"Break Copy" Duplication: A Model for Chromosome Fragment Formation in *Saccharomyces cerevisiae*

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ABSTRACT

Introduction of a chromosome fragmentation vector (CFV) into the budding yeast *Saccharomyces cerevisiae* results in a targeted homologous recombination event that yields an independently segregating chromosome fragment (CF) and an alteration in the strain's karyotype. Fragmentation with an acentric CFV directed in a centromere-proximal orientation generates a CF that contains all sequences proximal to the targeting segment and results in loss of the endogenous targeted chromosome to yield a 2N-1+CF karyotype. In contrast, fragmentation with a centric CFV directed in a centromere-distal orientation generates a CF that contains all sequences distal to the targeting segment and retention of the endogenous targeted chromosome to yield a 2N+CF karyotype. We have termed this phenomenon "break copy" duplication. Using yeast strains in which the centromere had been transposed to a new location, it was demonstrated that the centromere inhibited break copy duplication. These data suggest that CF formation is the product of an unscheduled DNA replication event initiated by the free end of the CFV and is analogous to a "half" double-strand break gap-repair reaction. We suggest that break copy duplication may have evolved as a mechanism for maintenance of ploidy following DNA breakage.

THE maintenance of ploidy is essential for survival of a species. Mechanisms exist to ensure that during mitosis daughter cells receive identical sets of chromosomes. Mechanisms also exist to ensure maintenance of ploidy after DNA damage. Double-stranded breaks (DSB), for example, must be efficiently repaired to avoid catastrophic results. It is generally believed that a DSB is repaired by a process that requires homologous recombination (RESNICK 1976). The DSB repair model for recombination suggests that following exonuclease digestion, a free end of the DSB invades an homologous region on the sister chromosome and initiates a unidirectional replication fork. This continues until the migrating D-loop reaches the other free end of the DSB where a new and opposing replication fork is initiated. Once replication is complete, the two predicted "Holliday" junctions resolve and the DSB is effectively repaired (HOLLIDAY 1964; SZOSTAK *et al.* 1983).

When exogenous linear DNA with homologous free ends is introduced into yeast, it integrates into the genome with high efficiency (ORR-WEAVER *et al.* 1981; ORR-WEAVER and SZOSTAK 1983). It is conceivable that the presence of these exogenously added homologous free ends is similar to the presence of an endogenous chromosomal free end that results after a DSB. Yeast cells, in fact, will attempt to repair the exogenous free end by homologous recombination. The observation that free ends are highly recombinogenic has been extraordinarily beneficial, permitting easy DNA-mediated manipulation of the yeast genome, including gene disruptions, replacements, and transplacements (reviewed by ROTHSTEIN 1991). Methods have also been developed for creating large terminal deletions that permit the generation of chromosomal variants useful for defining the minimal set of structural elements necessary for proper chromosome replication and efficient segregation (SUROSKY and TYE 1985; MURRAY *et al.* 1986; MURRAY and SZOSTAK 1986; SUROSKY *et al.* 1986). Further modification of these techniques has permitted the physical positioning of cloned DNA fragments on yeast chromosomes and on yeast artificial chromosomes (YACs) (VOLLRATH *et al.* 1988) in a process termed "chromosome fragmentation." Chromosome fragmentation has since become a standard tool for generating YAC deletion series useful in gene mapping and in the determination of exon structure (PAVAN *et al.* 1990; DASGUPTA *et al.* 1993).

In this article, we analyze the basis for observations originally made using chromosome fragmentation to physically map genetic loci on yeast endogenous chromosomes. The primary observation was that the fate of a targeted chromosome varied depending upon whether chromosome fragmentation was directed towards (proximal to) or away from (distal to) the centromere (*CEN*) (VOLLRATH *et al.* 1988). The chromosome fragmentation vectors used in these studies are similar to

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those described by VOLLRATH (1988). Briefly, the CFVs contain unique cloning sites, the yeast selectable marker *URA3,* the ochre-suppressing tRNA gene *SUP11* for visual monitoring of chromosome fragment stability, and sequences from the Y′ subtelomeric repeat for recombination and healing to yeast telomeres. To generate a chromosome fragment, a unique DNA segment is subcloned into the CFV; the CFV is then linearized between the DNA segment and the Y′ element with a unique endonuclease, transformed into yeast, and stable Ura+ colonies are selected. The chromosome fragments generated in this manner are linear DNA molecules that contain a centromere, telomeres at both ends, and consist of all DNA sequence from the site of fragmentation to the telomere (VOLLRATH *et al.* 1988).

There are several models that might explain how chromosome fragments are generated. They include the following: (1) gene conversion of all sequences between the unique chromosomal target segment and the chromosomal telomere onto the CFV, and (2) a double crossover event (reciprocal recombination) between the chromosome and the CFV, with one crossover occurring at telomere sequences and the second crossover occurring at the target segments. Although either of these two models seems plausible, neither can adequately account for all of the chromosome fragmentation products described here. A third possibility is that CF formation results from a replicative repair event initiated after strand invasion by the CFV. In this article, we provide evidence that CF formation results from duplication of the chromosome and is therefore more reminiscent of a replicative repair model than either a gene conversion or cross-over model. We have termed this phenomenon "break copy" duplication. Other groups, including VOELKEL-MEIMAN and ROEDER (1990a,b) and MALKOVA *et al.* (1996) have proposed similar DNA replicative repair models, referred to as "break and replicate" and "break-induced replication," respectively, to explain their results. VOELKEL-MEIMAN and ROEDER (1990a,b) demonstrated that introduction of a *HOT1* site resulted in an increase in mitotic recombination and tracts of gene conversion that extended up to 77 kb from the *HOT1* site; while MALKOVA *et al.* (1996) demonstrated that cleavage of chromosome III at the *MAT***a** locus by the HO endonuclease often resulted in homozygosity of all markers distal to the break. In both cases, extensive amounts of DNA replication must have occurred. While we suggest that CFs are formed by an unscheduled DNA replication event, additional evidence is presented demonstrating that chromosome duplication occurs primarily in a centromere distal, but not in a centromere proximal direction, because in a proximal direction unscheduled DNA replication is inhibited by the centromere. We further suggest that break copy duplication and inhibition of break copy duplication by the centromere might represent a biologically important mechanism for maintenance of ploidy following a double-strand break.

MATERIALS AND METHODS

Yeast strains and transformations: All yeast strains used in this study, listed in Table 1, were congenic derivatives of S288C. The "transpocentric" strain, J101-T55A, was obtained from S. ROEDER and is described elsewhere (LAMBIE and ROEDER 1986, 1988). The "heterotranspocentric" strain, YDM10, was constructed by mating J101-T55A with YPH47. Media and growth of yeast strains were as described (ROSE *et al.* 1990). All yeast cultures were grown at 30°. Transformation of yeast with DNA was performed by the lithium acetate procedure as previously described (ITO *et al.* 1983).

Chromosome fragmentation vectors: The CFVs, acentric pYCF1 and centric pYCF2 are derivatives of YRp14 (GERRING *et al.* 1991). They are the precursors, respectively, to the CFVs, pYCF3 and pYCF4, whose construction has been previously described (VOLLRATH *et al.* 1988). The fragments, D8B and H9G, used for constructing the D8B CFVs and H9G CFVs, respectively, were obtained from C. NEWLON (see NEWLON *et al.* 1991). These fragments, derived from chromosome III, are *Bam*HI restriction fragments generated during the construction of a restriction map of a circular derivative of chromosome III. To construct the D8B CFVs, a 5.0-kb *Bgl*II fragment from a D8B-containing vector was subcloned into the *Bam*HI/*Bgl*II sites of either pYCF1 or pYCF2. The resultant unique *Bgl*II site, which is located between the Y′ subtelomeric repeat and the D8B fragment in the pYCF vectors, was used was for linearization before transformation. To construct the H9G CFVs, a 1.5-kb *Bgl*II fragment from a H9G containing vector was subcloned into the *Bam*HI/*Bgl*II sites of either pYCF1 or pYCF2. The D8B isochromosome fragmentation vector was constructed by subcloning two identical 5.0-kb *Bgl*II fragments from D8B into the *Bam*HI/*Bgl*II sites of pYCF2. The fragments were oriented such that *Bgl*II was unique and lay between the two D8B fragments.

Chromosome fragmentation and pulsed-field gel analysis: Chromosome fragmentation was performed as described (VOLLRATH *et al.* 1988; GERRING *et al.* 1991). Typically, 3 μg of linearized DNA was transformed into yeast cells. Transformants were selected and colony purified by plating on selective, minimal media plates. In experiments involving the heterotranspocentric strains, it was observed that continuous selection for the CF resulted in the formation of stable CFs derived from fragmentation events where an acentric CF had been generated. We speculate that gene conversion of the *CEN* from the other chromosome III resulted in the conversion of unstable acentric CFs to stable monocentric CFs. To avoid this problem, single colonies were selected directly off of the transformation plate and grown in nonselective conditions to permit loss of unstable acentric CFs. DNA was prepared for pulsed-field gel electrophoresis as described (VOLLRATH *et al.* 1988; GERRING *et al.* 1991) and analyzed on either a BRL Hex-A-Field contour-clamped homogeneous electric field apparatus or a home-made orthogonal-field-alternating gel apparatus (SCHWARTZ and CANTOR 1984).

RESULTS

Restructuring yeast chromosomes using centric and acentric chromosome fragmentation vectors: The general method of chromosome restructuring at defined sites in the yeast genome by chromosome fragmenta-

TABLE 1

***S. cerevisiae* yeast strains**

Yeast strain	Genotype	Reference
YPH34	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1*	This study
YPH45	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ1*	SIKORSKI and HIETER (1989)
YPH47	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1*	SIKORSKI and HIETER (1989)
YPH49	*MAT***a**/*α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1*	GERRING *et al.* (1991)
J101-T55A	*MAT***a** *ura3-52 his3-11,15 his4-260 T5 ade1 lys1-1 arg4-17 leu2-3,112 ΔCEN3 PGK1 bik1::CEN3-211(oriR)*	LAMBIE and ROEDER (1986, 1988)
YDM10	*MAT***a**/*α ura3-52/ura3-52 LYS2/lys2-801 his3-11,15/HIS3 his4-260/HIS4 T5 ade1/ADE1 ADE2/ade2-101 arg4-17/ARG4 leu2-3,112/LEU2 TRP1/trp1-Δ1 ΔCEN3/CEN3 bik1::CEN3-211(oriR)/BIK1*	This study

tion is diagrammed in Figure 1. A unique DNA segment is cloned in both orientations into a "centric" (centromere containing) CFV and an "acentric" (centromere lacking) CFV. The resultant four vectors have a unique endonuclease site between the telomeric segment and the subcloned unique DNA segment to allow linearization of the recombinant vectors before transformation into yeast cells. The two orientations (ori) of the unique DNA segment, labeled ori-p (for *CEN*-proximal) and ori-d (for *CEN*-distal), denote the orientation of the recombinogenic end with respect to the centromere of the endogenous target chromosome. In effect, the linear molecule contains a "one-ended" double-strand break upon transformation into yeast, such that the telomeric end is essentially blocked from undergoing integrative recombination, while the unique end is recombinogenic. Homologous recombination of either an acentric or centric CFV derivative in the ori-d configuration is expected to yield a chromosome fragment carrying all sequences distal to the unique target segment. In the ori-d configuration, the acentric CFV is expected to yield an acentric (unstable) CF, while the centric CFV will yield a monocentric (stably segregating) CF. Similarly, homologous recombination of either an acentric or centric CFV derivative in the ori-p configuration is expected to yield a CF carrying all sequences proximal to the unique target segment. In the ori-p configuration, the acentric CFV would yield a monocentric (stable) CF, while the centric CFV would yield a dicentric (unstable) CF. Thus, for any unique DNA segment in the genome, only the acentric CFV ori-p and the centric CFV ori-d configurations are expected to yield stably segregating monocentric CFs.

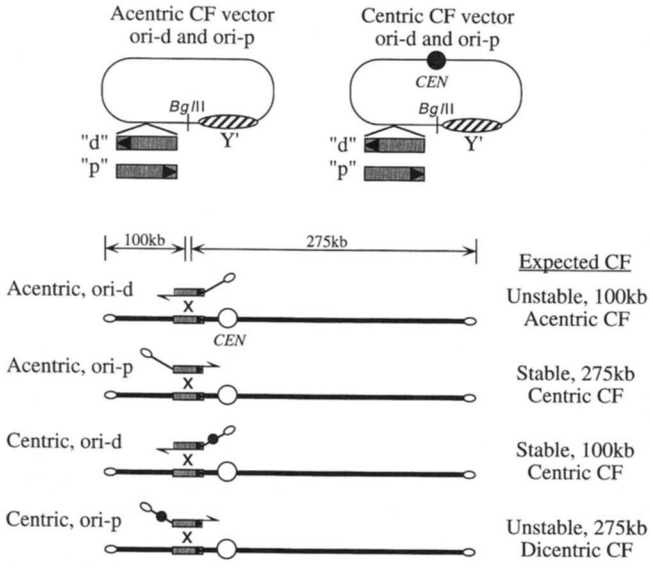

FIGURE 1.—Schematic of chromosome fragmentation vectors and expected products. A unique DNA segment is subcloned in both the proximal (ori-p, p) or distal (ori-d, d) orientations into either an acentric CFV or a centric CFV. The CFVs are linearized with the unique endonuclease *Bgl*II that cleaves between the unique DNA segment and the subtelomeric Y′ repeats. Transformation into yeast is expected to yield stable CFs only with the acentric (ori-p) CFV and the centric (ori-d) CFV. Transformation with the acentric (ori-d) should yield a highly unstable acentric CF, and transformation with the centric (ori-p) should yield a highly unstable dicentric CF. Distances shown are for fragmentation events at D8B, discussed below. ▨, unique DNA element D8B (black triangle indicates genomic orientation); ○, endogenous chromosomal *CEN;* ●, vector *CEN;* open oval, telomere; stippled oval, subtelomeric Y′ repeat.

Fates of haploid and diploid cells differ after transformation with acentric and centric chromosome fragmentation vectors: Early observations from this laboratory with CFVs used to fragment at the ARG4 (chromosome VIIIR), *CEN4*-adj, (adjacent to *CEN4*; chromosome IVL) or RNase H (chromosome XIIIR) loci indicated that the fate of haploid and diploid cells differed when transformed with acentric (ori-p) or centric (ori-d) chromosome fragmentation vectors (Table 2). In cases of chromosome fragmentation by acentric CFVs, transformation efficiencies were consistently low (zero to 10 colonies/μg input DNA) in haploid cells and high (>100 colonies/μg input DNA) in diploid cells. Tetrad analysis of stably transformed diploid strains demonstrated that most transformants could

TABLE 2

Varying fate of haploid cells fragmented with centric versus acentric fragmentation vectors

Acentric/Centric fragmentation vector	Fragmenting site	Chromosomal location	Transformation efficiency[a]		Spore viability of individual transformants[b,c]	
			Haploid	Diploid	4:0	2:2
Acentric ori-p	*RNase H*	XIIIR	Low	High	0/6	6/6
Acentric ori-p	*ARG4*	VIIIR	Low	High	1/6	5/6
Acentric ori-p	*CEN4*, adj.[d]	IVL	Low	High	1/6	5/6
Centric ori-d	*RNase H*	XIIIR	High	High	6/6	0/6
Centric ori-d	*ARG4*	VIIIR	High	High	5/6	1/6
Centric ori-d	*CEN4*, adj.	IVL	High	High	5/6	1/6

[a] Transformation efficiency: low, 0–10 colonies/μg input DNA; high, >100 colonies/μg input DNA.

[b] Diploid cells were sporulated after transformation with either acentric or centric fragmentation vectors and spore viability was monitored.

[c] For each transformant eight to 12 tetrads were analyzed. In each case, dissected tetrads from a given transformant yielded predominantly either 4:0 or 2:2 viability.

[d] The fragmenting site was immediately adjacent (adj.) to *CEN4*.

give rise to only two viable spores. These results were consistent with the possibility that recombination of an acentric CFV with the target chromosome resulted in a terminal deletion, deleting all sequences distal to the site of fragmentation. The generation of a chromosomal terminal deletion can be adequately explained by both gene conversion and reciprocal recombination models. In the case of the gene conversion model (the nonreciprocal exchange of genetic information), all sequences between the unique DNA segment and the telomere on the target chromosome would be gene converted to vector sequence. In the case of the reciprocal recombination model (the reciprocal exchange of genetic information), all sequences between the unique DNA segment and the telomere on the target chromosome would be exchanged with sequences from the vector. In the latter situation, the result would be a CF containing all sequences proximal to the unique DNA segment and a broken chromosome that would be rapidly lost. Both the gene conversion model and the reciprocal exchange model are expected to result in loss of all genetic material distal to the unique DNA segment. The loss of a large segment of genetic material is expected to be lethal to a haploid cell and nonlethal to a diploid cell (assuming the resultant partial monosomy is tolerated).

Regardless of the mechanism by which these proximal CFs were being formed, it seemed reasonable that the generation of "distal" CFs would occur by a similar process. However, high transformation efficiencies were observed in both haploid and diploid cells transformed with distal CFVs (Table 2). The high transformation efficiencies were independent of the target sites, since fragmentation at either *RNase H*, *ARG4* or *CEN4* gave similar results. Furthermore, tetrad analysis of independently transformed diploid cells typically resulted in four viable spores. These results were consistent with the notion that duplication of the distal chromosome segment was occurring, yielding partial disomes in haploids and partial trisomes in diploids.

Acentric CFVs (ori-p) result in a 2N-1+CF karyotype, whereas centric CFVs (ori-d) result in a 2N+CF karyotype: Since the fate of haploid and diploid cells differed when transformed with acentric or centric CFVs, we speculated that the fate of the targeted chromosome also differed. It seemed possible that whereas loss of all sequences distal to the site of fragmentation occurs with acentric CFVs (ori-p), perhaps a gain of all sequences distal to the site of fragmentation occurs with centric CFVs (ori-d). To test this hypothesis, however, it was necessary to determine which chromosome had been targeted for fragmentation and to determine the subsequent fate of that chromosome. This was accomplished by taking advantage of a chromosome III-length polymorphism, due to the presence of one or more copies of a repetitive element on the right arm of chromosome III. Several haploid strains were analyzed by PFGE for chromosome III-length polymorphism. Two strains, YPH34 *MAT*α and YPH45 *MAT***a**, were selected because their chromosome IIIs (termed IIIα and III**a**, respectively) were readily distinguishable by PFGE. These strains were mated and the resulting diploid, YPH49, was used for subsequent experiments.

A random and unique DNA segment from chromosome III, named D8B (NEWLON *et al.* 1991), was chosen as the target site. The D8B sequence lies ~10–15 kb to the left of *CEN3*. Both acentric (YCF1/D8B ori-p) and centric (YCF2/D8B ori-d) CFVs were constructed. These vectors were linearized with *Bgl*II and transformed into YPH49. The karyotypes of stable independent transformants were analyzed by PFGE. In Figure 2 (left panel, lanes 1–6), the karyotypes of six trans-

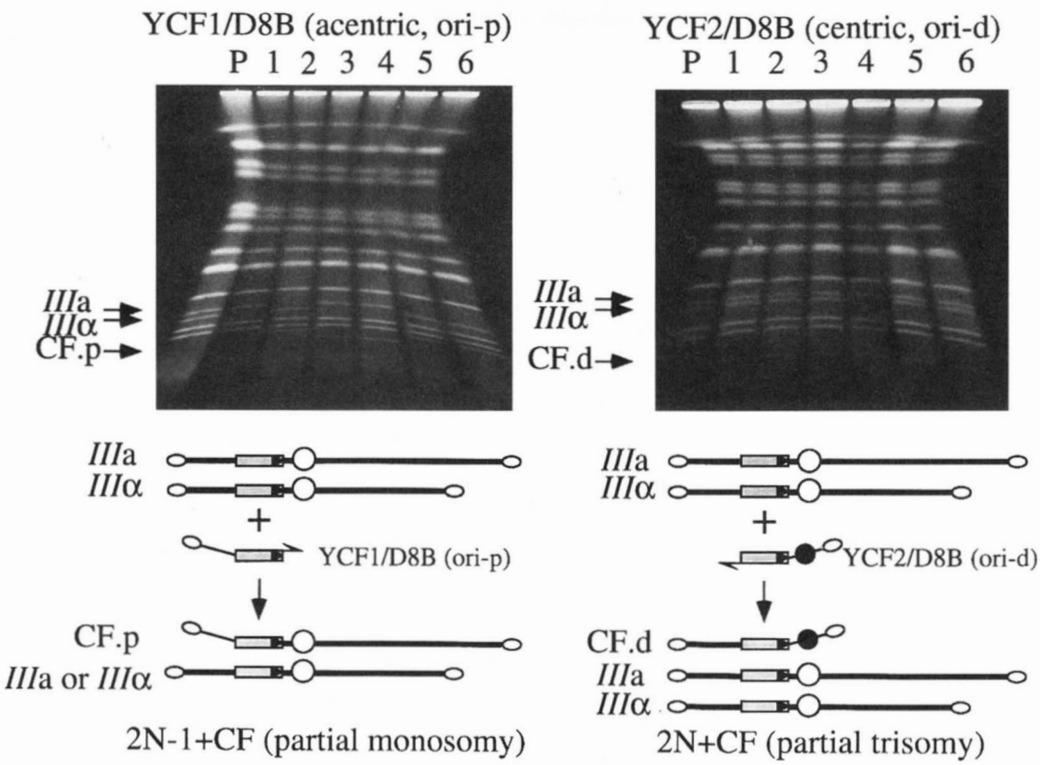

FIGURE 2.—Fate of a diploid cell varies after transformation with acentric and centric CFV. Top, karyotypic analysis by PFGE of YPH49 transformed with the acentric ori-p CFV (YCF1/D8B) (left side) or the centric ori-d CFV (YCF2/D8B) (right side). Lanes indicated are as follows: P, diploid parent (YPH49); 1–6, independent transformants from each fragmentation vector, respectively. Chromosome III from the *MAT***a** or *MAT*α original haploid parent are indicated by IIIa or IIIα, respectively. The proximal CF (left) is indicated by CF.p and migrates just below band 1; the distal fragment (right) is indicated by CF.d and also migrates below band 1, but is smaller than CF.p. Below each PFG a schematic of the fragmentation event is diagrammed. Left, the diploid strain YPH49, polymorphic for chromosome III length as indicated, was transformed with the linearized acentric CFV fragmenting in a *CEN*-proximal direction. The result was loss of the targeted chromosome (IIIa or IIIα) and gain of a proximal fragment to yield a partial monosome (2N-1+CF). Right, YPH49 was transformed with the linerized centric CFV fragmenting in a *CEN*-distal direction. The result was gain of a distal CF and retention of the targeted chromosome to yield a partial trisome (2N+CF). ▨, unique DNA element D8B (black triangle indicates genomic orientation); ○, endogenous chromosomal *CEN;* ●, vector *CEN*; open oval, telomere.

formants carrying acentric CFV (YCF1/D8B ori-p)-derived chromosomal fragments are shown. All six transformants gave rise to a proximal CF (indicated by CF.p) that migrated at either 180 or 200 kb. The proximal CFs contained the right arm chromosome III-length polymorphism. The sizes of the CFs vary depending upon which chromosome III had been fragmented. In each of the six transformants, one of the two chromosome IIIs (III**a** or IIIα) was visibly absent. It was evident that a relationship existed between the size of the CF and the missing chromosome. The longer CF (lanes 2, 5, and 6) was present only when the longer chromosome III (III**a**) was absent. Likewise, the shorter CF (lanes 1, 3, and 4) was present only when the shorter chromosome III (IIIα) was absent.

In contrast, transformation of YPH49 with the centric CFV (YCF2/D8B ori-d) caused quite different karyotypic outcomes. A fragment (indicated by CF.d) was visible in each of the six transformants (right panel, lanes 1–6), but loss of the target chromosome was seen in only one of the six transformants (lane 4, chromosome IIIα). No difference in CF length was expected since the length of the left arm of chromosome III distal to D8B is similar between chromosome III**a** and IIIα (~100 kb). Therefore, consistent with data presented above, fragmentation with the acentric CFV resulted in the gain of a chromosome fragment and the loss of the targeted chromosome (a 2N-1+CF karyotype); whereas fragmentation with the centric CFV resulted in gain of a CF and no loss of the targeted chromosome (a 2N+CF karyotype). In separate experiments, haploid strains transformed with the centric CFVs (ori-d) also showed generation of an identical, distal CF to give a 1N+CF karyotype (data not shown). In this and other fragmentation experiments, the loss or gain of genetic material distal or proximal to the sites of fragmentation was verified by Southern blot analysis using probes derived from genes found either distal or proximal to those sites (data not shown).

Quantitation of target chromosome duplication *vs.*

loss: To quantitate more accurately the efficiency of chromosome duplication *vs.* chromosome loss, 53 independent acentric CFV (ori-p) and 138 centric CFV (ori-d) transformants were analyzed by PFGE (Table 3). Target chromosome loss coupled with CF gain (2N-1+CF) was observed in 92% of the acentric CFV (ori-p) transformants analyzed, while the remaining 8% gained a CF but did not lose the target chromosome (2N+CF). By contrast, 89% of the centric CFV (ori-d) transformants were 2N+CF, while the remaining 11% were 2N-1+CF.

Since the D8B locus lies on the left arm of chromosome III, we wanted to test whether duplication or loss of genetic material also occurs when fragmentation is targeted on the right arm of chromosome III. A random and unique DNA segment, designated H9G (NEWLON *et al.* 1991) that lies ~70 kb to the right of *CEN3,* was targeted using acentric CFVs (ori-p) and centric CFVs (ori-d). Similar percentages of chromosome loss or chromosome duplication were obtained when fragmenting at H9G as compared with D8B. Of the 11 acentric CFV (ori-p) transformants analyzed, eight had a 2N-1+CF karyotype, while only three were 2N+CF. Of the 26 centric CFV (ori-d) transformants analyzed, 21 had a 2N+CF karyotype, while only five were 2N-1+CF. It is clear, therefore, that the predominant outcome of chromosome fragment formation is either duplication of all sequences distal to the site of fragmentation (with centric ori-d CFVs) or retention of all sequences proximal to the site of fragmentation (with acentric ori-p CFVs).

Formation of isochromosomes: The data presented above strongly suggest that chromosome fragmentation with a centric CFV in a centromere distal orientation results in duplication of all sequences distal to the site of fragmentation. To invoke a reciprocal recombination model or a gene conversion model to explain these data, it is necessary to propose that fragmentation occur in G2 (after replication of sister chromatids) and that the fragment then cosegregate or non-disjoin with the sister chromatid. An alternative model, which does not necessitate fragmentation in G2 followed by non-disjunction, is that replication of chromosomal sequences occur by an unscheduled DNA replication event. To assess this possibility, a test was designed to determine whether a chromosome arm could be reduplicated during *CEN*-distal chromosome fragmentation. The idea was to determine if a chromosome fragment could be generated demanding that both the left and right arms of the CF be derived from the same unique DNA target segment. The resultant chromosome fragment would be an "isochromosome" (IsoCF) and would consist of a centromere flanked by two identical chromosomal arms. To accomplish this, a centric CFV containing two copies of the D8B locus, both oriented in the *CEN*-distal direction, was created (Figure 3A). This vector

TABLE 3

Quantitation of chromosome break copy duplication

Vector	No. transformants analyzed	Karyotype[a]	
		2N+CF	2N-1+CF
Acentric YCF1/D8B ori-p	53	4 (8)	49 (92)
Centric YCF2/D8B ori-d	138	123 (89)	15 (11)

[a] Karyotype was determined by PFGE. Values in parentheses are percentages.

was called an isochromosome fragmentation vector (IsoCFV). It was predicted that isochromosome formation would not be possible in a haploid strain if reciprocal exchange were the mechanism. This would require, after S-phase, that both sister chromatids donate their respective distal fragments onto the CFV. In haploid cells, this is certain to be a lethal event. Gene conversion is not possible in this situation because the IsoCFV does not contain end-points that would permit a conversion event.

To first determine if an IsoCF could be generated, the IsoCFV (YCF2/D8B-D8B ori-d, ori-d) was linearized between the cloned D8B segments and transformed into the diploid strain YPH49. The transformation efficiency was ~14–25% that of *CEN*-distal or *CEN*-proximal CFVs and did not differ significantly from a linearized plasmid designed to delete the D8B region (Figure 3A). Transformation efficiency of a circular *CEN* plasmid and a plasmid designed to gap-repair the D8B region are shown for comparison. Seventeen of the 27 stable IsoCFV transformants (total from two independent experiments) were analyzed by PFGE. Sixteen had a CF of the expected 200 kb length equal to twice that of the D8B *CEN*-distal CF (100 kb) (data not shown). Of those 16 analyzed, nine had lost one of the two chromosome IIIs (2N-1+IsoCF) and seven retained both chromosome IIIs (2N+IsoCF). Southern blot analysis confirmed that these chromosome fragments were indeed derived from sequences distal to D8B (data not shown). While the rate of target chromosome III loss was higher than seen with the *CEN*-distal CFV (YCF2/D8B), it is possible that the initiation of two replication forks, either one behind the other on the same chromosome, or on separate chromosomes, could compromise the stability of the targeted chromosomes. Since the rate of target chromosome loss with YCF2/D8B (ori-d) was 11% (Table 3), it was expected that the rate of chromosome loss with YCF2/D8B-D8B (ori-d, ori-d) would be at least two times greater.

To demonstrate that chromosome duplication was occurring, the ability to generate isochromosomes in haploid strains was tested. The YCF2/D8B-D8B (ori-d, ori-d) IsoCFV was transformed into the haploid strain YPH45 and stable transformants were selected. The

A

	Chromosome restructuring vectors	Predicted structural alteration	# of stable transformants 1	2
i		Circular *CEN* plasmid	1100	1600
ii		*CEN*-distal CF formation	45	106
iii		*CEN*-proximal CF formation	25	75
iv		Gap-repaired plasmid	50	138
v		Chromosomal deletion	18	15
vi		Isochromosome formation	12	15

B

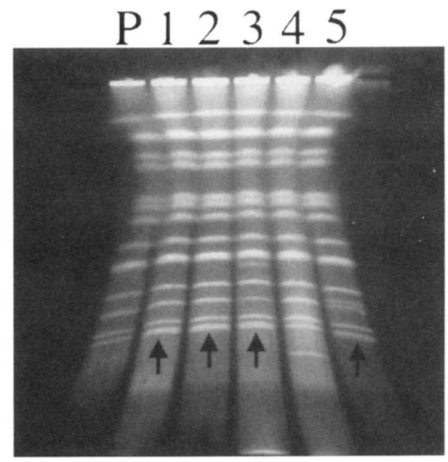

FIGURE 3.— (A) Schematic of chromosome restructuring vectors with associated transformation efficiencies. (Left) A schematic of the DNA transformed is shown with the predicted structural alteration (middle). In all situations the target site was D8B on chromosome III. In each experiment, 3 μg of DNA was transformed into the diploid YPH49. (Right) The number of stable transformants from two independent experiments is indicated. The transformation efficiency of a circular *CEN* plasmid is shown for comparison. In our experience, ~100% (i), 75–90% (ii, iii, iv), and 60–80% (v, vi) of stable transformants yield the predicted structure as determined by PFGE and Southern blotting. ▨, unique DNA element D8B; closed circle, vector *CEN*; open oval, telomere. (B) PFGE analysis of YPH47 transformed with the isochromosome fragmentation vector YCF2/D8B-D8B (ori-d, ori-d). Lane P, parent; lanes 1–5, independent transformants. The IsoCF is indicated by the arrow and comigrates just below band 1. Note the increase in ethidium bromide staining.

transformation efficiency in such experiments was typically low (<5 stable colonies/μg input DNA), but as shown in Figure 3B, isochromosomes were indeed generated. In this particular experiment four of the five transformants analyzed (lanes 1–3 and 5) had generated an isochromosome. The IsoCFs, noted by arrows, migrate just beneath band 1 (chromosome I $\approx$ 230 kb) at ~200 kb and are twice the size of a D8B distal CF.

Taken together, these experiments clearly demonstrate that transformation of a haploid strain with a centric YCF2/D8B (ori-d) generates a partial disome, and in a diploid generates a partial trisome. Transformation of a haploid with an IsoCFV (YCF2/D8B-D8B) also generates a partial trisome. The simplest explanation for these results is that the CFV containing either one or two free ends with homology to D8B invades the endogenous chromosome and initiates either one or two replication forks that proceed to duplicate to the end of the chromosome.

Break copy duplication: As described above, two of the models that might potentially explain how a linearized chromosome fragmentation vector recombines with the endogenous target chromosome to generate a chromosome fragment are gene conversion of chromosomal sequences to CFV sequences, and reciprocal recombination between the chromosome and the CFV (SUROSKY and TYE 1985; MURRAY and SZOSTAK 1986; VOLLRATH *et al.* 1988). Neither of these models, however, adequately accounts for the chromosome fragmentation products described here. We postulate, rather, that *CEN*-distal CF formation is the product of a unidirectional replication fork, initiated by the CFV that, we speculate, resembles a half-gap or break. This replication fork proceeds to duplicate all sequences distal to the site of initiation. The term break copy duplication is used to describe this model. In addition, we suggest that break copy duplication occurs at high frequency in a *CEN*-distal direction, but not in a *CEN*-proximal direction, because during unscheduled DNA replication the replication fork becomes blocked at the centromere.

Break copy duplication is inhibited by the centromere: The acentric CFVs (ori-p) targeting D8B, H9G, *RNase H,* or *ARG4* (resulting predominantly in a 2N-1+CF karyotype) have in common the fact that fragmentation is directed toward the centromere. If break copy duplication occurs in both *CEN*-distal and *CEN*-proximal directions, but in the *CEN*-proximal direction that duplication is blocked by a *cis*-element, then a candidate for that *cis*-element would be the centromere. To test this hypothesis, the centromere was moved into a new location. A diploid strain was constructed in which one of the two chromosome III centromeres had been transposed into a site distal to D8B (Figure 4). A haploid "transpocentric" strain, J101-T55A, was obtained from SHIRLEEN ROEDER (Yale University). J101-T55A was constructed using a double gene replacement technique in which the normal *CEN* from chromosome III was deleted and a 211-bp subclone of *CEN3* was simultaneously inserted into the *HIS4* locus [for construction of J101-T55A, see LAMBIE and ROEDER (1986, 1988)]. The *HIS4* locus is on the left arm of chromosome III, distal to the D8B locus. This haploid strain was mated to strain YPH47 that contains *CEN3* in the

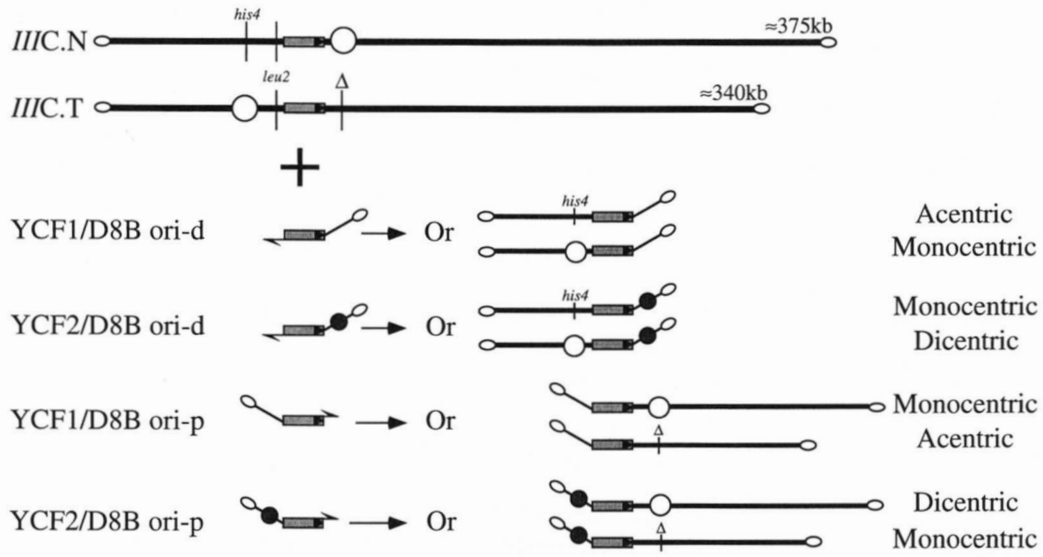

FIGURE 4.—Schematic of the chromosome IIIs from the heterotranspocentric strain. The heterotranspocentric strain YDM10 was created by mating the haploid strain YPH47 (*MAT***a**) with J101-T55A (*MATα*). The centromere of chromosome III from YPH47, indicated by IIIC.N, is located in its normal position. The centromere of chromosome III from J101-T55A, indicated by IIIC.T, has been transposed into the HIS4 locus distal to D8B. Transformation with one of the four CFVs will result in the formation of either a stable monocentric CF, an unstable acentric CF, or an unstable dicentric CF. ▨, unique DNA element D8B (black triangle indicates genomic orientation); ○, endogenous chromosomal *CEN*; ●, vector *CEN*; open oval, telomere.

normal chromosome III position. The chromosome IIIs from J101-T55A and YPH47 are herein called chromosome IIIC.T (transpocentric) and IIIC.N (normal), respectively. Since YPH47 and J101-T55A are polymorphic for chromosome III length, the fate of the targeted chromosome could be determined by PFGE. This "heterotranspocentric" diploid strain (YDM10) was transformed with linearized centric (YCF2), or acentric (YCF1) CFVs with the target D8B site cloned in either orientation (ori-p and ori-d). Even though each of the four fragmentation vectors can target either the normal (IIIC.N) or transpocentric chromosome (IIIC.T), stable transformants will arise only when a monocentric fragment has been generated (Figure 4). Analysis of chromosome fragment length by PFGE enabled the unambiguous assignment of a CF to the chromosome from which it was generated and permitted an accurate determination of the targeted chromosome's fate. If the model of centromere inhibition of break copy duplication were correct, then transposition of the centromere to a new location should result in transposition of the target chromosome loss phenotype.

The heterotranspocentric strain YDM10 was transformed with each of the four CFVs. Transformants were analyzed by PFGE for the presence CFs, the lengths of those CFs, and maintenance of the targeted chromosomes (Table 4). Transformation with the acentric YCF1/D8B (ori-d) should generate a stable transformant only when the transpocentric chromosome serves as the target. All 40 transformants analyzed contained a CF of the predicted length (100 kb) and 70% had a 2N-1+CF karyotype. In each case, the IIIC.T chromosome was lost. Most transformants with the centric YCF2/D8B ori-d vector, on the other hand, yielded a 2N+CF karyotype. In each case, the CF was of the predicted 100 kb length. As was expected, 81% of the acentric YCF/D8B ori-p transformants had a 2N-1+CF karyotype.

These results clearly demonstrate that transposition of the centromere to a new location results in transposition of the target chromosome loss phenotype. The centromere, therefore, is sufficient to inhibit break copy duplication, presumably by inhibiting the progression of replication forks during these unscheduled DNA replication events.

DISCUSSION

In this article we propose a model, termed break copy duplication, to describe the phenomenon observed when chromosome fragmentation vectors are introduced into yeast. The model arose from differences observed in the fate of strains transformed with acentric or centric CFVs. Stable transformants could be efficiently created in diploid cells, but not haploid cells, transformed with acentric CFVs. Transformation with centric distal CFVs, however, gave a different result as both haploid and diploid strains were stably transformed at equivalent levels. Subsequent analysis of transformants demonstrated that acentric CFVs resulted in the gain of a CF and loss of the targeted chromosome (2N-1+CF); whereas centric CFVs usually resulted in gain of a CF without loss of the targeted chromosome (2N+CF). Thus, the break copy duplica-

TABLE 4

Quantitation of break copy duplication in a heterotranspocentric diploid strain

Vector	No. of stable transformants with CF/total[a,b]	Karyotype		Chromosome III lost in the 2N-1+CF transformants	
		2N+CF[b]	2N-1+CF[b]	IIIC.N.	IIIC.T
Acentric YCF1/D8B ori-d[c]	40/40 (100)	12 (30)	28 (70)	0	28
Centric YCF2/D8B ori-d	16/20 (75)	12 (75)	4 (25)	2	2
Acentric YCF1/D8B ori-p	27/27 (100)	5 (19)	22 (81)	22	0
Centric YCF2/D8B ori-p	21/24 (88)	10 (48)	11 (52)	2	9

[a] Chromosome fragment gain and chromosome loss were determined by PFGE.

[b] CF formation not considered if not of the predicted size. It was expected that some centric YCF2 transformants would generate unstable dicentric CFs that might rearrange to form CFs of aberrant sizes. Values in parentheses are percentages.

[c] Orientation is relative to the normal centromere position.

tion model maintains that introduction of a linear DNA molecule (Figure 5i) or half-gap invades a chromosomal target and (ii) initiates an unscheduled DNA replication event manifested by a unidirectional replication fork that continues until it reaches either (iii, right panel) the end of the chromosome or (iii, left panel) a centromere. If a centromere is encountered, the replication fork becomes blocked. Resolution of the predicted "Holliday" junction (iv) and selection for the chromosome fragment results in the gain of a chromosome fragment and either loss (*CEN*-proximal) or retention (*CEN*-distal) of the targeted chromosome. It is hypothesized that an endonucleolytic break, or nick, must also occur, perhaps near the centromere, resulting in loss of the endogenous chromosome (iii, left panel).

The break copy duplication model described here is similar to the double-strand-break repair model proposed by SZOSTAK *et al.* (1983) as a mechanism for mei-

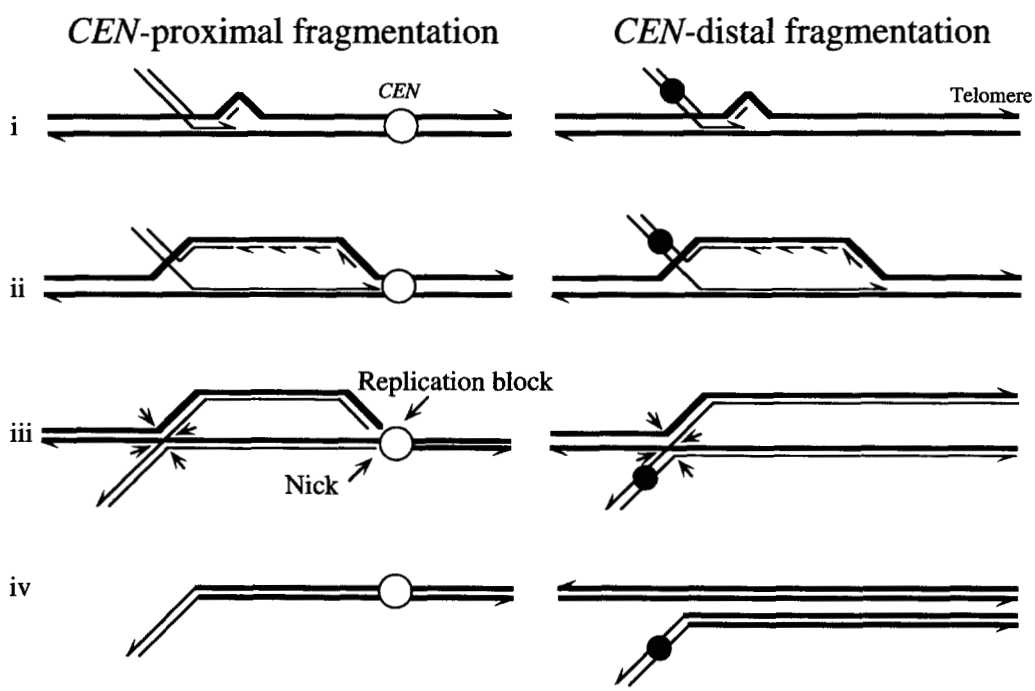

FIGURE 5.—Break copy duplication: a mechanism for the generation of chromosome fragments. Transformation with a linearized CFV leads to (i) invasion of the chromosomal duplex at the target site (ii), which initiates a unidirectional replication fork. We postulate that this replication fork proceeds to duplicate the chromosome until (left, iii) a centromere is encountered or until (right, iii) the end of the chromosome is reached. If a centromere is encountered (left, iii), the unscheduled DNA replication event is blocked. Resolution of the predicted Holliday junction (iii; indicated by arrows) and selection for the chromosome fragment yield (left, iv) loss of the target chromosome and gain of a CF or (right, iv) retention of the target chromosome and gain of a CF. It is predicted that a nick (left, iii) must occur somewhere along the target chromosome to permit chromosome loss.

otic recombination. This model argues that a double-strand break is made in one duplex, and nucleolytic degradation then expands the gap. Invasion into the intact duplex followed by two-sided replicative repair fixes the double-strand break. The primary difference between break copy duplication and double-strand-break repair is that in break copy duplication only one-sided, or half-gap, replicative repair is initiated, as opposed to two-sided replicative repair. This is similar to the "break and replicate" model proposed by VOELKEL-MEIMAN and ROEDER (1990a,b) to explain the extended tracts of gene conversion seen downstream of *HOT1*, a *cis*-acting element required for RNA polymerase I transcription. *HOT1* promoted an increase in mitotic interchromosomal gene conversion, and chromosomes containing *HOT1* were shown to preferentially receive genetic information during gene conversion. It was postulated that a double-strand break occurs at the *HOT1* site, resulting in a free 3′ end that invades the homologous chromosome and initiates replicative repair. Of significance to the break copy duplication model was the extensive amount of gene conversion, as far as 77 kb from *HOT1*, observed. In this paper, duplication events extend up to 230 kb, but we have observed duplications extending as long as 365 kb (D. M. MORROW, unpublished observations). Work in other systems, including bacteriaphage T4, also supports a mechanism of double-strand break repair that includes an extensive amount of DNA replication (MOSTIG 1983; GEORGE and KREUZER 1996). Recently, MALKOVA *et al.* (1996) described a phenomenon observed during HO endonuclease-induced cleavage of chromosome III at the *MAT* locus. They demonstrated that diploid *S. cerevisiae* strains were capable of repairing an HO endonuclease-induced double-strand break at the *MAT***a** site of chromosome III. This repair was *RAD52* dependent, and *RAD51* independent. Interestingly, 35% of the *rad51*Δ diploid strains were capable of repairing the broken chromosome, resulting in homozygosity of markers distal to the break on the right arm of chromosome III, but heterozygosity of markers on the left arm of chromosome III. MALKOVA *et al.* speculated that a mechanism requiring DNA replication, initiated by invasion of a broken end into the homologous chromosome, could account for the products they observed. They called this phenomenon break-induced replication (BIR). We speculate, as did MALKOVA *et al.*, that the models of break copy duplication, BIR, and break and replicate are similar, if not identical. These data, taken together, support the notion that replicative repair is not only an essential component of double-strand-break repair, but that it is an extremely efficient process that can extend hundreds of kilobases.

Perhaps the most convincing data presented here that duplication of DNA is the primary model for the creation of a chromosome fragment is the demonstration that isochromosome fragments can be generated efficiently. When a haploid cell, with a single copy of the DNA segment distal to D8B, was transformed with the isochromosome fragmentation vector, a strain was produced that contained three copies of DNA distal to D8B. The simplest model to account for this increase in segmental aneuploidy is chromosome arm duplication initiated by each of the two targeting segments of the fragmentation vector.

Our data establish that break copy duplication is a highly efficient process in a *CEN*-distal direction, but not in a *CEN*-proximal direction. The primary difference between the two directions is the presence of a centromere. We specifically tested the role of the centromere in the inhibition of break copy duplication by using a strain where the centromere had been transposed into a new location, distal to the target site. Transposition of the centromere resulted in transposition of the target chromosome loss phenotype. We conclude that the centromere is sufficient for inhibiting break copy duplication.

One interesting result from the analysis of chromosome fragmentation in the heterotranspocentric strain was the finding that more YCF2/D8B ori-p transformants than expected had a 2N-1+CF karyotype. Approximately 50% showed loss of a chromosome III, and of those, the IIIC.T chromosome was lost predominantly. One possible explanation is that other *cis*-elements, situated between D8B and H9G, might be present. It has been rigorously demonstrated that the yeast centromere consists of a nuclease protected core surrounded on either side by highly organized nucleosomal arrays of chromatin (BLOOM and CARBON 1982). This highly organized structure is not propagated if foreign DNA (*e.g.*, bacterial sequences) or yeast DNA from other regions is placed next to the centromere. This suggests that the specific DNA sequence surrounding the centromere contributes to these nucleosomal arrays. Perhaps in the heterotranspocentric strain, highly organized chromatin remains, contributing to the target chromosome loss phenotype. An additional explanation could be that a hot-spot for Holliday junction resolution lies in the region between D8B and H9G. Further investigation is required, however, to address these possibilities.

The centromere and its associated kinetochore proteins represent an important and privileged site on yeast chromosomes that is responsible for complexing to microtubules and ensuring both proper chromosome movement and segregation (see review by PLUTA *et al.* 1995). The core of the centromere consists of a 125-bp region containing centromere DNA elements I, II and III (FITZGERALD-HAYES *et al.* 1982) that bind the kinetochore's protein components. The 211-bp element from *CEN3* transposed by LAMBIE and ROEDER (1986, 1988) contains this 125-bp core. Using these centromere-

transposed strains, LAMBIE and ROEDER were able to demonstrate that cross-over events and meiotic gene conversion events are strongly inhibited in regions containing centromeres. GREENFEDER and NEWLON (1992) used two-dimensional gel analysis to show that during mitotic S-phase, replication forks pause and thereby accumulate when they encounter a centromere. A correlation existed between the nuclease resistant core and the replication fork pause suggesting that protein components of the centromere were responsible for the pausing. These data support the notion that centromeres are indeed privileged sites on yeast chromosomes and support our contention that unscheduled DNA replication or break copy duplication is inhibited by centromeres. Although replication fork pausing is not directly tested here, break copy duplication is. We suggest that the transpocentric results demonstrate that the centromere is not only sufficient for inhibition of break copy duplication, but that the centromere and perhaps sequences immediately flanking the centromere are likely the only regions capable of inhibiting break copy duplication.

Interestingly, the break copy duplication model also accounts for observations made by VOELKEL-MEIMAN and ROEDER (1990a,b) and by MALKOVA *et al.* (1996). Though sites proximal to *HOT1* were occasionally gene converted, VOELKEL-MEIMAN and ROEDER did not observe gene conversion extending proximally through the centromere. They postulated that the centromere-containing fragment preferentially serves as the primer for replicative repair. Theoretically, either the telomere- or centromere-containing fragments could serve as primers for replicative repair; but if replication in a *CEN*-proximal direction was blocked by the centromere as occurs during break copy duplication, then only centromere fragment initiated repair would be observed. VOELKEL-MEIMAN and ROEDER, in fact, speculated that this was a possibility. A similar argument can be made for the findings of MALKOVA *et al.* They did not observe BIR extending centromere proximally (*i.e.*, toward *CEN3*). It is likely that BIR was inhibited by the centromere.

The inhibition of break copy duplication by the centromere may reflect a more general level of replication control. It is generally believed that centromeres replicate early in S-phase (MCCARROLL and FANGMAN 1988), and thus it is conceivable that replication at other times is not permitted. Regulation of the cell cycle stage when centromere replication is permitted might ensure that only the centromere side of a broken chromosome can serve as a primer and be the recipient of genetic information. Perhaps consistent with this hypothesis, we observed some variability in the percentage of transformants that did not lose a chromosome but did gain a CF when transformed with acentric CFVs. One explanation is that a greater percentage of the transformation competent cells were in a stage of the cell cycle in which replication through a centromere was permitted.

One important consequence of the findings described here is the possibility that during integrative transformations, including one-step gene replacement (ROTHSTEIN 1983; BAUDIN *et al.* 1993), complete duplication of the chromosome could occur. In fact, we have observed that duplication does occur in a percentage of transformants (data not shown). It is therefore essential to analyze multiple transformants when doing integrative transformations. It is also important that analysis of transformants be done by Southern blotting, since polymerase chain reaction analysis might demonstrate the presence of a correctly integrated DNA fragment, but might not show the presence of a duplicated chromosome.

Why would a cell have evolved a mechanism to inhibit duplication in a *CEN*-proximal direction? It is interesting to speculate that if such a mechanism did not exist and a cell developed a double-strand break, the initiation of replicative repair in both *CEN*-distal and *CEN*-proximal directions would result in a trisomic strain. Inhibition of unscheduled DNA replication in one orientation (in the *CEN*-proximal direction) assures that only one arm of a broken chromosome (the *CEN*-containing arm) serves to initiate DNA replication. The results reported here may, therefore, have important implications for how cells maintain ploidy during DNA repair of broken whole chromosomes and for chromosome evolution.

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