e) A hypothetical organization of fission yeast centromeres. See DISCUSSION for explanation.

in different wild-type strains with a normal frequency. Strong constraints may exist to maintain perfect sequence identity between the inverted sequences. However, the essential functional role of the inverted repeat requires clarification, for example, by introducing sequence asymmetry with the use of mutagenesis and determining if asymmetry is converted.

Another important feature of these inverted repeats is that among different chromosomes, they are largely nonhomologous to each other. In this sense, *imrs* constitute a class of chromosome-specific repeats. They are not unique but, with the exception of the small regions containing tRNA-encoding sequences, are rare repeats, duplicated only once in the genome. In addition, the

central domain has unusual chromatin structure. No nucleosomal pattern was obtained by MNase digestion, consistent with the results of Polizzi and Clarke (1991). Note that budding yeast centromeres are also nonnucleosomal (Bloom and Carbon, 1982), although their sizes are exceedingly small by comparison. We suppose that the central region (*cnt* + *imr*) of *S. pombe* *cen*s is bound by sets of proteins that inhibit the formation of nucleosome-like structures, because a nucleosome-like pattern can be formed on these sequences when present on ARS plasmids. It is of great interest to identify proteins that specifically bind to the central *cen* regions. Such proteins might be classified into two classes, namely, those commonly bound to the central regions

or those bound to only one particular region of the centromere.

The properties of the outer regions are in sharp contrast to those of the central regions. The outer repeat units are commonly present in all of the centromeres. They are somewhat similar to the alphoid sequences of mammalian centromeres (Willard, 1990). The dg element contains motifs reminiscent of alphoids. By sequence comparison, we found that sequences that resemble the 17-bp CENP-B box sequence (CTT/AC/TGT/GTGGAAAC/AGGG/AA) are plentiful in dg; there are 22 TGGAAA. However, it remains to be determined whether mammalian centromeres contain a low copy number sequence in the middle of the alphoid repeats, and, if it exists, whether it plays an essential role for the centromere. Hadlaczký *et al.*, (1991) reported low-frequency human DNA that might be implicated in centromere function. CENP-B protein that binds to alphoid DNAs is thought to play an important role in kinetochore formation (Bernat *et al.*, 1990, 1991; Simerly *et al.*, 1990; Zinkowski *et al.*, 1991). Although various individual copies of dg (and dh) are highly homologous to each other, they are never 100% identical: 98.1% identity between dgI and dgIIa and 98.9% between dgIIa and dgIIb (Nakaseko *et al.*, 1986). The total size of the outer regions is variable; 20 kb for *cen1*, ~60 kb for *cen2*, and >100 kb for *cen3*. The chromatin of the *otrs* appears to be regular and nucleosomal (Chikashige *et al.*, 1989; Polizzi and Clarke, 1991). We presume that structural differences between the central and outer centromere regions reflect functional differences.

Truncated minichromosomes were previously made and characterized (Niwa *et al.*, 1989; Hahnenberger *et al.*, 1989, 1991; Clarke and Baum, 1990; Matsumoto *et al.*, 1990). The *otr* repeat sequences were shown to be essential for the maintenance of minichromosomes. In the present paper, we examined whether the central region is required for the maintenance of minichromosomes. Experiments creating deletions of *cen* sequences in artificial minichromosomes showed that, first, the central region may be important for the *cen* function. The truncated minichromosome, DC3640, in which a major portion of *cnt* and proximal part of *imr* were substituted with *ura4⁺* gene, was unstable. Second, judging from the behavior of RMCdel1, one intact *imr* sequence with *cnt* is not sufficient to confer chromosome stability, suggesting that a pair of inverted sequence (*imr*) is necessary for the function. However, the stability of RMCdel37 and DC3600 indicates that a complete *imr* set is not required. Considering the absence of sequence homology between *imr*'s of different centromeres, with exception of tRNA sequences, it is conceivable that the overall inverted structure may be important for function rather than specific sequences. Alternatively, specific sequences may play an essential role for interacting with proteins in SPB or the spindle. Besides the mitotic and meiotic function of the central region,

we provided additional results showing the importance of *otr* sequences for mitotic and meiotic chromosome segregation. In the present study, we showed that not only the outer region but also the central region appears to be essential for the *cen* function.

We previously reported the successful application of FISH to visualize centromeric and nucleolar DNA in fission yeast (Uzawa and Yanagida, 1992). In the present report, we present the visualization of individual centromeres; in mitotically arrested cells, three centromeres were seen as distinct dots. In interphase cells, they are located in a cluster at the nuclear periphery. This is reminiscent of the clustering of centromeres observed in *Drosophila* embryonic cells (Hiraoka *et al.*, 1990). During mitosis, the centromere DNAs are situated in the middle of the short mitotic metaphase spindle (Uzawa and Yanagida, 1992). After spindle extension in anaphase B, the centromeric DNAs are located at both ends of the spindle, i.e., adjacent to the SPBs. Hence, the presence of anaphase A (shortening the distance between the pole and the centromere) was convincingly demonstrated in fission yeast. In an analogous fashion, the clustered interphase centromeres may locate near to the SPB. These cytological observations by FISH shed light for first the time on the behavior of centromeres during the fission yeast cell division cycle.

A possible scheme for the organization of fission yeast centromeres is presented in Figure 9(e). A hypothesis is that the outer repetitive regions may be required for interaction with the corresponding regions of other centromeres to form a higher-order complex structure in the interphase nucleus. The central regions, on the other hand, may be required for positioning individual *cents* at specific spatial sites in the interphase nucleus or on the metaphase plate. The central regions may serve as an anchoring domain of individual chromosomes. These regions form specific nonnucleosomal inverted configurations that might be protruded from the other parts of the centromeres and easily recognized for anchoring (e.g., to the region nearby the nuclear periphery in interphase). Without the central domains, chromosomes might behave highly unstably not only in mitosis but also in the interphase. This model fits with the fact that the central regions contain chromosome-specific sequences, whereas the outer regions are common centromere domains. A region where kinetochore microtubules bind to remains an open question. Either or both central and outer regions possibly interact with the spindle apparatus. The number of the kinetochore microtubules per chromosome has not been established in fission yeast; however, there are probably not many judging from electron microscopy of thin sectioned mitotic cells (Kanbe *et al.*, 1990). In short, both the central and outer regions of the fission yeast centromeres are essential stability factors, but their functions may be largely different. Detailed understanding of their function requires further investigation. But, based on these

observations, fission yeast centromeres offer an excellent model for the behavior of the complex centromeres typical of higher eukaryotes. It remains to be seen whether higher eukaryotic centromeres have organizations similar to that of fission yeast.

ACKNOWLEDGMENTS

We thank Drs. H.L. Levin and J.D. Boeke for *S. pombe* strains. This work was supported by the grants from the Ministry of Education, Science and Culture, the Science and Technology Agency, and the Ministry of Agriculture, Fishery, and Forestry of Japan.

REFERENCES

- Bernat, R.L., Borisy, G.G., Rothfield, N.F., and Earnshaw, W.C. (1990). Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement at mitosis. *J. Cell Biol.* 111, 1519–1533.
- Bernat, R.L., Delannoy, M.R., Rothfield, N.F., and Earnshaw, W.C. (1991). Disruption of centromere assembly during interphase inhibits kinetochore morphogenesis and function in mitosis. *Cell* 66, 1229–1238.
- Blackburn, E.H., and Szostak, J.W. (1984). The molecular structure of centromeres and telomeres. *Annu. Rev. Biochem.* 53, 163–194.
- Bloom, K.S., and Carbon, J. (1982). Yeast centromere DNA is a unique and highly ordered structure in chromosomes and small circular minichromosomes. *Cell* 29, 305–317.
- Chikashige, Y., Kinoshita, N., Nakaseko, Y., Matsumoto, T., Murakami, S., Niwa, O., and Yanagida, M. (1989). Composite motifs and repeat symmetry in *S. pombe* centromeres: direct analysis by integration of *Not I* restriction sites. *Cell* 57, 739–751.
- Clarke, L., Amstutz, H., Fishel, B., and Carbon, J. (1986). Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* 83, 8253–8257.
- Clarke, L., and Baum, M.P. (1990). Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences. *Mol. Cell. Biol.* 10, 1863–1872.
- Clarke, L., and Carbon, J. (1985). The structure and function of yeast centromeres. *Annu. Rev. Genet.* 19, 29–56.
- Compton, D.A., Yen, T.J., and Cleveland, D.W. (1991). Identification of Novel centromere/kinetochore associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* 112, 1083–1097.
- Cotterel, G., Shero, J.H., Hieter, P., and Hagemann, J.H. (1989). A 125-base-pair CEN6 DNA fragment is sufficient for complete meiotic and mitotic centromere function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9, 3342–3349.
- Earnshaw, W.C. (1991). When is a centromere not a kinetochore? *J. Cell Biol.* 99, 1–4.
- Earnshaw, W.C., and Bernat, R.L. (1991). Chromosomal passengers: toward an integrated view of mitosis. *Chromosoma* 100, 139–146.
- Fan, J.B., Chikashige, Y., Smith, C.L., Niwa, O., Yanagida, M., and Cantor, C.R. (1988). Construction of a *Not I* restriction map of the fission yeast *Schizosaccharomyces pombe* genome. *Nucleic Acids Res.* 17, 2801–2818.
- Fishel, B., Amstutz, H., Baum, M., Carbon, J., and Clarke, L. (1988). Structural organization and functional analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 8, 754–763.
- Hadlaczy, G., Praznovsky, T., Cserpan, I., Kereso, J., Peterfy, M., Kelemen, I., Atalay, E., Szeles, A., Szelei, J., Tubak, V., and Burg, K. (1991). Centromere formation in mouse cells cotransformed with human DNA and a dominant marker gene. *Proc. Natl. Acad. Sci. USA* 88, 8106–8110.
- Hagan, I.M., and Hyams, J.S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* 89, 343–357.
- Hahnenberger, K.M., Baum, M.P., Polizzi, C.M., Carbon, J., and Clarke, L. (1989). Construction of functional artificial minichromosomes in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* 86, 577–581.
- Hahnenberger, K.M., Carbon, J., and Clarke, L. (1991). Identification of DNA regions required for mitotic and meiotic functions within the centromere of *Schizosaccharomyces pombe* chromosome I. *Mol. Cell. Biol.* 11, 2206–2215.
- Hieter, P., Mann, C., Snyder, M., and Davis, R.W. (1985). Mitotic stability of yeast chromosome: a colony colour assay that measures nondisjunction and chromosome loss. *Cell* 40, 381–392.
- Hiraoka, Y., Agard, D.A., and Sedat, J.W. (1990). Temporal and spatial coordination of chromosome movement, spindle formation, and nuclear envelope breakdown during prometaphase in *Drosophila melanogaster* embryos. *J. Cell Biol.* 111, 2815–2828.
- Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes β -tubulin: a cold sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement during mitosis. *Cell* 39, 349–358.
- Horio, T., Uzawa, S., Jung, M.K., Oakley, B.R., Tanaka, K., and Yanagida, M. (1991). The fission yeast γ -tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99, 697–700.
- Kanbe, T., Hiraoka, Y., Tanaka, K., and Yanagida, M. (1990). The transition of cells of the fission yeast β -tubulin mutant *nda3-311* as seen by freeze-substitution electron microscopy. Requirement of functional tubulin for spindle pole body duplication. *J. Cell Sci.* 96, 275–282.
- Koshland, D., Kent, J.C., and Hartwell, L.H. (1985). Genetic analysis of the mitotic transmission of minichromosomes. *Cell* 40, 393–403.
- Kuhn, R.M., Clarke, L., and Carbon, J. (1991). Clustered tRNA genes in *Schizosaccharomyces pombe* centromeric DNA sequence repeats. *Proc. Natl. Acad. Sci. USA* 88, 1306–1310.
- Levin, H.L., Weaver, D.C., and Boeke, J.D. (1990). Two related families of retrotransposons from *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 10, 6791–6798.
- Maniatis, T., Fritsch, E.A., and Sambrook, J. (1982). *Molecular Cloning*. New York: Cold Spring Harbor Laboratory Press.
- Masumoto, H., Masukata, H., Muro, Y., Nozaki, N., and Okazaki, T. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 109, 1963–1973.
- Matsumoto, T., Murakami, S., Niwa, O., and Yanagida, M. (1990). Construction and characterization of centric circular and acentric linear chromosomes in fission yeast. *Curr. Genet.* 18, 323–330.
- Maundrell, K., Hutchison, A., and Shall, S. (1988). Sequence analysis of ARS elements in fission yeast. *EMBO J.* 7, 2203–2209.
- Mitchison, T.J. (1988). Microtubule dynamics and kinetochore function in mitosis. *Annu. Rev. Cell Biol.* 4, 527–549.
- Moroi, Y., Peebles, C., Fritzler, M.J., Steigerwald, J., and Tan, E.M. (1980). Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA* 77, 1627–1631.

- Murakami, S., Matsumoto, T., Niwa, O., and Yanagida, M. (1991). Structure of the fission yeast centromere cen3: direct analysis of the reiterated inverted region. *Chromosoma* 101, 214–221.
- Murray, A.W., and Szostak, J.W. (1983). Construction of artificial chromosomes in yeast. *Nature* 305, 189–193.
- Murray, A.W., and Szostak, J.W. (1985). Chromosome segregation in mitosis and meiosis. *Annu. Rev. Cell Biol.* 1, 289–315.
- Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O., and Yanagida, M. (1986). Chromosome walking shows a highly homologous repetitive sequence present in all the centromere regions of fission yeast. *EMBO J.* 5, 1011–1021.
- Nakaseko, Y., Kinoshita, N., and Yanagida, M. (1987). A novel sequence common to the centromere regions of *Schizosaccharomyces pombe* chromosomes. *Nucleic Acids Res.* 15, 4705–4715.
- Newlon, C.S. (1988). Yeast chromosome replication and segregation. *Microbiol. Rev.* 52, 568–601.
- Niwa, O., Matsumoto, T., Chikashige, Y., and Yanagida, M. (1989). Characterization of *Schizosaccharomyces pombe* minichromosome deletion derivatives and a functional allocation of their centromere. *EMBO J.* 8, 3045–3052.
- Niwa, O., Matsumoto, T., and Yanagida, M. (1986). Construction of a minichromosome by deletion and its mitotic and meiotic behaviour in fission yeast. *Mol. & Gen. Genet.* 203, 397–405.
- Polizzi, C., and Clarke, L. (1991). The chromatin structure of centromeres from fission yeast is distinct, with differentiation of the central core which correlates with function. *J. Cell Biol.* 112, 191–201.
- Rothstein, R.J. (1983). One-step gene disruption in yeast. *Methods Enzymol.* 101, 202–211.
- Rothstein, R.J. (1985). Cloning in yeast. In: *DNA Cloning*, ed. D. Glover, Oxford: IRL Oxford Press.
- Sanger, F., Nicken, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- Shiozaki, K., and Yanagida, M. (1991). A functional 125-kDa core polypeptide of fission yeast DNA topoisomerase II. *Mol. Cell. Biol.* 11, 6093–6102.
- Simerly, C., Balczon, R., Brinkley, B.R., and Schatten, G. (1990). Microinjected kinetochore antibodies interfere with chromosome movement in meiotic and mitotic mouse oocytes. *J. Cell Biol.* 111, 1491–1504.
- Takahashi, K., Murakami, S., Chikashige, Y., Niwa, O., and Yanagida, M. (1991). A large number of tRNA genes are symmetrically located in fission yeast centromeres. *J. Mol. Biol.* 218, 13–17.
- Tanaka, K., and Kanbe, T. (1986). Mitosis in the fission yeast *Schizosaccharomyces pombe* as revealed by freeze-substitution electron microscopy. *J. Cell Sci.* 80, 253–268.
- Uemura, T., Morikawa, K., and Yanagida, M. (1986). The nucleotide sequence of the fission yeast DNA topoisomerase II gene: structural and functional relationships to other DNA topoisomerases. *EMBO J.* 5, 2355–2361.
- Uemura, T., and Yanagida, M. (1984). Isolation of type I and II DNA Topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO J.* 3, 1737–1744.
- Uzawa, S., and Yanagida, M. (1992). Visualization of centromeric and nucleolar DNA in fission yeast by fluorescence in situ hybridization. *J. Cell Sci.* 101, 267–275.
- Wevrick, R., Earnshaw, W.C., Howard-Peebles, P.N., and Willard, H.F. (1990). Partial deletion of alpha satellite DNA associated with reduced amounts of the centromere protein CENP-B in a mitotically stable human chromosome rearrangement. *Mol. Cell. Biol.* 10, 6374–6380.
- Willard, H.F. (1990). Centromeres of mammalian chromosomes. *Trends Genet.* 6, 410–416.
- Woods, A., Sherwin, T., Sasse, R., MacRae, T.H., Baines, A.J., and Gull, K. (1989). Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* 93, 491–500.
- Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. *Gene* 33, 103–119.
- Yen, T.J., Compton, D.A., Earnshaw, W.C., and Cleveland, D.W. (1991). CENP-E, a human centromere associated protein related from chromosomes at the onset of anaphase. *EMBO J.* 10, 1245–1254.
- Zimmer, M., Welser, F., Oraler, G., and Wolf, (1987). Distribution of mitochondrial introns in the species *Schizosaccharomyces pombe* and the origin of the group II intron in the gene encoding apocytochrome b. *Curr. Genet.* 12, 329–336.
- Zinkowski, R.P., Meyne, J., and Brinkley, B.R. (1991). The centromere-kinetochore complex: a repeat subunit model. *J. Cell Biol.* 113, 1092–1110.