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Gross chromosome rearrangements in fission yeast

(分裂酵母を用いた染色体の安定維持機構の解析)

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PhD thesis

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1.Summary

Centromere contains repetitive sequences in many eukaryotes and is crucial to faithful segregation of chromosomes.   Fission yeast centromere is composed of a unique core sequence surrounded by inverted repeats.   Although the stability of repetitive sequences in telomere and rDNA regions has been extensively studied. little is known about the centromere repeats.   Here. we have developed a fission yeast system to detect gross chromosomal rearrangements (GCRs). using a minichromosome that contains a complete set of centromere sequences.   Pulse field gel electrophoresis and comprehensive genome hybridization of the GCR products that formed spontaneously under the normal growth condition reveal that two types of GCR events are detected in this system.   One is translocation between homologous chromosomes. and another type is formation of isochromosomes in which the one arm of chromosome has been replaced by a copy of the other.   Remarkably, the isochromosome formation is associated with rearrangement of the centromere repeats.   The Rad3[ATR] kinase is found to suppress both the translocation and isochromosome formation.   These data suggest that instability of the centromere repeats can cause isochromosome formation.

Contents

2. General introduction

All living organisms including human consist of cells. The genetic information called gene is translated into the protein that is composed of the cell. So the genetic information is vital for maintaining our lives. Genes are encoded by DNA and are packed into chromosome. Chromosome is DNA-protein complex and has a large amount of DNA. For example, the length of human chromosome is 48 million base pairs (1 base pair is one DNA molecule). Bacteria has usually ring chromosome and the eukaryote including yeast to human has linear chromosome. Chromosome is folded compactly and packed into the nucleus where is far smaller compared with the size of the chromosome in the cell.

To maintain the genetic information during the cell proliferation, chromosomes must be replicated accurately and a pair of chromosomes called sister chromatids must be segregated equally to daughter cells (Figure 1). There are three elements needed for these processes. Replication origin is the site for initiating DNA replication and a number of the replication origins are distributed throughout the chromosome in order to replicate the whole chromosome in a short time. Centromere is only a site in the chromosome for forming the kinetochore (DNA - protein large complex that connects chromosome and spindle) - spindle (microtubule that is needed for pulling the sister chromosome to the separating cells) attachment and vital for equal segregation. Telomere is the site of the linear chromosome end. Telomere is formed by the repetitive DNA sequences and usually protected against the DNA degradation and DNA damage recognition by a lot of proteins because telomere is similar structurally to the
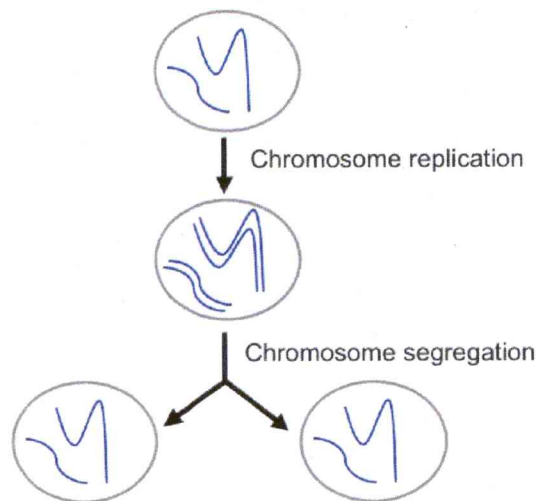
Stable maintenance of genetic information

Chromosome replication

Chromosome segregation

**Figure 1 Stable maintenance of genetic information is important for all living organism**
In order to maintain the genetic information stably, Chromosome (contains genetic information) must be replicated accurately and segregated equally to duaghter cellduring cell proliferation

DNA double strand break.

Cancer becomes one of the major causes of death and serious problems in modern society. Establishing the cancer treatment is immediate subject. Cancer cells grow limitlessly and finally destruct the human body leading to death. Recently, understanding the mechanism of tumorigeneis progressed and it is thought that the alteration of genetic information causes cancer.

Chromosome stability is important for the cell survival. Chromosome instability including gross chromosome rearrangements (GCRs) is one of the major characteristics of cancer and genetic diseases. In the figure 2, the translocation between chromosome 7 and chromosome 9 is observed in cancer cells. Such a translocation is suggested to cause tumorigenesis by the destruction of tumor-suppressor genes or amplification of oncogenes by the fusion of the actively transcribed genes and oncogenes. Aneuploidy is also observed in the cancer cell (Figure 2) and is suggested to causes tumorigenesis by the imbalanced expression of a subset of genes. Furthermore, some genetic diseases are associated with chromosome instability. For example, an additional chromosome 21 in human cell causes dawn syndrome. Although a lot of information about the relationship between chromosome instability and diseases has been obtained, the molecular mechanism leading to GCR such as translocation and aneuploidy remains unknown.

The fission yeast, *Schizosaccharomyces pombe* is a one of a model organism. Fission yeast belongs eukaryote and is a unicellular fungus. Fission yeast has advantage for using genetic technique easily such as gene knockout. Furthurmore, the

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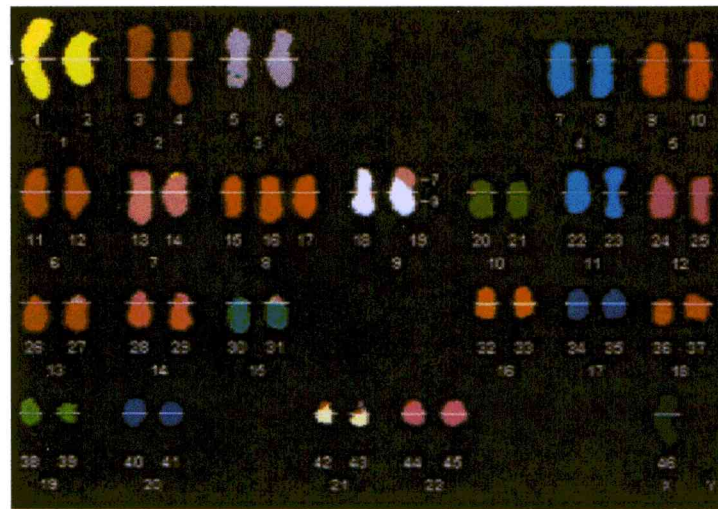
Cited from Elenbaas B et al., 50-65: 15, Genes & Development 2001

Figure 2 Gross chromosome rearrangements (GCRs) and aneuploidy are associated with tumorigenesis and genetic diseases

Translocation between chromosome 7 and chromosome 9 and trisomy of chromosome 8 are observed in cancer cell. Gross chromosome rearrangements such as translocation induces destruction of the tumor suppressor gene and fusion of the oncogene and actively transcribed gene.

size of fission yeast genome is not so large compared with human genome but has common characteristics of chromosome structure to human. For example, centromere size is large 40 – 110kb and is composed of highly repetitive sequences (Figure 3). This feature is common to human centromere that also has repetitive sequences and the size is so large 0.3Mb to 5Mb(Cleveland et al., 2003). These characteristics make fission yeast excellent model organism for chromosomal research.

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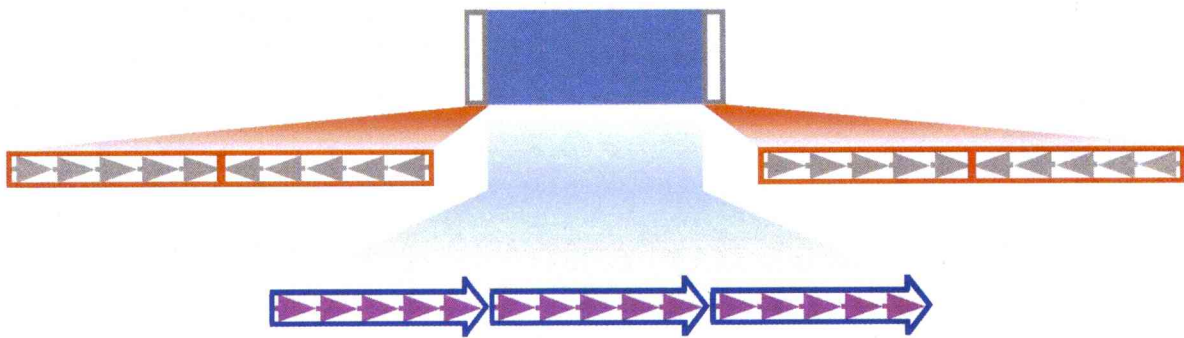
Budding yeast

~125 bp, -repeat

Fission yeast

40~110 kb, +repeat

Human

300~5000 kb, +repeat

Modified the figure from Schueler MG. and Sullivan BA., 301-313: 7, Annu Rev. Genomics Hum. Genet. 2006

Figure 3 Centromere structure of budding yeast, fission yeast and human
Centromere of budding yeast, fission yeast and human is shown. Characteristics of
centromere of fission yeast are similar to those of human. Fission yeast centromere
is large (40-110kb) and contains inverted repeat sequence. Human centromere is
very large (300-5000kb). Core centromere contains tandem repeat sequence and
pericentromere also contains repeat sequence but the repeat direction is not uniform.

3. Introduction

Precise replication of chromosome DNA and faithful transmission of the genetic material to daughter cells are crucial to maintain the genetic information throughout cell divisions (Bell and Dutta, 2002; Nasmyth, 2002; Tanaka, 2005). However, external stresses such as ionizing radiation and ultraviolet light or internal stresses such as replication errors and reactive oxygen can introduce some damage into DNA. The damaged DNA can be cured by various kinds of processes including recombinational repair, nucleotide excision repair, base excision repair, and mismatch repair (Friedberg, 2001; Lindahl, 2000; Paques and Haber, 1999). DNA structure checkpoint that, can be divided into DNA damage and replication checkpoints, detects aberrant DNA structures and coordinates the repair and the cell cycle progression (Carr, 2002; McGowan and Russell, 2004; Nyberg et al., 2002). Prior to the onset of mitosis, spindle checkpoint ensures correct attachment of mitotic spindle and kinetochore, so that unequal segregation of sister chromatids is prevented (Hardwick, 1998). Many elaborate systems collaborate in cells to maintain the genetic information.

Gross chromosomal rearrangement (GCR) such as translocation and deletion is one of the hallmarks of cancer cells, suggesting that chromosome instability is intimately associated with the tumorigenesis (Lengauer et al., 1998). An isochromosome, a chromosome with homologous arms that are mirror images of one another, is one of GCR products. In humans, isochromosomes are observed in genetic diseases including cancer (Mertens et al., 1994). Since isochromosome formation can be associated with loss of tumor suppressor genes or amplification of oncogenes, it is implicated in the tumorgenic process of neoplasms. In

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Candida albicans, the most prevalent human fungal pathogen, formation of an isochromosome

V is associated with resistance to azole, one of a limited number of available antifungal drugs

(Selmecki et al., 2006). Although centromere cleavage is proposed to account for

isochromosome formation (Jin et al., 2000), the sequence complexity of centromere hampers the

underlying molecular mechanism.

GCR events have been extensively studied using the budding yeast, *Saccharomyces*

cerevisiae (for a review see (Kolodner et al., 2002)). DNA repair and checkpoint regulation

play important roles to suppress GCRs (Myung et al., 2001a; Myung et al., 2001b). Not only

trans-factors but also cis-elements that affect chromosome stability have been found. The

number of replication initiation along a chromosome affects chromosome stability (Huang and

Koshland, 2003; van Brabant et al., 2001). DNA elements such as inverted repeat, transposons

and tRNA genes can induce GCR events (Admire et al., 2006; Lemoine et al., 2005; Lobachev

et al., 2000; Umezu et al., 2002). In the fission yeast, *Schizosaccharomyces pombe*, it has been

observed that a site-specific DNA double-strand break or replication fork barrier causes GCRs

(Lambert et al., 2005; Prudden et al., 2003).

Centromere is crucial for proper segregation of chromosomes, and it can be divided

into two regions, kinetochore and pericentromeric regions (Pidoux and Allshire, 2005; Schueler

and Sullivan, 2006). Kinetochore domain is the place where chromosome and mitotic spindle

is attached, whereas pericentromeric region is in heterochromatic state and play a role in

cohesion of sister centromeres (Bernard et al., 2001). The budding yeast centromere is

extremely short (125 bp) and contains no obvious repeats (Cheeseman et al., 2002). In

humans, centromere spans 300-5,000 kb and contain extensive copies (1,500->30,000) of a

171-bp sequence element termed α satellite (Cleveland et al., 2003). In the centromere core, α

satellites are arranged in extremely homogeneous higher-order, multimeric repeat units. While,

in pericentromeric region, the stretch of α satellites is frequently interrupted by interspersed

elements such as LINEs, SINEs and LTRs, and the α satellites are not uniformly oriented

(Schueler and Sullivan, 2006). In fission yeast, centromeres span 40-110 kb and consist of a

unique central core (*cnt*) surrounded by pairs of inverted repeats (*imr*, *otr* and *irc*) (Grewal and

Jia, 2007). It is known that DNA repeats in telomere and rDNA regions are maintained by

sophisticated manners (Autexier and Lue, 2006; Kobayashi, 2006). However, little is known

about the DNA repeats present in centromere.

Here, I develop a new system to detect GCR events using a fission yeast

minichromosome ChL that is derived from chromosome III (ChIII). The detailed analyses of

the GCR products show that translocation between homologous chromosomes, ChL and ChIII,

and isochromosome formation are detected in my system. Interestingly, the formation of

isochromosomes is found to be associated with rearrangement of the centromere repeat,

suggesting that the centromere repeat can cause the isochromosome formation.

4. Results

An assay system to detect gross chromosomal rearrangements (GCRs) in fission yeast

Fission yeast has three chromosomes, ChI to ChIII (Wood et al., 2002). Gross chromosomal rearrangements (GCRs) are the genetic alteration at the chromosomal level. When an essential gene is eliminated by GCR, the cell will be dead preventing the analysis of the GCR event. Thus, to monitor chromosome instability, I decided to use a minichromosome that is dispensable for cell proliferation. It seems that the minichromosome Ch16 that is derived from ChIII is useful to this purpose, since it contains an intact centromere and telomere sequences, and is transmitted to daughter cells with a high fidelity (Matsumoto et al., 1987; Niwa et al., 1986). To monitor the chromosome stability, I introduced three genetic markers onto Ch16, creating ChL (Fig. 4A). *LEU2* was introduced into a centromere-proximal site on the left arm of the minichromosome, whereas *ura4$^+$* and *ade6$^+$* genes were introduced in the middle of the right arm. In a *leu1 ura4 ade6* strain background, the presence of ChL is manifested as Leu$^+$ Ura$^+$ Ade$^+$. If GCR events associated with specific loss of *ura4$^+$* and *ade6$^+$* markers take place, the cell becomes Leu$^+$ Ura$^-$ Ade$^-$ (Fig. 4B, GCR). While, loss of ChL results in Leu$^-$ Ura$^-$ (Fig. 4B, Chromosome loss). To obtain the clones that have experienced GCR or chromosome loss, the yeast strain harboring ChL was grown on the rich medium, YE3S. To detect Ura$^-$ cells, single colonies on YE3S plates were picked, suspended in distilled water and plated onto YE3S and 5FOA media supplemented with Leu and Ade (5FOA+LA) (Fig. 4C). The Ura$^-$ colonies formed on 5FOA+LA plates were further examined by replica plating onto the media lacking

A ChL minichromosome

LEU2   ura4+ ade6+

cen3

$\smile$ 110 $\smile$ $\smile$ 110 $\smile$ $\smile$ 170 $\smile$ 8 $\smile$ 140 $\smile$
(kb)

B   (Leu+ Ura+ Ade+)

Gross Chromosomal   Chromosome Loss
Rearrangement (GCR)

(Leu+ Ura- Ade-)   (Leu- Ura-)

C   YE3S

YE3S   5FOA+AL
[Total]   [Ura-]

EMM+UA   EMM+U
[Leu+ Ura-]   [Leu+ Ura- Ade+]

D
[Leu+ Ura- Ade-] = [Leu+ Ura-] − [Leu+ Ura- Ade+]
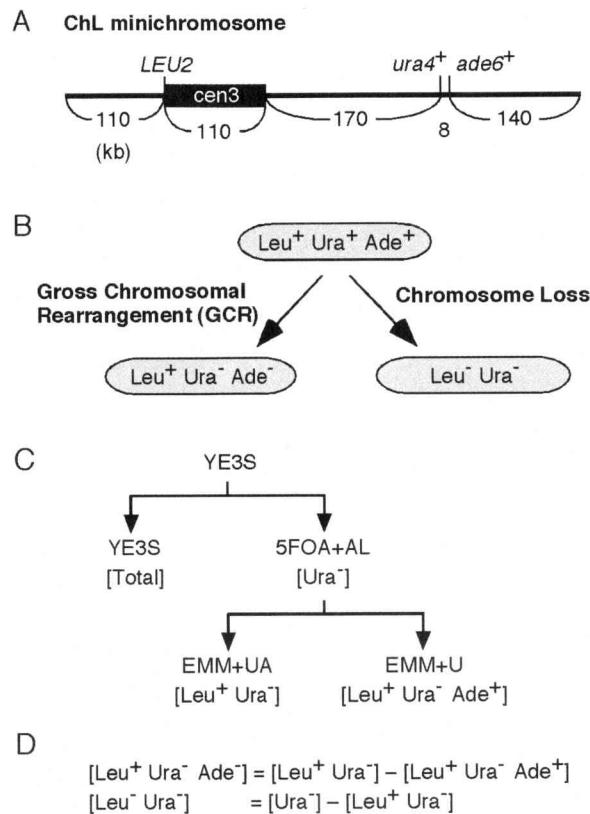[Leu- Ura-]   = [Ura-] − [Leu+ Ura-]

Figure 4 An assay system to study chromosome stability using ChL minichromosome.
(A) ChL minichromosome was created by the introduction of *LEU2*, *ura4+* and *ade6+* markers
onto Ch16 (Niwa et al., 1986). Indicated are positions of the three markers integrated and the
centromere. (B) The parental ChL gives Leu+ Ura+ Ade+ prototroph to *leu1 ura4 ade6* cells.
Gross chromosomal rearrangements (GCRs) associated with specific loss of the region encom-
-passing ura4+ and ade6+ lead to Leu+ Ura- Ade- (the left arrow). Loss of ChL results in
Leu- Ura- (Ade-) (the right arrow). (C) A schematic view of a protocol to determine the rate of
GCRs and chromosome loss. A single colony formed on YE3S, that contains Leu Ura and Ade,
was suspended in distilled water and plated onto YE3S and 5FOA+AL media. After 4-5 days'
incubation at 30。C, the colonies were counted to determine the number of total and Ura- cells.
The Ura- colonies formed on 5FOA+AL plates were replicated onto EMM+U and EMM+UA
plates and incubated for additional 3-4 days, to determine the number of Leu+ Ura- and
Leu+ Ura- Ade+ cells, respectively. The numbers of Leu+ Ura- Ade- (GCRs) and Leu- Ura-
(chromosome loss) cells were calculated as shown in (D). A, Ade; L, Leu; U, Ura.

Leu (EMM+UA) or both Leu and Ade (EMM+U). The numbers of Leu⁺ Ura⁻ Ade⁻ cells (i.e.

GCR) and Leu⁻ Ura⁻ (i.e. chromosome loss) were calculated as shown in Fig. 4D.

Analysis of GCR products by pulse field gel electrophoresis

From 15 independent clones of Leu⁺ Ura⁻ Ade⁻ and the parental strain (P), chromosomal DNA

was prepared and separated by pulse field gel electrophoresis (PFGE) (Fig. 5). DNA staining

with ethidium bromide (EtBr) showed that all the clones contained minichromosomes in

addition to ChI-III (Fig. 5A-C, the left panels). However, the length of the minichromosomes

was different form that of the parental ChL, indicating that GCRs rather than simple gene

conversions have occurred in Leu⁺ Ura⁻ Ade⁻ clones. To see more details about the GCR

product, DNA in the gel was transfer to a nylon membrane and hybridized with specific probes

indicated in Fig. 5D (Fig. 5A-C). When the hybridization was performed using probe LEU2

all the minichromosomes were detected (Fig. 5A, probe LEU2), demonstrating that they were

derived from ChL. From the results shown in Fig. 5A-C, the GCR products observed in this

assay can be categorized into two types (Fig. 5E). Type-I products contain regions A and B as

well as region C and rDNA, and are ~2.0 Mb long. It should be noted that ChIII contains

arrays of rDNA genes on its both ends. On the other hand, type-II products have lost regions

A and B, and are slightly smaller than the parental ChL (0.5 Mb). These results show that at

least two types of GCR products can be detected in this assay.

Translocation between homologous chromosomes, ChL and ChIII

The above results suggest that type-I GCR products are formed by translocation between ChL
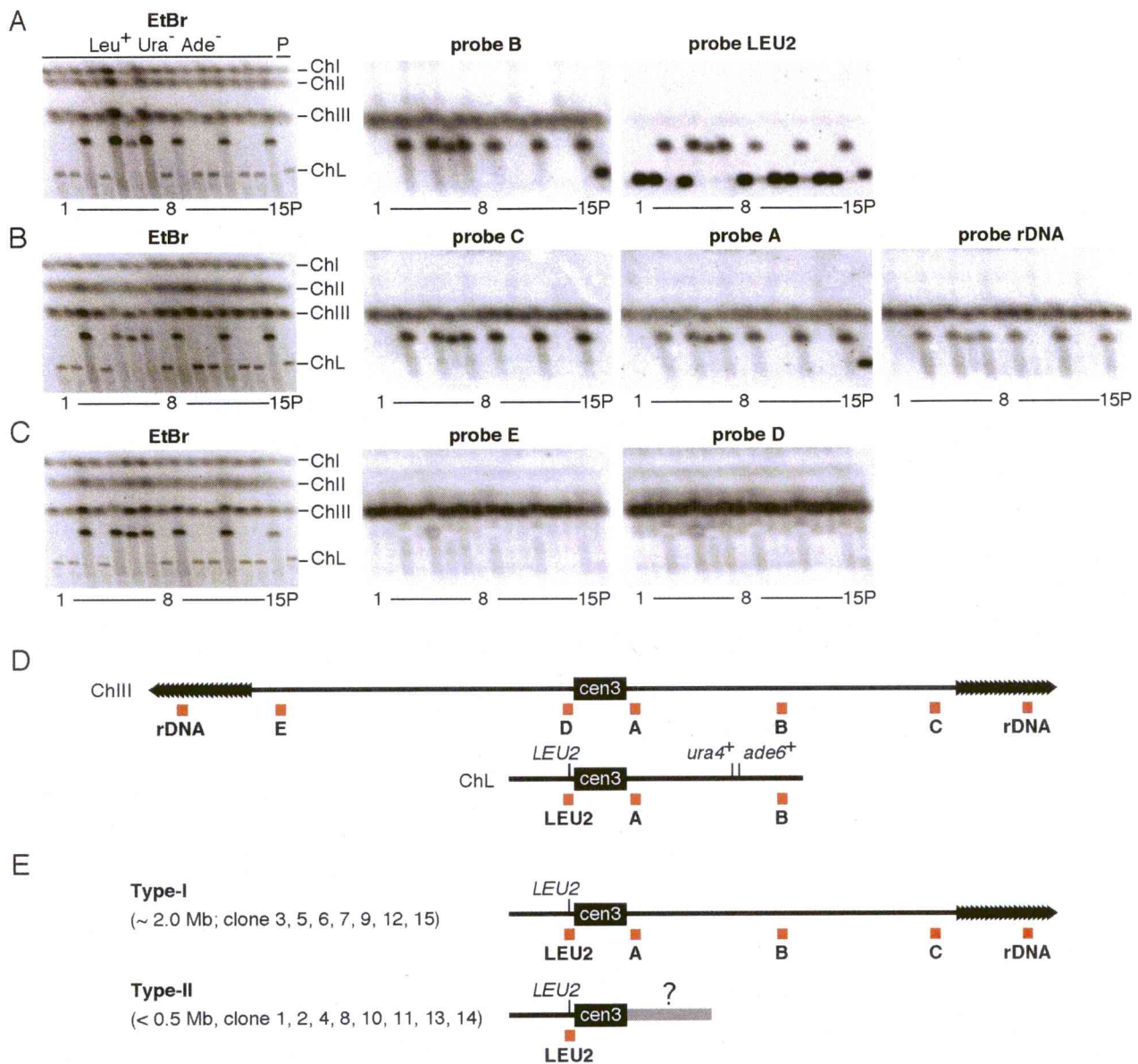
Figure 5 ChL chromosome has been rearranged in Leu⁺ Ura⁻ Ade⁻ clones.
(A-C) Chromosomal DNA was prepared from the parental strain (P, Parental; TNF1610) and 15 Leu⁺ Ura⁻ Ade⁻ GCR clones derived from different colonies on YE3S, separated by PFGE (pulse time: 1,800 sec in 2 V/cm for 72 hr) using 0.7% agarose gel in 1x TAE buffer, and stained with EtBr (the left panel). Positions of ChI, ChII, ChIII and ChL chromosomes (5.7, 4.6, 3.5 and 0.5 Mb, respectively) in the parental strain are indicated on the right of the EtBr panel. DNA in the agarose gel was transferred onto a nylon membrane and hybridized sequentially with the specific probes indicated on the top of each panel. (D) Positions of the probes used in Southern blotting are indicated as red boxes. ChIII and ChL are shown. (E) Type-I and Type-II GCR products observed in this assay. The size of the GCR products and the clone number are shown in parentheses.
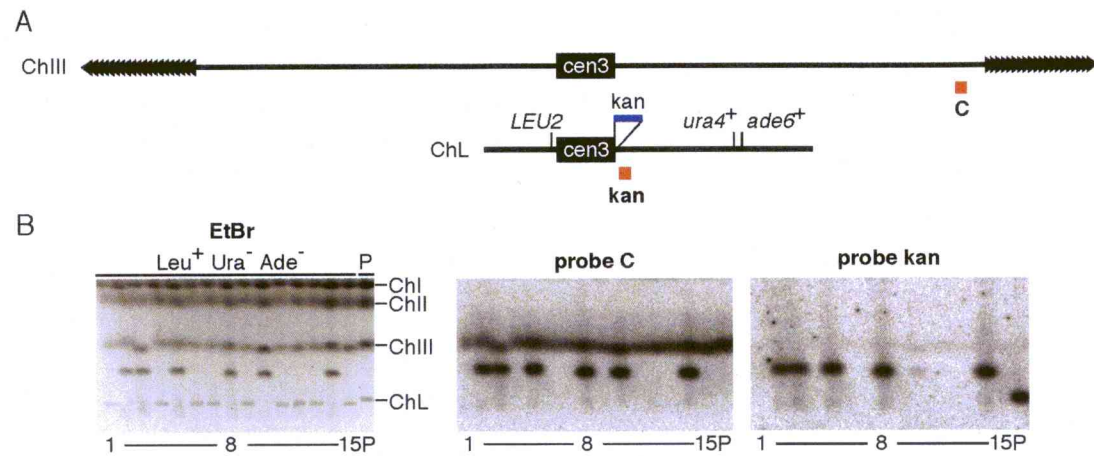
Figure 6 Type-I GCR products are associated with translocation between ChIII and ChL chromosomes.

(A) A schematic diagram of ChIII and a ChL derivative (P, Parental; TNF2076) that contains the kan gene (the blue box) on the right side of cen3. Positions of the probes used in Southern hybridization are indicated as red boxes. (B) Chromosomal DNA was prepared from the parental strain (TNF2076) and 15 Leu$^+$ Ura$^-$ Ade$^-$ clones derived from different colonies on YE3S, separated by PFGE (pulse time: 1,800 sec in 2 V/cm for 72 hr) using 0.8% agarose gel in 1x TAE buffer, and stained with EtBr (the left panel). DNA was transferred onto a nylon membrane and hybridized sequentially with the specific probe C and probe kan.

and ChIII.   To narrow the position where the translocation takes place, I introduced an

additional marker, the *kan* gene, into a centromere-proximal site o the right side of cen3 (Fig.

6A), and carried out the isolation of Leu$^+$ Ura$^-$ Ade$^-$ clones and the Southern analysis as

described above.   Out of 15 GCR clones, 6 clones contained type-I products judged by the

chromosome length and the presence of region C (Fig. 6B).   Among the 6 type-I products, 5

retained the *kan* marker.   Thus, in most cases type-I products are formed by the translocation

between the right arm regions (~170 kb, see Fig. 4A) of ChL and ChIII.   Loss of the *kan* maker

may be due to translocation between centromere regions.

The isochromosome produced around centromere

To determine the length of type-II GCR products that have lost the original right arm (Fig. 7),

chromosomal DNA was separated with Lambda DNA ladder by PFGE under the condition

where 50-800 kb DNA can be resolved.   DNA staining with EtBr showed that type-II products

were 320-400 kb although the parental ChL was ~540 kb (Fig. 7A).   Since the length of ChL

except the right arm is ~220 kb (Fig. 4A), some additional sequence of 100-180 kb must be

present on type-II products.   To see the additional sequence, the minichromosomes were

recovered from the gel and subjected to comprehensive genome hybridization (CGH) using an

oligonucleotide array (Fig. 7B).   As expected, the parental ChL consisted of a ~540 kb region

of ChIII (Fig. 4B, Parental ChL).   However, the ~220 kb region of ChL and no other sequences

were detected in a type-II product (Fig. 7B, Type-II).   These results suggest that type-II

product is the isochromosome in which the original right arm has been lost and replaced with a

copy of the left arm.   To test this possibility, from the 15 Leu$^+$ Ura$^-$ Ade$^-$ clones and the
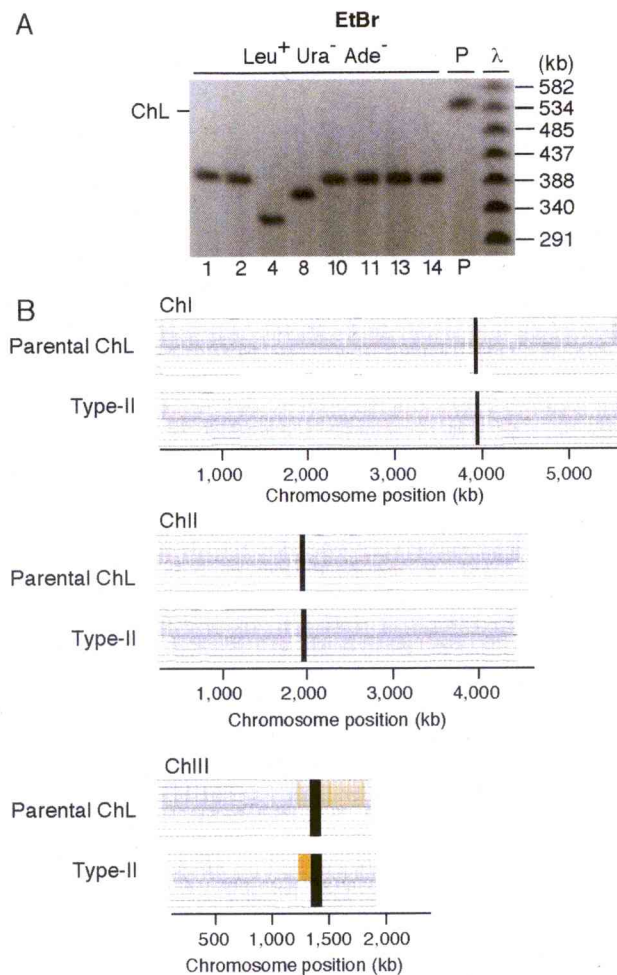
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Figure 7 Analysis of type-II GCR products by comprehensive genome hybridization.
(A) To determine the chromosome size of type-II products, chromosomal DNA prepared from
the parental strain (P, Parental; TNF1610) and type-II GCR clones shown in Fig. 2 were separated
by PFGE (pulse time: 5 to 120 sec linear, 4.5 V/cm for 48 hr) using 1.0% agarose gel in 0.5x TBE
buffer and stained with EtBr. The position of the parental ChL is indicated on the left of the panel.
Sizes of Lambda DNA ladder (New England Biolabs) are indicated on the right. The clone numbers
are shown on the bottom. (B) By a DNA microarray technique, comprehensive genome hybridization
(CGH) was carried out for the parental ChL and a type-II GCR product. Each bar represents the mean
of 11 oligonucleotide probes within adjacent 250-bp windows. Orange bars represent significant binding
Chromosome position corresponds to the fully assembled virtual contigs in Sanger Center S. pombe
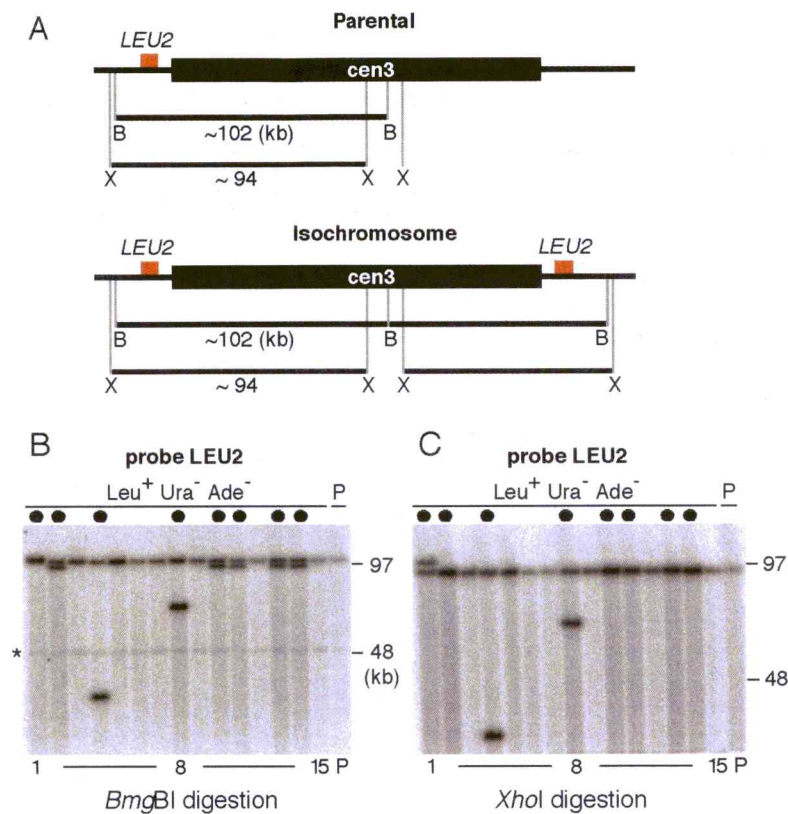Genome Database. The Y-axis scale is log2.

Figure 8 Type-II GCR products are isochromosomes.
(A) The parental ChL contains a single *LEU2* (the red box) on the left of cen3. However, if type-II GCR products were isochromosomes, they have two copies of *LEU2*, one on the left and another on the right arm. Positions of *Bmg*BI and *Xho*I restriction sites are indicated as B and X, respectively. (B) Chromosomal DNA was prepared from the parental strain (P, Parental; TNF1610) and 15 Leu[+] Ura[-] Ade[-] clones shown in Fig. 2, digested with *Bmg*BI, separated by PFGE, transferred onto a nylon membrane, and hybridized with the LEU2 probe. (C) Chromosomal DNA was digested with *Xho*I. Type-II GCR clones are indicated by closed circles on the top of each panel. It is estimated that clones 2, 10, 11, 13 and 14 contain the same number (i.e. ~12 copies) of *otr3* tandem repeats on both sides of *cnt3* while clones 1, 4 and 8 contain 2, 7 or 13 *otr3*s, respectively, on the right side.

parental strain, chromosome DNA was digested with *Bmg*BI (B) or *Xho*I (X), separated by

PFGE, and the restriction fragment containing *LEU2* was detected by Southern blot.   When the

isochromosome is formed around centromere, the *LEU2* marker will be duplicated, and the

LEU2 restriction fragments are detected as two separate bands as far as they are significantly

different in the length (Fig. 8A, Parental versus Isochromosome).   Fig. 8B&C show that, in the

case of type-II products that are indicated by closed circles at the top of the panel, at least one

the two digestions creates two separate *LEU2* bands.   These results show that type-II GCR

products are the isochromosome that contains essentially the same sequence on both arms.

The difference lengths of the restriction fragments among type-II clones (Fig. 7A and Fig. 8)

may be due to the differences in the centromere repeats (see also below).

The isochromosome formation is associated with rearrangement of the centromere repeat

Cen3 consists of a unique central sequence, *cnt3* (4.9 kb), and surrounded by inverted repeats,

imr3 (5.4 kb), *otr3* (6.3 kb) and *irc3* (2.2 kb) (Grewal and Jia, 2007) (Fig. 9A).   There are ~12

and 2 copies of *otr3*s on the left and right sides of *cnt3* on ChL, respectively (Supplemental data

Figure S1).   The nucleotide sequences of the two *imr3*s are completely identical (Takahashi et

al., 1992), while small differences between the repeats of *otr3*s or *irc3*s.   To diagnose the

integrity of centromere of isochromosomes, PCR analysis was carried out using the rearranged

and parental ChLs as templates.   440-bp region of *irc3*s was amplified using a pair of primers,

digested with *Apa*I, and separated by conventional agarose gel electrophoresis (Fig. 9A).

Because of the nucleotide difference, an additional *Apa*I site is present in the PCR fragment

amplified from the right *irc3* (*irc3-R*) compared to that from the left (*irc3-L*).   Four restriction
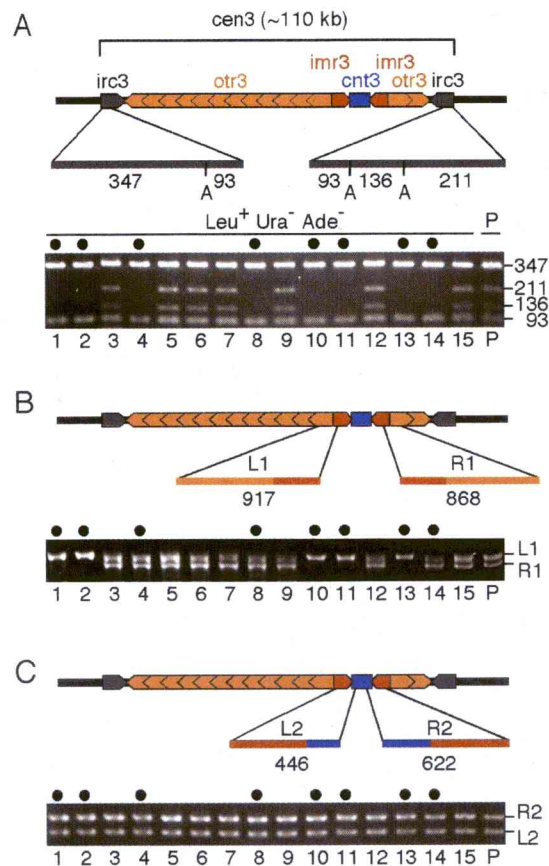
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Figure 9 Isochromosomes are associated with rearrangement of the centromere repeats.

Fission yeast cen3 is consist of a unique central sequence called *cnt3* (blue) and inverted repeats of three different sequences *imr3* (red), *otr3* (orange) and *irc3* (gray). The parental (P, Parental; TNF1610) and rearranged ChLs were separated by PFGE, recovered from the agarose gel and used as templates in PCR amplification. A region of *irc3* on the left and right sides was amplified using irc3-R and irc3-F primers, digested with *Apo*I, separated by 2.0% agarose gel electrophoresis, and stained with EtBr. Because of nucleotide polymorphism, the amplified regions in the left and right irc3s contain one and two ApoI sites, respectively. (B) Centromere regions encompassing the junction of *imr3* and *otr3* was amplified using imr3-out and dh primers, separated by 2.0% agarose gel electrophoresis, and stained with EtBr. Because of small deletions on the right side, 917 (L1) and 868-bp (R1) fragments are amplified from the left and right, respectively. (C) Centromere regions encompassing the junction of *cnt3* and *imr3* was amplified, using a combination of three primers, cnt3-L, cnt3-R and imr3-in2, separated by 1.2% agarose gel electrophoresis, and stained by EtBr. 446 (L2) and 622-bp (R2) regions are amplified from the left and right, respectively. Type-II GCR clones are indicated by closed circles on the top of each panel. The clone number is shown at the bottom of each panel.

fragments were produced from the parental and type-I ChLs. However, the two fragments specific to *irc3-R* (136 and 211-bp) were not produced from any of the 8 type-II ChLs (Fig. 9A, type-II products are indicated by closed circles), indicating that the isochromosomes have lost the original *irc3-R* sequence. I extended this assay to *otr3* by amplifying the junction of *imr3* and *otr3* (Fig. 9B). Because of small deletions, two fragments of different sizes (L1 and R1) are amplified using a pair of primers from the left and right, respectively. Only the L1 fragment was amplified from 5 of the 8 type-II ChLs, indicating that some isochromosomes have lost the right *otr3* too. When the junction of *cnt3* and *imr3* were amplified, the two fragments (L2 and R2) corresponding to the left and right junction, respectively, were detected in all the cases examined (Fig. 9C). These data show that the isochromosome formation is associated with rearrangement of the centromere repeat.

Rad3^{ATR} kinase suppresses GCRs

Mammalian ATR (ataxia telangiectasia- and Rad3-related) kinase is the central player in DNA structure checkpoint control. ATR knockout mice accumulate chromosomal breaks and become embryonic lethal, suggesting an essential role for ATR even in the absence of exogenous stresses (Brown and Baltimore. 2000). To see whether Rad3, the fission yeast homolog of ATR, affects chromosome stability in the absence of external stresses, the rates of GCRs and chromosome loss that occur spontaneously were determined using the wild-type and the *rad3* deletion (*rad3Δ*) strains (Table. 1). In the wild type, GCRs were found to occur at 2.9×10^{-5} per cell division, which is comparable to those observed in budding yeast using artificial chromosomes (YACs) (Huang and Koshland, 2003). The loss rate of ChL is similar

Table 1. Rates of GCR and chromosome loss in the wild type and *rad3* mutant strains.

Strain	Sample#	Genotype	GCR		Chromosome loss	
			Rate (x10^{-5})	Relative	Rate (x10^{-5})	Relative
TNF1610	45	wild type	2.9 | 2.5~ 3.2|	1.0	3.4| 2.6~ 4.1|	1.0
TNF1700	31	*rad3Δ*	21.7 |18.4~28.4|	7.4	33.7 |25.0~ 41.2|	10.0
TNF1728	15	*rad3D2230A*	32.0 |28.1~38.2|	10.9	72.0 |60.5~218.0|	21.4
TNF1730	15	*rad3N2235K*	57.6 |37.3~82.0|	19.6	150.0 |94.0~287.0|	44.5
TNF1732	15	*rad3D2249E*	19.2 |16.2~29.3|	6.5	41.7 |30.5~ 64.9|	12.4

Fluctuation analysis was carried out to determine rates at which GCR or chromosome loss takes place per cell division. The number of independent colonies examined is listed under Sample#. The mean value of the rates is indicated, and 95% confidence interval is shown in | |. The relative rate compare to that of wild type is indicated.

to that determined using Ch16 (Niwa et al., 1986). Compared to the wild type, the rates of

GCR and chromosome loss were significantly increased in *rad3Δ* cells, indicating Rad3 is

required to maintain the minichromosome. Both type-I and type-II products were observed in

15 Leu⁺ Ura⁻ Ade⁻ clones isolated from *rad3Δ* cells (data not shown). Introduction of the

kinase-dead mutations (*rad3D2230A*, *rad3N2235K* or *rad3D2249E*) also increased the rate of

GCR and chromosome loss (Table 1). Thus, the kinase function of Rad3 may play an

important role to maintain chromosome stability in the absence of external stresses.

5. Discussion

Gross chromosomal rearrangements (GCRs) can cause cell death because of loss of the genes essential for cell proliferation, which hampers the study of the GCR mechanism. Here, using an extra minichromosome ChL, I have developed a fission yeast system to detect GCRs that occur spontaneously. Detailed analyses of the rearranged chromosomes produced in the wild type showed two different types of GCRs: Translocation between homologous chromosomes ChL and ChIII, and formation of isochromosomes. Remarkably, the isochromosome formation was associated with rearrangements of the centromere repeat. Both the translocation and isochromosome formation were suppressed by Rad3^{ATR} kinase, the central player in DNA structure checkpoint.

In my assay, the GCR (i.e. Leu$^+$ Ura$^-$ Ade$^-$) clones are isolated based on the specific loss of both the two markers ($ura4^+$ and $ade6^+$) onto the right arm of ChL during the growth on nonselective media. Pulse field gel electrophoresis (PFGE) showed that the length of ChLs in the GCR clones were different from that of the parental ChL, indicating that simple gene conversions do not account for loss of the markers. Gene conversion between allelic loci occurs at the rate of 1~4x10^{-6} per cell division in vegetatively growing cells (Virgin et al., 2001), and the mean length of the conversion tract is ~1 kb (Grimm et al., 1994). Since $ura4^+$ and $ade6^+$ on ChL are ~8 kb apart from each other, the chance of the co-conversion must be far less than the rate of Leu$^+$ Ura$^-$ Ade$^-$ formation determined in my assay (i.e. the GCR rate, 2.9x10^{-5}). Simultaneous loss of two genetic makers near telomere sometimes results in deletion of a chromosome end combined with *de novo* addition of telomere sequence (Chen et al., 1998).

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However, such kind of GCR products was not seen in this study, probably because chromosomal rearrangement between homologous sequences is prevailing when they are available.

About a half of the GCR products were larger (~2.0 Mb) than the parental (~540 kb). However, the rate of this type of GCRs (type-I) is likely to be underestimated, since I noticed that the cells harboring type-I products grew less well compared to those containing the parental ChL or isochromosomes (type-II). Southern blot analysis showed that type-I products contained the sequence unique to the right arm of ChIII, and also rDNA that is present right next to ChIII telomeres. As ChL is a derivative of Ch16 that has been created by truncation of ChIII (Niwa et al., 1986), type-I is the products formed by translocation between homologous chromosomes, ChL and ChIII. In most cases the breakpoint is located in the 170-kb region o the right arms, since a marker introduced adjacent to the right edge of cen3 was retained in 5 of 6 type-I products. Translocation between homologs is accompanied with loss of heterogeneity (LOH), which is one of the ways to eliminate wild-type alleles of tumor suppressor genes (Cavenee et al., 1983). A similar type of translocation was induced by DNA double-strand breaks (DSBs), and it was shown by pedigree analysis that the translocation occurs either by reciprocal crossing over in late S or G2 phase or nonreciprocal break-induced replication (BIR) mechanism (Cullen et al., 2007; Prudden et al., 2003). The spontaneous translocation observed in this study may be initiated by DSBs produced at fragile sites on a chromosome arm (Cha and Kleckner, 2002). tRNA gene is one of the fragile sites (Admire et al., 2006; Ivessa et al., 2003), and the minichromosome has three of them on the right arm (~70, 110, 220 kb from centromere). DNA degradation from a chromosome end may also leads the translocation,

although BIR rather than crossing over occurs in this case because the DSB has only one end.

Another type (type-II) of the GCR products observed in this study has lost the right arm sequences. The length of type-II products determined by PFGE is longer than that estimated by comprehensive genome hybridization (CGH) (320-400 versus ~220 kb). This apparent discrepancy can be solved if the right arm had been replaced by a copy of the left. Indeed, I found that the *LEU2* marker integrated on the left side of cen3 was duplicated. These data demonstrate that type-II products are the isochromosomes that form around cen3. Different lengths among the isochromosomes are likely to be due to the copy number of tandem repeats (*otr3*) present in cen3. The fission yeast centromere is composed of a unique central core (*cnt*) surrounded by the inverted repeats; innermost repeats (*imr*), outer repeats (*otr*), and also *irc* except cen2 where no *irc* is recognized. In all the 8 isochromosomes examined, the right edge of cen3 (i.e. *irc3-R*) had been altered whereas the *cnt3/imr3* junctions were preserved, indicating that the breakpoint of the isochromosome locates somewhere in the centromere repeat (i.e. *imr3*, *otr3* and *irc3*) but not in *cnt3*. Interestingly, about a half of the isochromosomes retain at least a part of the original *otr3-R* and another half has lost it. The breakpoint may be located in *imr3* in the former case while it is in *otr3* or *irc3* in the latter. These data show that the isochromosome formation is associated with rearrangement of the centromere repeat and that the breakpoint is not limited to a specific repeat.

Centromere can be divided into two sub-regions with respect to the function; the kinetochore (*cnt* and *imr*) where attachment of microtubules to chromosomes takes place during mitosis and the pericentromeric heterochromatin region (*otr* and *irc*). Incorporation of histone variants (e.g. CENP-A) occur in kinetochore, and specific modifications of histones (e.g. H3

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K9-methylation) take place in pericentromeric heterochromatin. In *imr* and *irc*, the chromatin

boundary of pericentromeric heterochromatin is present (Cam et al., 2005; Scott et al., 2006).

The tRNA genes are also embedded in *imr* of any centromere. Thus, it is possible that aberrant

DNA is produced in centromere and causes isochromosome formation. Consistent to this idea,

two-dimensional electrophoresis has shown that cruciform DNA is produced in centromere of

fission yeast (Smith et al., 1995). In addition, pausing of replication forks has been seen in

centromere of budding yeast (Greenfeder and Newlon, 1992). It cannot be formally excluded

the possibility that the initial event (e.g. DSB formation) occurs on a chromosome arm or

telomere rather than centromere. However, I think it unlikely because a DSB is repaired using

the sequence adjacent to the break before extensive degradation of broken end (Paques and

Haber, 1999). Interestingly, in humans, most pericentromeric heterochromatin regions have

been subjected to a complex series of segmental duplications during the course of evolution

(She et al., 2004). Isochromosomes are observed in various types of genetic diseases including

cancer (Mertens et al., 1994). Thus, rearrangement of the centromere repeats may be one of a

driving force of the evolution and one that enforces tumorigenesis.

DNA sequences of *imr* inverted repeats on a chromosome are the same while they

are different among the three chromosomes in fission yeast (Takahashi et al., 1992), suggesting

an interaction between the inverted repeats in centromere. It has been observed that, in

budding yeast, an intramolecular loop (C-loop) is produced around centromere in mitosis (Yeh

et al., 2008). C-loop formation may facilitate the interaction between the inverted repeats in

the fission yeast centromere. The isochromosomes observed in this study appear to be

produced via recombination between the inverted repeats surrounding a central sequence *cnt*. I

propose that BIR accounts for the isochromosome formation. Since, intramolecular crossing over between a pair of the inverted repeats only flip the orientation of centromere and does not result in an isochromosome. It was seen that the fission yeast homolog of mammalian ATR checkpoint kinase, Rad3 is required to suppress GCRs in my assay. The kinase activity of Rad3 appears to be important for the GCR suppression, as amino acid substitution in the kinase domain caused chromosome instability as well as a deletion of Rad3. The assay system described here seems to be useful to study the molecular mechanism underlying isochromosome formation and translocation.

6. Materials and methods

Yeast strains and media

The fission yeast strains used in this study are listed in Table S1. Cells were grown in complete (YE) or minimum (EMM) medium supplemented with the indicated amino acid at a final concentration of 225 μg/ml (Moreno et al., 1991).

Construction of the ChL chromosome

To construct the ChL chromosome, *LEU2*, *ura4*$^+$ and *ade6*$^+$ genes were integrated into Ch16 (Niwa et al., 1986) as follow. A 1.0 kb region flanking the *ubcp4*$^+$ gene was amplified using III_1057F and III_1057R primers by PCR, and digested with *Msc*I and *Hind*III. The resulting 0.4 kb *Msc*I-*Hind*III fragment was introduced between *Hinc*II and *Hind*III sites of pBluescriptII SK$^+$(Stratagene), creating the plasmid pTN593. A 2.0 kb region flanking the *chk1*$^+$ gene was amplified using III_1062F and III_1062R primers, digested with *Sac*I and *Hind*III and the resulting 0.4 kb *Sac*I-*Hind*III fragment was introduced between *Sac*I and *Hind*III sites of pTN593, creating pTN594. A 2.2 kb *Hind*III fragment containing *LEU2* from pREP81 (Maundrell, 1993) was introduced into the *Hind*III site of pTN594, creating pTN595. A 3.1 kb *Sac*I-*Xho*I fragment containing the *ubcp4::LEU2::chk1* construct from pTN595 was introduced into an yeast strain harboring Ch16, and the transformants were selected using a medium lacking Leu, creating the yeast strain TNF844. A 1.7 kb *Pvu*II-*Hind*III fragment containing 5'-half of *ade6*$^+$ from pTN435 was introduced into TNF844 strain, and the transformants were selected using a medium lacking Ade, creating TNF1383 strain. 0.3 and 0.5 kb regions flanking the ORF, *spcc1322.09*, were amplified using a primer pair, III_1310A and

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III_1310B+ura4AN5, and another pair, III_1310C+ura4AN3 and III_1310D, respectively.

The second amplification was carried out in the presence of the two PCR products, pTN445

plasmid containing $ura4^+$ and a pair of primers, III_1310A and III_1310D. The PCR product

of 2.5 kb was introduced into TNF1383 strain, and the transformants were selected using a

medium lacking Ura, creating TNF1610 strain.

A ChL derivative that contains the *kanMX6* gene on the right arm proximal to cen3

was created as follows. 0.2 kb regions flanking the ORF, *spcc4B3.18*, were amplified using a

primer pair, irc3R7and irc3R8+kanAN3, and another pair, irc3R9+kanAN5 and irc3R10. The

second amplification was carried out in the presence of the two PCR products, pFA6a-kanMX6

(Bahler et al., 1998) and a pair of primers, irc3R7 and irc3R10. The PCR product of 1.8 kb

fragment was introduced into TNF1610 strain, and the transformants were selected using a

medium containing G418 (Nacalai tesque) at a final concentration of 50 μg/ml, creating

TNF2076 strain. The sequence of the oligonucleotide primers used in this study is listed in

Table S2. Correct integration was confirmed by PCR or by Southern blotting.

Pulsed field gel electrophoresis (PFGE) and Southern hybridization

In the case where DNA was not treated with restriction enzymes, chromosomal DNA was

prepared basically as described before (Smith et al., 1987). 1.6×10^8 cells were collected from

log-phase culture, washed with 50 mM EDTA twice, and suspended in 1 ml of CSE1 solution

[20 mM citrate phosphate (pH5.6), 1.0 M sorbitol, 50 mM EDTA] supplemented with

Zymolyase 20T (Seikagaku) and Lysing enzyme (Sigma) at the final concentration of 0.25

mg/ml each. After incubation at 30°C for 30 min with rotation, the spheroplasts were collected

by centrifugation at 500 rpm for 15 min (MX-201 micro centrifuge, TOMY), washed with 0.5

ml of CSE2 solution [20 mM citrate phosphate (pH5.6), 0.9 M sorbitol, 50 mM EDTA], and

resuspended with 200 μl of CSE2. After addition of 200 μl of 1.6% low-melting agarose LM

(Nacalai tesque) solution in H_2O that was pre-incubated at 50°C, the spheroplast suspension was

transferred into molds (Bio-Rad) to make four agarose plugs. The four plugs were incubated

at 60°C for 2 h in 2 ml of ES solution [0.25 M EDTA, 1% SDS], and then incubated at 50°C for

24 h in 2 ml of ESP solution [0.5 M EDTA, 1.0% N-lauroyl sarcosine (Sigma), 1.0 mg/ml

Proteinase K (Merck)]. The plug was washed with TE10:1 [10 mM TrisHCl (pH8.0), 1 mM

EDTA] three times, and kept at 4°C. Electrophoresis was carried out using Certified

Megabase agarose gel and CHEF-DRII system (Bio-Rad) under the condition indicated in the

figure legends. After treatment of the gel with 0.25 M HCl for 1 h, with 0.6 M NaCl, 0.2 M

NaOH for 30 min twice, and with 50 mM sodium phosphate (pH6.5) for 1 h, DNA was

transferred to a Nytran nylon membrane (Schleicher & Schuell).

In the case where chromosomal DNA was digested with *Bmg*BI or *Xho*I restriction

enzyme, 6.25 x 10^8 cells were collected, washed with TE10:25 [10 mM TrisHCl (pH8.0), 25 mM

EDTA] and then with SP1 buffer [1.2 M sorbitol, 50 mM citrate phosphate (pH5.6), 40 mM

EDTA (pH8.0)], and stored at -80°C before use. The cells were resuspended with 1.25 ml SP1

buffer. After addition of 0.25 ml of SP1 containing Lyticase (Sigma) at 3.5 mg/ml, the cell

suspension was incubated at 37°C for 30-60 min. The spheroplasts were collected by

centrifugation at 2,000 rpm for 3 min (GRX-250 centrifuge, TOMY) and suspended with 150 μl

of SP1. After addition of 200 μl of low-melting agarose LM, the spheroplasts were transferred

into molds. The plug was incubated overnight at 50°C in 5 ml of DB buffer [1.0 mg/ml

Proteinase K (Merck), 1.0% N-lauroyl sarcosine (Sigma), 25 mM EDTA], and then stored at

4°C in TE10:50 [10 mM TrisHCl (pH8.0), 25 mM EDTA]. The plug was treated with 10-20 U

of a restriction enzyme at 37 °C for 3 h. The plug was melted by incubation at 70°C for 10

min. After addition of 2 μl of 0.5 mg/ml RNase A (Sigma) and 2 U of β-agarase I (New

England Biolabs), the solution was incubated at 42°C for 1 h, followed by addition of 20 U of

restriction enzyme and overnight incubation at 37°C. After centrifugation at 13,000 rpm for

10 min (MX-201, TOMY), DNA in the supernatant was alcohol precipitated. DNA was

applied to PFGE (pulse time: 1 to 6 sec, 6 V/cm, 22 h in 0.5x TBE buffer). 0.8 and 0.7%

agarose gels were used for *Bmg*BI and *Xho*I cases, respectively. Following UV irradiation

(300 mJ), the gel was pretreated as described above prior to blotting. The probe for Southern

hybridization was prepared using Megaprime DNA labeling system (GE Healthcare) with α-^{32}P

-dATP. Radioactive signals were detected using a BAS2500 phosphorimager (Fuji film).

The rate of gross chromosomal rearrangements and loss of the ChL chromosome

Rates of GCRs and the minichromosome loss were determined by a fluctuation analysis using

the method of the median (Lea and Coulson, 1949; Lin et al., 1996). 95% confidential interval

was determined as described (Dixon and Massey, 1969).

PCR analysis of the centromere region of ChL

ChL chromosomes separated by PFGE were recovered from the gel using QIAquick gel

extraction kit (Qiagen). The PCR products were separated by agarose gel electrophoresis in 1x

TAE buffer. The oligonucleotide primers used are listed in Table S2.

Comprehensive genome hybridization (CGH) analysis

DNA microarray analysis was carried out as described before (Hayashi et al., 2007), using ChL

chromosomes recovered from pulse field agarose gel using QIAquick extraction kit (Qiagen).

Plasmids

A 1.4 kb region near SPCC11E10.02c was amplified using ChLR-F and ChLR-R primers and digested with *Eco*RI and *Hae*III. The resulting 1.3 kb *Eco*RI-*Hae*III fragment was introduced between *Eco*RI and *Eco*RV sites of pBluescript II SK$^+$, creating pTN672, and used to prepare the probe B. pTN445 harboring a 3.0 kb *Xba*I-*Bam*HI fragment containing the *ura4$^+$* gene on SK$^+$, was digested with *Hind*III and self-ligated, creating pTN753. A 0.5 kb *Xba*I-*Hind*III fragment from pTN753 was used as to prepare the probe E. A 2.0 kb fragment near the *chk1$^+$* gene was amplified using III_1062F and III_1062R primers and digested with *Cla*I and *Hind*III. The resulting 0.7 kb fragment was introduced between *Cla*I and *Hind*III sites of SK$^+$, creating pTN754, and used to prepare the probe D. A 2.2 kb region near *spcc4B3.18* was amplified using cen3R-F and cen3R-R primers and digested with *Eco*RI. The resulting 0.6 kb *Eco*RI fragment was cloned into the *Eco*RI site of pBluescript II SK$^+$, creating pTN755, and used to prepare the probe A. A 1.0 kb region near the *ade5$^+$* gene was amplified using ChIIIR-F and ChIIIR-R primers and digested with *Eco*RI and *Xba*I. The resulting 0.8 kb *Eco*RI-*Xba*I fragment was cloned between *Eco*RI and *Xba*I sites of SK$^+$, creating pTN756, and used to prepare the probe C. A 2.2 kb *Hind*III fragment from pREP1 (Maundrell, 1993) was used to prepare the probe LEU2. A 10.4 kb *Hind*III fragment from YIp10.4 (Toda et al., 1984) was used to prepare the probe rDNA.

7. Acknowledgements

8. Supplemental Data

Table S1 The fission yeast strains used in this study

Strain	Genotype
TNF844	h^-, ade6Δ-D, ura4-D18, leu1-32, ChL [ubcp4::LEU2::chk1, ade6-M216]
TNF1383	h^-, ade6Δ-D, ura4-D18, leu1-32, ChL [ubcp4::LEU2::chk1, ade6$^+$]
TNF1610	h^-, ade6Δ-D, ura4-D18, leu1-32, ChL [ubcp4::LEU2::chk1, spcc1322.09::ura4$^+$, ade6$^+$]
TNF2076	h^-, ade6Δ-D, ura4-D18, leu1-32, ChL [ubcp4::LEU2::chk1, kanMX6:spcc4B3.18, spcc1322.09::ura4$^+$, ade6$^+$]
TNF1700	h^-, ade6Δ-D, ura4-D18, leu1-32, rad3::kanMX6, ChL [ubcp4::LEU2::chk1, spcc1322.09::ura4$^+$, ade6$^+$]
TNF1728	h^-, ade6Δ-D, ura4-D18, leu1-32, rad3D2230A, ChL [ubcp4::LEU2::chk1, spcc1322.09::ura4$^+$, ade6$^+$]
TNF1730	h^-, ade6Δ-D, ura4-D18, leu1-32, rad3N2235K, ChL [ubcp4::LEU2::chk1, spcc1322.09::ura4$^+$, ade6$^+$]
TNF1732	h^-, ade6Δ-D, ura4-D18, leu1-32, rad3D2249E, ChL [ubcp4::LEU2::chk1, spcc1322.09::ura4$^+$, ade6$^+$]

Table S2 Nucleotide sequences of the oligonucleotides used in this study.

| Name	Nucleotide sequences
III_1057F	5'-AAACGATAATGGGCTTGAAAATGGG
III_1057R	5'-ATCTTTAATAGGTCCTGAAGCCGGC
III_1062F	5'-AGCAAATATCAATGGTTGCGAATGC
III_1062R	5'-GTTTATCCCAATACTGGAATGCTGC
III_1310A	5'-CAGAATGTCGTTCGATCAATATTGG
III_1310B+ura4AN5	5'-GCCAGTGGGATTTGTAGCTAGGTCTTGTCTA-GTTGATGAGTTTCG
III_1310C+ura4AN3	5'-GGCGTTTTATGTCAGAAGGCTAGAGCAATG-TTAGAGTAGTGAGCG
III_1310D	5'-TTGGTCTTATTCATCATCAACAGG
irc3R7	5'-GTTTATTGAAGATTTAACAATATGC
irc3R8+kanAN3	5'-CGATACTAACGCCGCCATCCTCAATCTTTTT-CTGTAAAGAACAGG
irc3R9+kanAN5	5'-CCCGGCGGGGACGAGGCAAAAAGTCATTG-ATGAATCATGATGC
irc3R10	5'-TTTGTAGCAGAATCTTGGGAAGTCG
irc3-F	5'-CATTAAAAATCAACAAGTCTTGTCC
irc3-R	5'-GAAACATTTTTGAGTGTTGTTCAGG
imr3-in	5'-AAGTTTTGATGCTCAACAAATGGC
imr3-out	5'-GTGGTTAGAATTGGACAAGC
dh	5'-CGTGTAAATAAGGGACAAATAGG
cnt3-L	5'-AACCGCAACAAACGATTAGC
cnt3-R	5'-CGGAATTAGAAAGATTGATGATTTG
cen3R-F	5'-CCGCAGCCGTAATCGTCAAAGATGC
cen3R-R	5'-TGCTTTAACTTATATGAACTATCGG
ChLR-F	5'-CCAAGGATGCCTTATTGGTAATGG
ChLR-R	5'-CCGGAATTCTGACATTTTAATAACG
ChIIIR-F	5'-AGTTATAGGAAACCGACTGAAACCG
ChIIIR-R	5'-TATCGCAAGAGCACTAAAATTCCCG

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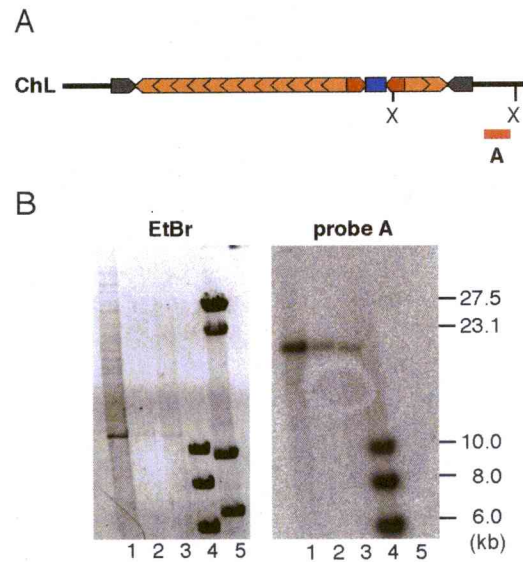
Figure S1 Measurement of the *otr3* repeat number on the right side of cen3.
(A) Positions of *Xho*I restriction sites on the right side of cen3 are indicated. X, *Xho*I.
(B) Chromosomal DNA was prepared from TNF1610, TNF664 and 972 strains, digested
with *Xho*I, separated by PFGE (pulse time: 0.5 to 1.3 sec in 6 V/cm for 16.5 h; 1.0% agarose
gel in 0.5x TBE buffer), transferred onto a nylon membrane, and hybridized with probe
A shown in (A) and in Fig. 2. Lane 1, TNF1610; lane 2, TNF664 (*h-*, *ade6M-210*, *his2*, *Ch16*);
lane 3, 972 (*h-*); lane 4, 1 kb DNA ladder (New England Biolabs); lane 5, *Hin*dIII digested Lambda
DNA. Lengths of the standard are indicated on the right. The restriction fragment detected by
Southern blot was determined to be 21.0 kb. Since the sequence other than *otr3* in this fragment is
9.5 kb in the length, two copies of *otr3* (6.3 kb each) must be present on the right side of cen3.

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10. Achievement

1. 中村健一、中川拓郎、升方久夫
「分裂酵母の人工染色体を用いた染色体安定性の解析系の構築」
第２２回 DNA 複製、分配ワークショップ ポスター発表

2. 中村健一、村上成文、升方久夫、中川拓郎
「分裂酵母を用いた染色体安定性の解析」
日本遺伝学会 第７６回大会 口頭発表

3. 中村健一、村上成文、升方久夫、中川拓郎
「分裂酵母を用いた染色体安定性の解析」
第７６回 日本分子生物学会 年会 ポスター発表

4. 中村健一、村上成文、升方久夫、中川拓郎
「分裂酵母ミニ染色体を利用した染色体安定性の解析」
第２２回 染色体ワークショップ ポスター発表

5. 中村健一、村上成文、升方久夫、中川拓郎
「DNA チェックポイントの染色体の安定な維持における役割」
第 77 回 日本分子生物学会 年会 ポスター発表

6. Ken-ichi Nakamura, Hisao Masukata, Takuro Nakagawa
「DNA checkpoint proteins are required for   chromosome stability in fission yeast」
2006 FASEB summer research conferences, California, USA

7. 中村健一, 加藤由起, 岡本亜矢, 伊藤武彦, 高橋達郎, 升方久夫, 白髭克彦,
中川拓郎
「分裂酵母を用いた染色体の安定維持機構の解析」
第 77 回 日本分子生物学会 年会 ポスター発表