CHROMOSOME BREAKAGE IN YEAST CAUSED BY SISTER-CHROMATID RECOMBINATION IN AN INTEGRATED 2-MICRON PLASMID

by

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A pure yeast plasmid consisting of 2-micron DNA and a 9.4 kb fragment with *HIS4* has been chromosomally integrated at the *his4* locus in a haploid *his4-24* strain. The integration was induced with sodium azide, whereafter colonies were screened for improved maintenance of histidine prototrophy. DNA was prepared from a strain with partially stabilized histidine prototrophy, and restriction fragments containing *HIS4* sequences were analysed. The results were consistent with the notion that the plasmid had integrated by homologous recombination.

Size-selected *Bam*HI restriction fragments from the strain with the integrated plasmid were cloned in *Escherichia coli*. The clones were screened by colony hybridization and the plasmid from one clone which contained 2-micron DNA sequences as well as *HIS4* sequences was mapped. The structure of the insert showed that it was derived from the acentric fragment which is formed when an unequal sister-chromatid recombination occurs between the inverted repeats of the integrated 2-micron DNA. We suggest that this rearrangement frequently takes place, a hypothesis which was supported by the finding of a high rate of lethality in the culture.

This hypothesis was further confirmed by analysis of the genetic consequences of the rearrangement in a diploid. The results show that monocentric fragments are formed by breakage of the dicentric fragment during mitosis. The monocentric fragments are frequently lost, but are also often repaired by recombination or by resynthesis of the missing part of the chromosome, using the homologous chromosome III as a template.

Although some replication and disjunction of the acentric fragment appears to take place, we were able to maintain it by selection only for a limited number of generations.

1. INTRODUCTION

Two-micron DNA is a circular, double-stranded DNA species occurring widely in *Saccharomyces* strains, with about 50 copies per cell. Its molecular biology has been recently reviewed by BROACH (2). Because of its non-mendelian inheritance 2-micron DNA is called a plasmid, although both genetic (22,32) and biochemical (25,31,37) evidence suggests that it resides in the nucleoplasm. It has a size of six thousand base pairs and its most prominent structural feature is a non-tandem inverted repetition of six hundred base pairs, which subdivides the rest of the plasmid into two unique regions. Intramolecular recombination, promoted by the plasmid-coded gene *FLP*, frequently takes place between the two repeated regions. Due to this recombination the wild type plasmid is present in the yeast in equal amounts of two interconvertible forms that differ in the orientation of one unique region with respect to the other. The recombination occurs at a specific site within the repeated region. Two-micron DNA does not have nucleotide sequence homology with the chromosomes at levels detectable by molecular hybridization (4) and the natural plasmid has never been observed to integrate into the chromosomes.

Two-micron DNA or subsegments carrying its origin of replication are widely used as parts of genetic vectors, since they enable the vector to replicate autonomously in yeast.

During experiments on the transformation of yeast without the use of bacterial plasmids (20) we observed a meiotically induced integration of a plasmid consisting of a full copy of 2-micron DNA with a 9.4 kb *Pst*I insert carrying the *HIS4* gene. This integration was due to homologous recombination between sequences of the 9.4 kb *Pst*I fragment and the chromosomal sequences at *his4*.

The original clone from this spore was lost (21), but rather than trying to obtain another occasional meiotically induced integration of the plasmid, we applied recombinogenic treatment in order to induce the integration in vegetative cells. An unstable chromosome with integrated 2-micron DNA was obtained and the nature of this instability is the subject of the present paper. Parts of the results have been reported previously in a summarized form (21).

2. MATERIALS AND METHODS

2.1. Media and growth

The media used for growth of yeast have been described (20). *Escherichia coli* was grown in LB (1% tryptone, 0.5% yeast extract, 1% sodium chloride, 0.1% glucose). Plates were solidified with 2% agar. Ampicillin (Sigma) was used at a concentration of 50 $\mu\text{g}\cdot\text{ml}^{-1}$. Growth of *E. coli* was at 37 °C, except when the strain contained a plasmid derived from the thermosensitive vector pJC75-58, in which case 30 °C or 20 °C were used.

2.2. Plasmids, strains and crosses

The following plasmids were used: pJC75-58 (6); pHH1, which is pBR322 with a full copy of 2-micron DNA inserted in the *Pst*I site, was obtained from H. HARTMANN and J. COLLINS; pC500 and pC502 (17). Yeast strains employed are given in Table I. The *E. coli* strain used was HB101. Crosses and tetrad analysis were carried out according to HAWTHORNE and MORTIMER (14). The two *his4* mutant alleles *his4-24* and *his4-644* were distinguished by their different complementation behavior (10) when mated to strains carrying the *his4C* allele *his4-280*.

2.3. Induction of plasmid integration with sodium azide

The procedure of NILSSON-TILLGREN and KAHN (26) was used: Yeast cells grown to early stationary phase in YPD were washed three times in distilled water and resuspended in 0.1 M-sodium citrate pH 4.75. Sodium azide was added to a final concentration of 50 mM. The cell suspension was shaken in a closed screw-cap tube for 18 hours at 25 °C. The treatment was stopped by adding one tenth volume of 1 M-sodium citrate. Before plating, the cells were washed three times in distilled water.

2.4. DNA purification

The preparation of total yeast DNA (20) and plasmid DNA from *E. coli* (17, 19,38) has been described.

2.5. Transformation

The procedures for transformation of yeast (15) and *E. coli* (5) have been described.

Table I

Yeast strains used in this study

Name	Genotype	Source
C75-M23	<i>MATα ade2 his4-24</i>	Genetic crosses (20)
C78-H23/1	<i>MATα ade2 his4-24</i> [pC504:HIS4]	(20)
C79-S976	Stabilized derivative of C78-H23/1	This study
C79-993	<i>MATα his4-644 leu2 thr4 ura4</i>	Genetic crosses (27)
C82-1799	<i>MATα his4-29 leu2 thr4 trp1 arg4</i>	Genetic crosses
C79-917	<i>MATα his4-29 ura4 arg4-16 trp1</i> [TRP 1]	(21)

2.6. In vitro packaging of DNA and transduction

In vitro packaging of ligated DNA into bacteriophage lambda particles and subsequent transduction into *E. coli* were performed according to COLLINS and HOHN (7). The biological activity of endogenous DNA in the packaging cell extract was destroyed by ultraviolet irradiation.

2.7. Enzymes, electrophoresis and isolation of DNA fragments from agarose gels

Restriction endonucleases, T4 DNA ligase and DNA polymerase I were purchased from Boehringer Mannheim Biochemicals and Bethesda Research Laboratories Inc. Gel electrophoresis of DNA (20) was in 0.4% or 0.7% agarose. Restriction fragments of specific sizes were purified from the agarose by gel electrophoresis into hydroxyapatite (36).

2.8. Colony hybridization

Colony hybridization was carried out according to GRUNSTEIN and HOGNESS (12) with the modifications described by BRANDT (1).

2.9. In vitro labelling of DNA, transfer of electrophoretically separated DNA and hybridization

Labelling of DNA by nick translation was performed as described by RIGBY et al. (30), using ³²P-dTTP (Amersham Radiochemical Centre or New England Nuclear). DNA fragments separated in agarose gels were transferred to nitrocellulose filters (Millipore, HAWP 000 10) by a modification of the procedure of SOUTHERN (33) as previously described (17). Hybridization of labelled DNA to the DNA immobilized on nitrocellulose was performed as described by DEN-

HARDT (8) with the modifications of JEFFREYS and FLAVELL (18). Autoradiographic exposure was for 0.5-72 hours with or without Kodak X-Omatic intensifying screens at -80 °C.

2.10. Containment

Experiments involving recombinant DNA were registered with the committee on genetic engineering of the Danish National Research Councils. Yeast cells containing DNA sequences from pJC75-58 were handled under P1 conditions and *E. coli* cells containing yeast DNA sequences were handled under P2 conditions (13).

3. RESULTS**3.1. Recombinogenic treatment can induce partial stabilization of the unstable His⁺ phenotype associated with a 2-micron DNA-HIS4 plasmid**

The plasmid pC504 was constructed by inserting a 9.4 kb chromosomal PstI fragment from *S. cerevisiae* with *HIS4* into 2-micron DNA at the PstI site (17,20). The plasmid is able to replicate autonomously in yeast, but is lost at a high rate during growth. Even under selective conditions only about a quarter of the cells have retained the plasmid. We have devised a simple plate test which is useful to distinguish colonies with this level of instability from colonies with a more stable prototrophic phenotype: After growth on a histidine-free plate a replica is made onto the complete medium YPD, and the fraction of histidine prototrophic cells in each unstable colony will decrease to a few per cent. Upon a subsequent replica plating from the YPD plate to a new histidine-free plate each unstable colony will give rise to a patch of non-growing cells with growing papillae, while a stable, prototrophic

colony will show up by its confluent growth.

This plate assay was used to screen for stabilization of the His⁺ phenotype after recombinogenic treatment of cells harboring the plasmid, pC504. As a recombinogen, sodium azide was chosen. When used as described in section 2.3, it induces mitotic recombination in a diploid at a high rate without enhancing the reversion frequency of auxotrophic point mutations (26). Ten to the ninth cells of C78-H23/1 (*MAT α ade2 his4-24* [pC504: *HIS4*]) were treated with sodium azide to a survival of 10% and subsequently grown over night in 5 ml of liquid medium without histidine and a sample was plated to screen the colonies for stability as described above. Ten colonies with greatly enhanced stability were picked and tested further. Seven of the clones were completely stable histidine prototrophs and 3 segregated histidine auxotrophs at a low frequency: 3-20% during 7 generations of growth in YPD. These clones were hypothesized to be products of integration of pC504 at the *his4* locus and one of them, C79-S976, was investigated further. The fully stabilized clones may be regarded as results of mitotic gene conversion.

3.2. Restriction endonuclease analysis of the *his4* region of the partly stabilized His⁺ strain C79-S976

Chromosomal integration by homology of pC504 in C78-H23/1 will, if it takes place as a simple cross-over, yield the structure *his4* - 2-micron DNA - *HIS4* or *HIS4* - 2-micron DNA - *his4*. Either of these two configurations would seem consistent with the level of segregation of His⁻ cells exhibited by strain C79-S976. The type of arrangement can be tested by restriction endonuclease analysis of DNA from the yeast, using the 9.4 kb *HIS4* PstI fragment as hybridization probe.

Thus, total DNA was isolated from C79-S976. The culture was grown for about 30 generations in YPD and at the time of harvest a sample was plated on YPD for subsequent replication to histidine-free medium. Thirty-five per cent of the colony-forming units were His⁻. Four His⁺ colonies on the YPD plate were picked, grown up in liquid YPD and plated again on YPD for subsequent replica plating as before. Two still had the partly stabilized phenotype of C79-S976, where-

as the two others were completely stable. Thus the extracted DNA is from a population of roughly equal numbers of *HIS4*, *his4* and partially stable His⁺ cells.

Samples of this DNA were treated with restriction endonucleases EcoRI or BamHI, subjected to electrophoresis and the electropherogram was hybridized with ³²P-labelled *HIS4* DNA (pC502) and autoradiographed (Figure 1). As expected, bands characteristic for both *HIS4* and the 500 bp deletion *his4-24* were seen in C79-S976 (EcoRI digestion, lane h). BamHI does not cut in the cloned 9.4 kb PstI fragment, but leaves this sequence on a fragment about 21 kb long when total wild-type yeast DNA is digested (lane e). Since BamHI neither cuts 2-micron DNA, the expected structure, *HIS4* - 2-micron DNA - *his4-24*, for the partially stabilized cells, should be recognizable by hybridization with labelled *HIS4* sequences as a larger fragment. Since 2-micron DNA has a size of 6.3 kb the fragment should be 21-0.5+9.4+6.3, i.e. about 36 kb. Indeed a band of size 30-40 kb is seen, in addition to the band of about 21 kb (lane d).

3.3. Molecular cloning and mapping of an extended chromosomal sequence linked to 2-micron DNA

In order to investigate the detailed structure of the observed 30-40 kb BamHI fragment it was isolated by size fractionation and subsequent molecular cloning. Five hundred micrograms of DNA from C79-S976 were digested with restriction endonuclease BamHI and fractionated on 0.4% agarose gels. Fragments larger than 23 kb were ligated into cosmid pJC75-58 (6) employing a ratio of 2:1 (w/w) at a DNA concentration of 450 $\mu\text{g} \cdot \text{ml}^{-1}$, after linearization of the cosmid with BamHI. Two micrograms of the ligated DNA were packaged in vitro into bacteriophage λ particles and transduced into *E. coli* (7). Eighty-seven ampicillin resistant transductants were recovered. Colony hybridization with radioactive DNA probes showed that one of the transductants contained a plasmid with 2-micron DNA sequences as well as *HIS4* sequences, while another colony contained *HIS4* sequences only (Figure 2). The plasmid containing both 2-micron DNA and *HIS4* sequences, called pC505,

Figure 1. Detection and sizing of restriction endonuclease fragments carrying sequences belonging to the *HIS4* PstI fragment.

Total DNA from the partially stabilized strain C79-S976 (lanes a, d, and h), transformant *his4-29* [pC505:HIS4] strain C78-H26 (lanes c and g), *HIS4* strain S288C (lane e) and *his4-24* strain C75-M23 (lane f) as well as ccDNA from transformant C78-H26 (lanes b and i) were treated with restriction endonucle-

ases BamHI or EcoRI (lanes d-i) or left untreated (lanes a-c). After electrophoresis at 1.5 V $\cdot$ cm $^{-1}$ in 0.4% agarose (origin at top), the specific sequences were detected by molecular hybridization and autoradiography (33). The radioactive hybridization probe was pC502, a bacterial plasmid carrying the *HIS4* PstI fragment (17).

The 3.0 and 2.65 kb fragments in lanes h and i are characteristic for *HIS4* and the 5.2 kb fragment in lane h is characteristic for *his4-24*. The 21 kb band in lanes d-g is the *HIS4* BamHI fragment, whose mobility is not detectably affected by deletions up to 0.9 kb. By electrophoresis at a higher agarose concentration HOLMBERG et al. (16) estimated the size of this fragment to about 16 kb. For the identity of this and other bands cf. HOLMBERG et al. (16,17).

The 30-40 kb band in lane d indicates a chromosomally integrated state of pC504 in the partially stabilized strain C79-S976.

Figure 2. Screening of bacterial colonies, containing cosmids, for the presence of inserts with nucleotide sequences of the *HIS4* PstI fragment (a) and 2-micron DNA (b).

32 P-labelled probes of pC502 (a) and pHH1 (b) were hybridized to the DNA of the bacteria which had been lysed on nitrocellulose filters (12).

was expected to contain an insert of the structure *his4* - 2-micron DNA - *HIS4*. To confirm this, it was propagated, purified and mapped by restriction endonuclease cleavage. Figure 3 shows an ethidium bromide stained gel with fragments obtained using BamHI, PstI, EcoRI, SalI and Hind-III, singly and in combinations. We were not able to interpret the band pattern on the basis of

Figure 3. Restriction cleavage of plasmid pC505.

One half microgram samples of pC505 were treated with restriction endonucleases BamHI (lane a), BamHI + PstI (lane b), BamHI + EcoRI (lane c), PstI (lane d), PstI + EcoRI (lane e), EcoRI (lane f), Sall (lane g) and HindIII (lane h). The cosmid vector, pC75-58, cleaved with BamHI (lane i) and PstI (lane k) was included. Finally are shown PstI (lane l), EcoRI (lane m), PstI + EcoRI (lane n) and HindIII (lane o) cleaved ccDNA from strain C79-917. This strain contains two sorts of 2-micron DNA, as apparent from lane m. They correspond to those called Sep1 and Sep2 by CAMERON et al. (4). The strong 1.45 kb band in lanes l, m and o is due to the presence of a circularized *TRP1* EcoRI fragment (21) introduced by transformation. Electrophoresis was for 16 hours at 2 V $\cdot$ cm $^{-1}$ in 0.7 % agarose.

TABLE II

Sizes and hybridization behavior of restriction fragments of pC505. The restriction fragment patterns are given in Figure 3 and have been used to construct the map of Figure 5.

BamHI Fig.3, lane a	BamHI+PstI lane b	BamHI+EcoRI lane c	PstI lane d	PstI+EcoRI lane e	EcoRI lane f	Sall lane g	HindIII lane h
kb hybr.to	kb hybr.to	kb hybr.to	kb hybr.to	kb hybr.to	kb hybr.to	kb hybr.to	kb hybr.to
30 ^b 2 μ	10 ^{ab} HIS 4	10 ^b vector	12 ^b vector	6.25 vector	12 ^b vector	30 ^b vector	25 ^b vector
+HIS4	9 vector	4.2 ^a HIS4	9 ^a HIS4	5.0 vector	5.0 vector	+HIS4	+HIS4
12 ^b vector	7.2 2 μ	4.0 2 μ	7.0 2 μ	4.0 2 μ	4.2 ^a HIS4	11 HIS4	4.05 ^a HIS4
	3.05 ^a none	3.05 ^a vector	4.0 vector	3.1 ^a HIS4	4.0 2 μ	+2 μ	+2 μ
	2.15 vector	+HIS4	2.05 vector	2.7 ^a vector	3.05 ^a HIS4	1.53 ^a HIS4	2.7 2 μ
	0.92 vector	2.7 ^a HIS4		2.7 ^a +HIS4	2.7 ^a HIS4		1.28 ^a 2 μ
		2.35 2 μ		2.4 2 μ	2.35 2 μ		0.78 2 μ
		1.88 ^a none		2.1 vector	1.05 ^a HIS4		0.69 ^a HIS4
		1.08 ^a HIS4		1.2 ^a none	+2 μ		0.62 ^a HIS4
		+2 μ		0.85 ^a HIS4			0.46 ^a HIS4
							0.39 ^a HIS4

^a Band intensity indicates that more than one fragment of this size is formed from each plasmid molecule.

^b Approximate size.

Figure 5. Cleavage map of pC505, derived from the data of Table II.

B, H, R, S and P designate cleavage sites for restriction endonucleases BamHI, HindIII, EcoRI, Sall and PstI, respectively. Arrows indicate position and orientation of the natural inverted repeat in 2-micron DNA. Continuous lines designate yeast chromosomal sequences; the thick lines sequences of the *HIS4* PstI region. The broken line means 2-micron DNA and the dotted line shows where the sequence of cosmid pJC75-58 is. The orientation of this sequence has been chosen arbitrarily, as it could not be determined from the data.

Figure 4. Determination of the origin of restriction endonuclease fragments of plasmid pC505.

Three gels identical to that shown in Figure 3 were set up. Molecular hybridization with ^{32}P -labelled DNA probes (33) and subsequent autoradiography revealed the presence of specific sequences in each band. The lanes with the HindIII digest are shown. a: Staining with ethidium bromide. b: Hybridization with the *HIS4* PstI fragment cut out from pC502 and purified. c: Hybridization with a full copy of 2-micron DNA cut out from pHH1 and purified. d: Hybridization with the cosmid vector pJC75-58.

the assumption that the insert present in pC505 is the primary product of integration of pC504 at *his4*. It was therefore decided to hybridize identical electropherograms with each of the following probes: ^{32}P -labelled DNA of the 9.4 kb PstI fragment containing *HIS4*, ^{32}P -labelled pJC75-58 and ^{32}P -labelled 2-micron DNA sequences. The origin of each band could then be determined and is given in Table II. As an example for the identification of the bands, the HindIII lane of the stained gel is presented together with the HindIII lanes of the autoradiograms for the three hybridizations in Figure 4.

The map of pC505 shown in Figure 5 could then be constructed. The cloned BamHI fragment does not have the structure of a primary product of chromosomal integration of pC504. It does have some of the expected features: Two regions with sequences corresponding to the *HIS4* PstI fragment bracket a region of 2-micron DNA sequences and are flanked by regions hybridizing with neither 2-micron DNA nor the *HIS4* PstI fragment. However, both *HIS4* PstI regions carry the wild type allele, i.e. *his4-24* is absent. Furthermore, the two non-hybridizing chromosomal regions have identical maps, which together with the *HIS4* PstI regions and part of the 2-micron DNA sequences form an inverted repeat bracketing the rest of the 2-micron DNA. Thus, the 2-micron DNA sequence is not a simple copy of the plasmid. When pC504 was made, one of the unique regions of the 2-micron DNA was split into two parts by the insertion of the *HIS4* PstI fragment (17,20). In the cloned BamHI fragment (Figure 5) one of these two parts is present twice, being part of the large inverted repeat of pC505, whereas the other is missing. The other unique region is undisturbed and is the only sequence that has remained unique in the cloned BamHI fragment.

As might be expected, pC505 can transform yeast to histidine independence at the frequency characteristic of self-replicating plasmids.

3.4. Sister-chromatid recombination involving integrated 2-micron DNA

The sequence arrangement of the cloned BamHI fragment has evidently arisen by a recombinational event at the inverted repeat regions of 2-micron DNA linked to *HIS4* se-

Figure 6. Mitotic sister-chromatid recombination involving integrated 2-micron DNA.

Some restriction endonuclease cleavage sites are shown and abbreviated as in Figure 5. (R) means one of the two possible positions for the *EcoRI* site in the invertible part of the 2-micron DNA sequences. The small arrows indicate position and orientation of the natural inverted repeat in 2-micron DNA. C, B and A designate the functional regions of the *HIS4* gene. Thick line and broken line mean sequences of the *HIS4* PstI region and of 2-micron DNA, respectively.

quences. It is extremely unlikely that this recombination should have taken place in *E. coli*. First, two different *HIS4*-carrying cosmids would have to transduce the same cell and the multiplicity of transduction was low: Only two colonies with *HIS4* sequences were obtained from 10^9 cells. Secondly, recombination at the inverted repeat regions of a 2-micron DNA sequence is infrequent when they are in *E. coli* (cf. refs. 11,17). Rejecting this possibility we conclude that the cloned *Bam*HI fragment was cut out from a molecule existing in the C79-S976 culture. We propose that this DNA molecule was an acentric fragment derived from chromosome III by the recombinational event shown in Figure 6. The position of the *EcoRI* site in the repeated, non-hybridizing region of pC505 is identical to the position of an *EcoRI* site in the corresponding region adjacent to the *HIS4* PstI region (16). This fact, as well as the absence from the non-hybridizing region of other cleavage sites for the used enzymes, is consistent with the identity of the two regions. Unfortunately, the *SalI* site right

of the *HIS4* PstI region was indicated erroneously on the map of HOLMBERG et al. (16). It should be placed 3.5 kb further to the right as apparent from the data given in the same paper.

As will be discussed, the finding of the cloned *Bam*HI fragment does not mean that all 30-40 kb fragments detected in Figure 1 must have the same structure; most of them could have the structure of a primary product of chromosomal integration.

3.5. Frequent lethality in strain C79-S976

The sister chromatid recombination proposed in Figure 6 may well be expected to have lethal consequences at the following cell division. First, sequences distal to *his4* will be lost from one daughter cell and, secondly, the dicentric fragment will be in trouble, when its two centromeres move to opposite spindle poles.

Single cells of two subclones of strain C79-S976 were isolated on agar slabs by micro-manipulation and it was determined how many were able to form colonies. A comparison was

Table III

Influence of integrated 2-micron DNA on the survival of single cells isolated by micromanipulation.

Culture	Single cells isolated	Colonies formed	Fraction of dead cells
C79-S976, subclone 1	128	90	0.30
C79-S976, subclone 2	72	57	0.21
C75-M23 (control)	76	72	0.05

made to C75-M23, the strain that by transformation to His⁺ became C78-H23/1, i.e. a strain that should be isogenic to C79-S976, except for the integrated pC504 in the latter. Table III shows that there is several-fold higher lethality in C79-S976 than in C75-M23.

3.6. Mitotic segregation of linked genes

One way to follow the genetic consequences of events that are lethal in a haploid is to look at the event in a diploid.

C79-S976 was crossed to C79-993 (*MATa his4-644 leu2 thr4 ura4*), so that the segregation of four markers on chromosome III, two on each side of the centromere, could be followed (cf. Figure 7). The resulting diploid clones segregated His⁻ colonies at a much higher rate than did C79-S976 (Table IV). Clone 4 is interpreted as the product of mating between the tester strain C79-993 and a completely stabilized His⁺ derivative of C79-S976. The high segregation rates in the first three clones suggests that the *HIS4* gene is in most cases lost by an event which is lethal in a haploid, but not in a diploid. As already discussed, the sister-chromatid recombination depicted in Figure 6 is expected to be such an event. In addition to *his4*, other genes on chromosome III segregated, i.e. other wild type genes than

Figure 7. Map of chromosome III of *S. cerevisiae*. Only relevant markers are shown.

HIS4, on the chromosome with the integrated 2-micron DNA, were lost. The unlinked genes *ura4* and *ade2* segregated at normal, low frequency due to mitotic crossing over and gene conversion, although, by incidence, one of the clones was Ura⁻. Table IV shows that *LEU2*, which is located on the same arm as *HIS4*, was lost at a rate of more than 10% during the 6 generations and in more than half of the cases *THR4*, located on the other arm, was concomitantly lost. In order to obtain more information on the behavior of the right arm of chromosome III, some of the His⁻Leu⁻ colonies and some of the His⁻Leu⁺ colonies were picked and tested for mating type. The results are given in Table V. In most cases, when a colony was His⁻Leu⁻Thr⁺, it was a non-mater, whereas all His⁻Leu⁻Thr⁻ colonies were MAT^a. Furthermore, all His⁻Leu⁺ colonies were Thr⁺ and non-maters. The results of Tables IV and V can be summarized as follows: The main classes of segregants are His⁻Leu⁺ NM Thr⁺, His⁻Leu⁻ NM Thr⁻ and His⁻Leu⁻ MAT^a Thr⁻. While the first class may comprise the results of several different types of events, including the excision of pC504, the most obvious interpretation of the last class is the complete loss of the chromosome which carried 2-micron DNA. The most interesting class may be the His⁻Leu⁻ NM Thr⁺ segregants, which are still euploid, as evidenced by their non-mating phenotype. The *LEU2* gene, proximal to *HIS4*, has been lost, while MAT^a and *THR4* on the other arm have been retained. It raises the possibility that the dicentric fragment formed by sister-chromatid recombination (Figure 6) in the subsequent mitosis is broken into two monocentric fragments, one to each progeny cell, and that the monocentric fragments can get repaired in some way. It seemed of interest to find out whether the repair had resulted in the formation of a complete chromosome III and tetrad analysis was chosen as an obvious way to get information on this point. The two minor classes of His⁻Leu⁻Thr⁺ colonies expressing mating type may be due to mitotic recombination.

3.7. Tetrad analysis of subclones of the diploid containing integrated 2-micron DNA

Several NM Thr⁺ subclones of the clones presented in Tables IV and V were subjected to tetrad analysis. In some subclones the spore

Table IV

Mitotic segregation of phenotypes in the diploid C79-S976 (*MAT α HIS4 - 2 μ - his4-24 ade2*) $\times$ C79-993 (*MATa his4-644 leu2 thr4 ura4*) after 6 generations in non-selective medium.

Diploid clone no.	No. of colonies	His ⁺ Leu ⁺ Thr ⁺	His ⁺ Leu ⁺ Thr ⁺	His ⁺ Leu ⁻ Thr ⁺	His ⁺ Leu ⁻ Thr ⁻	Ura ⁻	Ade ⁻
1	1284	129	1016	50	89	1284	0
2	1663	166	1322	93	82	4	1
3	1765	86	1402	96	181	3	0
4	1735	1735	0	0	0	1	0

viability was lower than normal, but it seemed independent of the His and Leu phenotypes; most importantly, none of the subclones showed a 2:2 segregation for viability. The analysis of three subclones of clone 1, giving normal spore viability (81%, 94% and 91%, respectively) are presented in Table VI.

Subclone A is of the class, His⁺Leu⁻ NM Thr⁺, supposed to have undergone repair of a monocentric fragment. The data show that the repair resulted in a full, functional chromosome III with the constitution *his4-644 leu2 MATa THR4*, i.e., a break of the dicentric fragment occurred between *MATa* and *LEU2*. This break produced a presumably monocentric fragment which was repaired either by mitotic recombination with the homologue at the four-strand stage or by re-synthesis of most of the left arm of chromosome III, using the homologue as a template.

Subclone B, of the phenotype His⁺Leu⁺ NM Thr⁺, turned out to be more trivial. Here *his4-24* is still present, so it can be interpreted as the product of the excision of pC504, either by loop-out or by an unequal sister-chromatid exchange between a *HIS4* PstI region and a *his4* PstI region. Segregants of the phenotype His⁺Leu⁺ NM

Thr⁺ should also occur via the dicentric fragment, this being broken between *LEU2* and *his4-24* and subsequently repaired. They would give *his4-644* spores only.

Subclone C was like its parental clone 1 in its mitotic segregation of His⁺, Leu⁻ and Thr⁻. The tetrad analysis showed the presence of all three *his4* alleles. The 3 asci, with *his4-644* spores only, are interpreted to result from mitotic segregants in which the dicentric fragment was broken distal to *LEU2* and subsequently repaired. The tetrads with 2 *his4-644* spores are interpreted as products of plasmid excision, leaving behind either *his4-24* or *HIS4*.

The tetrad analyses suggest that the original cell of clone 1, as well as that of subclone C, had the structure *HIS4* - 2-micron DNA - *his4-24* at the *his4* locus and that the dicentric fragment formed in some of the cells (Figure 6) can be broken into fragments that are repaired by recombination or extensive DNA synthesis using the homologous chromosome as template.

3.8. Fate of the acentric fragment?

It was speculated that the acentric fragment carrying *HIS4* might be able to replicate in each

Table V

Phenotypes of a sample of the histidine auxotrophic segregants presented in Table IV.

Diploid clone no.	His ⁺ Leu ⁻ colonies picked	Leu ⁻ Thr ⁺ NM	Leu ⁻ Thr ⁺ MATa	Leu ⁻ Thr ⁺ MAT α	Leu ⁻ Thr ⁺ MATa	His ⁺ Leu ⁺ colonies picked	Leu ⁺ Thr ⁺ NM
1	76	19	1	1	53	10	10
2	69	27	0	3	34	19	19
3	87	29	3	0	52	59	59

Table VI

Tetrad analysis of three subclones of the diploid C79-S976 (*MAT α HIS4* - 2 μ -*his4-24 ade2*) $\times$ C79-993 (*MATa his4-644 leu2 thr4 ura4*)

Subclone A, phenotype His ⁻ Leu ⁻ Thr ⁺ , 13 tetrads					
	4:0	3:1	2:2	1:3	0:4
<i>his4-644/his4-24</i>	13				
<i>LEU2/leu2</i>					13
<i>THR4/thr4</i>			13		
<i>ADE2/ade2</i>			13		
<i>MATa/MATα</i>			13		
Subclone B, phenotype His ⁺ Leu ⁺ Thr ⁺ , 13 tetrads					
	4:0	3:1	2:2	1:3	0:4
<i>his4-644/his4-24</i>			13		
<i>LEU2/leu2</i>			13		
<i>THR4/thr4</i>			13		
<i>ADE2/ade2</i>		1	12		
<i>MATa/MATα</i>			13		
Subclone C, Phenotype His ⁺ Leu ⁺ Thr ⁺ , 20 tetrads					
	4:0	3:1	2:2	1:3	0:4
<i>HIS4/his4</i>		1	13 ^a		6 ^b
<i>LEU2/leu2</i>			19	1	
<i>THR4/thr4</i>			20		
<i>ADE2/ade2</i>			20		
<i>MATa/MATα</i>			20		

^a All were 2 *HIS4* (stable) : 2 *his4-644*.

^b Three tetrads were 4 *his4-644* : 0 *his4-24* and the other three were 2 *his4-644* : 2 *his4-24*.

S phase and that the copies might be able to disjoin more or less randomly into progeny cells. If that were the case, the acentric fragment should be maintainable by selection. To test this possibility we looked, in subclone C of the diploid analysed above, for cells that had lost *LEU2*, *MATa* and *THR4*, but had retained *HIS4*. In order to select for such cells, the experiment was carried out as a mating rescue. Thus, subclone C (*Ura*⁻) was mated to C82-1799 (*MATa his4-29 leu2 thr4 trp1 arg4*). None of the alleles *his4-29*, *his4-24* and *his4-644* complement one another. *Ura*⁺ *Trp*⁺ *Arg*⁺ *His*⁺ triploids (or rather 3n-1) could now be selected on minimal medium with leucine and threonine as the products of loss of *MATa* in subclone C and a subsequent mating of the resulting *MATa* cell with a cell of C82-1799.

Most of the triploids turned out to be *Leu*⁻*Thr*⁻, as expected for the complete loss of the dicentric fragment. As the plate did not contain histidine, there should be a strong selection for cells with the acentric fragment carrying *HIS4*. The colonies were of two classes: A trivial class of large colonies with a stable *His*⁺ phenotype; they were regarded as the result of gene conversion of *his4* by the *HIS4* gene of the acentric fragment. The other class consisted of small colonies. Upon re-streaking onto plates lacking histidine, only slight growth occurred and replica plating to new histidine-free plates gave small papillae that stopped growing. Repeated streaking yielded no growth at all, whereas streaking onto histidine-containing medium gave normal growth. Occasionally fast-growing sectors appeared in the first re-streakings; they had a stable *His*⁺ phenotype like the large colonies. We find it likely that the small colonies originate from cells with the acentric fragment. It is not clear why the fragment can be passed on to progeny cells for some generations and not later on. Attempts to recover the acentric fragment by its internuclear transfer in a *kar* cross (27) gave the same result.

4. DISCUSSION

As previously reported in summarized form (21), the present study shows that 2-micron DNA can integrate into a yeast chromosome if it has been joined in vitro to a sequence providing homology and that the integrated plasmid can give rise to a chromosomal rearrangement as depicted in Figure 6. We have furthermore studied the genetic consequences of having an integrated copy of 2-micron DNA in order to obtain information on the behavior of the chromosomal fragments formed.

4.1. Chromosomal rearrangement caused by integrated 2-micron DNA

The cloning of the *Bam*HI insert found in pC505 is evidence for the presence of the acentric fragment in the culture and thereby for the occurrence of the rearrangement depicted in Figure 6. This evidence is supported by the finding of high lethality in C79-S976 and the segregation of chromosome III markers in the diploid C79-S976 $\times$ C79-993. The rates of these events are consistent with the view that a large proportion of

the cells of the C79-S976 culture had undergone the sister-chromatid recombination (Figure 6). It is likely that a fairly large proportion of the 30-40 kb BamHI fragments which we found to contain *HIS4* sequences (Figure 1) had been cut out from the acentric fragment and the dicentric fragment, but that still the majority had the simpler structure of the primary product of integration of pC504.

The rate of the unequal sister-chromatid recombination is much higher than usually found for similar mitotic events, such as uneven sister-chromatid recombination in the *RDN1* cluster (29,35) or loss of an integrated plasmid (15). We have proposed (21) that this is due to the high efficiency of the *FLP* promoted recombination system, specific for the inverted repeat of 2-micron DNA (3), and which we suppose also works on integrated sequences. This view has recently been confirmed by FALCO et al. (9), who selected for chromosomal integration of plasmids containing either of the two repeated regions of 2-micron DNA. Such an integration does not by itself allow an unequal sister-chromatid recombination, but in strains with the natural 2-micron DNA plasmid ([*cir*⁺] strains), FALCO et al. (9) detected the additional integration of a full copy of 2-micron DNA into the insert. In that situation, three copies of the repeated sequence of 2-micron DNA are present in the chromosome in alternate orientation, allowing the unequal, inverted sister-chromatid recombination to take place. Only in strains with *FLP* function did FALCO et al. (9) observe the events which they interpreted as the result of the sister-chromatid recombination.

4.2. Behavior of the dicentric fragment

Already before the sister-chromatid recombination, in S or G2 phase, the two centromeres ending up in the dicentric chromosome must have been predestined to go to opposite poles in the following mitosis, since the chromatids of *different* chromosomes segregate together as a function of the generation in which they were synthesized (39). It may therefore be expected that, in every cell where the dicentric fragment is formed, the two centromeres will try to go to opposite poles. In maize endosperms this situation leads to breakage of the dicentric fragment and

fusion of the broken ends after DNA replication, whereby a new dicentric fragment is formed (23). Whether this full cycle can take place in our case is unknown. We have found, however, that in the diploid, the chromosome with 2-micron DNA is frequently lost. Also, in many segregants, we observed the loss of *LEU2* and the subsequent repair of the chromosome into its normal structure, i.e., with no lethal deletions etc. The repair resulted in a chromosome arm containing the *leu2* and *his4-644* information from the homologue. We interpret this to be due to the recombinogenic effect of free ends of double-stranded DNA found by ORR-WEAVER et al. (28) in studies of plasmid integration. McCUSKER and HABER (24) have found evidence that the recombinogenic effect of a free DNA end at the *MAT* locus can lead to the repair of chromosome III by recombination with the homologue or by copying the right arm of the homologue. FALCO et al. (9), studying the genetic effects of the integration of 2-micron DNA sequences at *ura3* and *leu2* have obtained effects, on those loci and on linked markers, similar to those described here. Like FALCO et al., we suggest that the broken DNA end invades the homologous chromosome and uses it either for recombination or as a template for the resynthesis of the large, missing part of the chromosome. We cannot distinguish the two types of mechanism.

4.3. Behavior of the acentric fragment

It was not possible to maintain the acentric fragment with *HIS4* by selective growth, i.e., in the absence of histidine. It is not known why some growth on the histidine-free plates occurred after the mating designed to rescue the acentric fragment. Some replication and disjunction of a *HIS4* carrying genetic element must have taken place, since each small colony could give rise to several papillae, but then growth stopped. Whether this stop was due to defective replication or increased failure of disjunction is not known. Small artificial, linear plasmids that have presumably no centromeric function are easily maintainable in yeast (34). One possible ad hoc interpretation of our results is that large chromosome fragments that do not contain a centromere can be pulled into the buds of a zygote, but not into the buds of normal vegetative cells.

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