The Effect on Chromosome Stability of Deleting Replication Origins

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The observed spacing between chromosomal DNA replication origins in *Saccharomyces cerevisiae* is at least four times shorter than should be necessary to ensure complete replication of chromosomal DNA during the S phase. To test whether all replication origins are required for normal chromosome stability, the loss rates of derivatives of chromosome III from which one or more origins had been deleted were measured. In the case of a 61-kb circular derivative of the chromosome that has two highly active origins and one origin that initiates only 10 to 20% of the time, deletion of either highly active origin increased its rate of loss two- to fourfold. Deletion of both highly active origins caused the ring chromosome to be lost in approximately 20% of cell divisions. This very high rate of loss demonstrates that there are no efficient cryptic origins on the ring chromosome that are capable of ensuring its replication in the absence of the origins that are normally used. Deletion of the same two origins from the full-length chromosome III, which contains more than six replication origins, had no effect on its rate of loss. These results suggest that the increase in the rate of loss of the small circular chromosome from which a single highly active origin was deleted was caused by the failure of the remaining highly active origin to initiate replication in a small fraction (approximately 0.003) of cell cycles.

Chromosomal sequences that enable plasmids to replicate in the nucleus of the yeast *Saccharomyces cerevisiae* have been identified. These have been termed autonomously replicating sequence or *ARS* elements. Fourteen *ARS* elements have been identified and mapped in a 200-kb region of chromosome III (4, 27, 28). By two-dimensional (2-D) gel electrophoresis techniques, 10 of the chromosome III *ARS* elements have been examined directly for chromosomal origin function. Replication origins have been mapped to within a few hundred base pairs of six of these *ARS* elements: *ARS305* (7, 9, 17); *ARS306* (7, 48); *ARS307*, *ARS308*, and *ARS309* (10); and *ARS310* (6). The other four *ARS* elements tested, *ARS301*, *ARS302*, *ARS303* (9), and *ARS304* (5), do not function as chromosomal origins at detectable levels (Fig. 1).

ARS elements are essential for chromosomal origin function. Deletion of a 200-bp fragment containing *ARS306* and point mutations in domain A of *ARS307* that abolish *ARS* activity also abolish chromosomal origin function when they are placed in their natural chromosomal context (7). In addition, multiple point mutations within the consensus sequence of the *HMR E ARS* also abolish the associated chromosomal replication origin function (33).

On yeast chromosomes, adjacent replication origins have an average spacing of 36 kb (26). A calculation based on reported fork rates (19, 32, 34) and an S phase of 25 min at 30°C (46) suggests that between 120 and 300 kb of chromosomal DNA could be replicated from a single replication origin if it initiated early in the S phase. Thus, the spacing between replication origins on yeast chromosomes appears to be at least four times shorter than is necessary for complete replication.

To determine whether all replication origins are required for normal chromosome stability, we have deleted replication origins from a 61-kb circular derivative of chromosome

III which contains only three origins (10), as well as from a full-length copy of chromosome III. The three origins on the ring chromosome are active to various degrees: two are used >90% of the time, and the third is used only 10 to 20% of the time (10). We find that deletion of either of the highly active origins from the ring chromosome increases its rate of loss but that deletion of the same origins from the full-length chromosome has no effect on stability.

MATERIALS AND METHODS

Strains. All yeast strains used in this study are listed in Table 1. *ARS* deletions were constructed in the 61-kb ring chromosome of strain CN16A and the *MAT α* chromosome of CF4-16B.

Strain CN16A was the product of a cross between strain 3A and strain ICH2. Chromosome III in ICH2 exists in two parts that resulted from an intrachromosomal recombination between Ty1-17 and Ty1-161 (40). This recombination resolved a dicentric chromosome created by integrating *CEN5* at the *HIS4* locus and yielded a 61-kb ring chromosome containing *CEN3* and other sequences between Ty1-17 and Ty1-161 (see Fig. 1) and a linear chromosome approximately 260 kb in length bearing the remaining chromosome III sequences, *CEN5* and YIp5. CN16A carries the 61-kb ring chromosome from ICH2 bearing the *LEU2* gene as well as the complete copy of chromosome III from strain 3A carrying a *leu2* allele.

To obtain ring-bearing strains for use with the *ade2-SUP11* reporter system (14), CN16A derivatives with a wild-type 61-kb ring chromosome or one bearing the *ars309 Δ 1* deletion were crossed to YPH47 and sporulated to produce PF1-7D, which carries the wild-type ring chromosome, and PF6-5B, which carries the ring with the *ars309 Δ 1* deletion. These strains were made [*psi*[−]] by growth on guanidine HCl (43), and a 0.7-kb *SUP11-1*-containing fragment (42) was inserted at a *Bgl*II site 2 kb to the left of *ARS307* in the 61-kb ring (see Fig. 2B) by the method

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FIG. 1. Maps of chromosome III. (A) Physical map of the 200-kb region extending from the left telomere to the *MAT* locus. This region of the chromosome has been analyzed for the presence of *ARS* elements (28), and the approximate positions of the 14 *ARS* elements are indicated by the numbers above the line. The positions of the centromere (*CEN3*) and several genes are also indicated. Tick marks on the map are at 10-kb intervals. (B) Physical map of the 61-kb ring chromosome created by an intrachromosomal recombination between *Ty1-17* and *Ty1-161* (40). The positions of the centromere, the hybrid *Ty* element, the three *ARS* elements, and several genes are indicated. Tick marks on the map are at 10-kb intervals.

described below for construction of *ARS* deletions. This *SUP11-1* construct was named *C2G::SUP11-1*.

The CF4-16B strain was constructed by C. Falco and is disomic for chromosome III. For use of the *ade2-SUP11-1*-

scoring system, [*psi*⁻] derivatives of the strains carrying single *ARS* deletions were isolated and the *SUP11-1* fragment was inserted at the *Bgl*II site centromere distal to *ARS307* of the *MAT* α chromosomes as described above (see Fig. 4).

Escherichia coli JA226 (8) was used for plasmid propagations.

Media. Yeast extract-peptone-dextrose (YEPD) medium (35) was supplemented with 20 mg of uracil and adenine per liter. Color assay medium contained 6.7 g of yeast nitrogen base without amino acids, 5 g of Casamino Acids, 20 mg of uracil, 4.5 mg of adenine, and 50 mg of tryptophan per liter. Yeast minimal medium (29) was supplemented with 10 mg of adenine, 20 mg of uracil, and 50 mg each of histidine, lysine, methionine, phenylalanine, tryptophan, and tyrosine per liter to make minimal complete medium. For plates, liquid media were supplemented with 15 g of agar per liter.

Preparation of DNA. Yeast genomic DNA was prepared either according to the method described by Newlon, et al. (28) or according to that described by Holm et al. (15). For analysis of replication intermediates by 2-D agarose gel electrophoresis, genomic DNA was isolated by the procedure described by Huberman et al. (16). Plasmid DNAs were prepared from *E. coli* strains by the alkaline lysis procedure described by Maniatis et al. (23).

Southern analysis. DNA was digested with one or more restriction enzymes (New England Biolabs) under conditions recommended by the supplier. Following electrophoresis in 0.7% agarose gels with Tris-borate-EDTA buffer (23), the separated DNA fragments were transferred bidirectionally (37) to a Nytran or BA85 nitrocellulose filter (0.45- μ m pore size; both obtained from Schleicher & Schuell). Probes were prepared by nick translation (23) or random primer labeling (Multiprime kit; Amersham Corporation) with [α -³²P]dCTP (3,000 Ci/mmol; Amersham). Hybridizations used procedures described previously (7).

Construction of *ARS* deletion plasmids. Plasmids carrying *ARS* deletions were constructed in the integrating vector, *YIp5* (38), by cloning pairs of fragments flanking *ARS*

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
CF4-16B ^a	<i>MAT</i> α <i>his4-280</i> <i>MAT</i> α <i>his4-290</i> <i>ade2-101</i> <i>ura3-52</i>	E. W. Jones
YNN216	<i>MAT</i> α <i>ade2-101</i> <i>ura3-52</i> <i>lys2-801</i> <i>MAT</i> α <i>ade2-101</i> <i>ura3-52</i> <i>lys2-801</i>	36
CN16A ^b	<i>MAT</i> α <i>leu2</i> <i>his4-38</i> <i>cry1</i> <i>ura3-52</i> <i>aro7</i> <i>trp1-289</i> <i>LEU2</i>	This study
3A	<i>MAT</i> α <i>cdc7-4</i> <i>ade1</i> <i>arg4</i> <i>his4</i> <i>leu2</i> <i>aro7</i>	This study
ICH2 ^c	<i>MAT</i> α <i>his4-38::HIS4</i> <i>CEN5</i> <i>URA3</i> <i>ura3-52</i> <i>trp1-289</i> <i>LEU2</i>	40
YPH47	<i>MAT</i> α <i>ura3-52</i> <i>lys2-801</i> <i>ade2-101</i> <i>trp1-Δ1</i>	36
PF1-7D ^b	Spore from cross of CN16A with YPH47 <i>MAT</i> α <i>his4-38</i> <i>leu2</i> <i>lys2-801</i> <i>trp1-289</i> <i>aro7</i> <i>ura3-52</i> <i>ade2-101</i> [<i>psi</i> ⁻] <i>C2G::SUP11</i> <i>LEU2</i>	This study
PF6-5B ^b	Spore from cross of 3-1 ^d with YPH47 <i>MAT</i> α <i>his4-38</i> <i>leu2</i> <i>ura3-52</i> <i>ade2-101</i> [<i>psi</i> ⁻] <i>C2G::SUP11</i> <i>ars309Δ1</i> <i>LEU2</i>	This study

^a CF4-16B is disomic for chromosome III.

^b This strain carries the 61-kb ring chromosome, marked with *LEU2*, as well as a full-length chromosome III.

^c This strain carries the 61-kb ring chromosome together with a linear derivative of chromosome III from which the 61-kb ring chromosome sequences are deleted.

^d See Table 2.

TABLE 2. Mitotic loss rates of 61-kb ring chromosome III derivatives

Strain ^a	ARS element(s) deleted	Liquid culture loss rate (losses/cell/generation) ^b	Ratio of loss rates (deletion/wild type)	Colony isolation loss rate (losses/cell/generation) ^b	Ratio of loss rates (deletion/wild type)
33-6	None	$1.59 \times 10^{-3} \pm 0.25 \times 10^{-3}$		$0.83 \times 10^{-3} \pm 0.48 \times 10^{-3}$	
33-4	ARS307	$4.11 \times 10^{-3} \pm 0.58 \times 10^{-3}$	2.58	$1.67 \times 10^{-3} \pm 0.75 \times 10^{-3}$	2.02
3-2	None	$0.76 \times 10^{-3} \pm 0.16 \times 10^{-3}$		$1.13 \times 10^{-3} \pm 0.43 \times 10^{-3}$	
3-1	ARS309	$2.62 \times 10^{-3} \pm 0.26 \times 10^{-3}$	3.45	$2.74 \times 10^{-3} \pm 0.62 \times 10^{-3}$	2.42
11w'	ARS307 and ARS309	$0.16 \pm .01$	210	ND	
PF1-7D	None	$3.62 \times 10^{-3} \pm 0.77 \times 10^{-3}$		$2.86 \times 10^{-3} \pm 0.82 \times 10^{-3}$	
PF6-5B	ARS309	$1.61 \times 10^{-2} \pm 0.13 \times 10^{-2}$	4.45	$9.12 \times 10^{-3} \pm 2.1 \times 10^{-3}$	3.18
PF6-5B-21a	ARS307 and ARS309	0.211 ^c	58	ND	

^a Strains 33-6, 33-4, 3-2, 3-1, and 11w' were derived from strain CN16a (Table 1). 33-6 and 33-4 are wild-type and ARS307 deletion popout strains, respectively, from a single transformant. 3-2 and 3-1 are wild-type and ARS309 deletion popout strains, respectively, from a single transformant. To make 11w', the ARS309 deletion was constructed in strain 33-4. The PF strains are *ade2-101* and [*psi*], and the 61-kb ring chromosomes in these strains are marked with *SUP11-1*. PF6-5B-21a was derived by constructing the ARS307 deletion in PF6-5B.

^b Values ($\pm$ standard deviation) obtained from fluctuation analysis as described in Materials and Methods, except for strain PF6-5B-21a. ND, no data.

^c Loss rate determined by time course experiment as described in Materials and Methods. Correlation coefficient, 0.986.

elements in their chromosomal orientations. The ARS307 deletion plasmid was constructed by cloning the 2.3-kb *Bam*HI-*Eco*RI fragment flanking the left side of ARS307 in YIp5 (see Fig. 2A). The resulting plasmid was then linearized by being cut with *Eco*RI and filled in with the Klenow fragment of DNA polymerase I, and a *Cla*I linker was attached. The 1.7-kb *Cla*I fragment flanking the right side of ARS307 was then cloned in the newly created *Cla*I site, and a plasmid carrying the *Cla*I fragment in the proper orientation was identified. This plasmid, Δ ARS307, carries a 0.5-kb deletion called *ars307- Δ 1*.

The plasmid Δ ARS309 was constructed by first cloning the 750-bp *Bgl*II-*Hind*III fragment that flanks the left side of ARS309 between the *Bam*HI and the *Hind*III sites of YIp5 (see Fig. 2B). The 1.4-kb *Hind*III fragment flanking the other side of ARS309 was then inserted in the *Hind*III site of the vector, and a plasmid with the *Hind*III fragment in its chromosomal orientation was identified. The resulting plasmid carries a 1.7-kb deletion called *ars309- Δ 1*.

Construction of chromosomal ARS deletions. To direct their integration into chromosome III, ARS deletion plasmids were linearized as indicated in Fig. 2 and used to transform the appropriate yeast strain by the LiAc procedure (18). Transformants carrying a single integrated copy of the plasmid were identified by genomic Southern analysis with the probes diagrammed in Fig. 2. Strains with the ARS deletion plasmid integrated into the desired copy of chromosome III were identified by cosegregation of the plasmid *URA3* gene with the chromosomal *LEU2* or *SUP11-1* gene. Strains that had spontaneously lost the plasmid (Ura⁻ "pop-outs") were selected by growth on 5-fluoroorotic acid (SCM Specialty Chemicals) as described by Boeke et al. (2). These strains were then screened by genomic Southern analysis to identify one carrying the desired ARS deletion and to identify an isogenic derivative carrying the wild-type ARS element (see Fig. 2).

2-D agarose gel electrophoresis. The 2-D agarose gel electrophoresis method described by Brewer and Fangman (3) was used for analysis of replication intermediates as described previously (7).

Measurement of mitotic loss rates. Mitotic loss rates were determined by either fluctuation analysis (21) or, for very unstable species, a direct measurement of the fraction of ring bearing cells as a function of time (31, 44).

Fluctuation analysis was applied to two different measurement techniques: the liquid culture procedure and the colony isolation procedure. For the liquid culture procedure, expo-

entially growing cultures were diluted to 10 cells per ml in YEPD medium, and 0.3-ml aliquots were distributed to 60 to 120 tubes. To monitor loss of the 61-kb ring chromosome, these 0.3-ml cultures were incubated in a rollerdrum at 30°C for six to seven generations and plated in their entirety to detect loss of *LEU2* or *SUP11-1*. To monitor loss of the *LEU2* marker, the cultures were plated on YEPD medium, and after 2 days, colonies were replica plated to plates lacking leucine. Colonies which grew on YEPD medium but not on plates lacking leucine were scored as having lost the ring chromosome. To monitor the loss of *SUP11-1*, cultures were plated on color assay plates. The appearance of red colonies signalled loss of the *SUP11-1* marker (14). (*SUP11-1* is an ochre suppressor tRNA capable of suppressing the *ade2-101* mutation. In the absence of the suppressor, *ade2* colonies become red because they accumulate a red pigment formed from the metabolic intermediate that is the substrate of the *ADE2* gene product.) Rates of loss were calculated using either the zero class of the Poisson distribution or the method of the median (21). Data used in the calculation were taken only from trials in which the total colonies per plate differed by threefold or less.

For measurements of loss of the full-length chromosome, disomic strains were allowed to grow for 11 generations, and each culture was divided among four color assay plates. Loss rates were calculated using the zero class of the Poisson distribution (21).

For the colony isolation procedure, exponentially growing cultures were plated on YEPD plates and allowed to grow for 8 to 9 generations in the case of 61-kb-ring strains or for 12 to 13 generations in the case of the chromosome III disomes. When the colonies were the appropriate size, they were excised from the agar with a sterile Pasteur pipette as described by Hegemann et al. (13), suspended in sterile water by being vigorously vortexed, and plated on either YEPD for subsequent replica plating to plates lacking leucine or directly onto color assay plates. The results were analyzed as described for the liquid culture assays.

For very unstable derivatives, the time course procedure was used for measuring loss rates. A colony from a plate lacking leucine was suspended in 10 ml of supplemented minimal medium and grown overnight at 30°C to 7×10^5 cells per ml. Leucine was added to a final concentration of 100 mg/liter, and 2 h later (defined as time zero) and at hourly intervals thereafter, aliquots were plated on color assay plates and plates lacking leucine. The fraction of Leu⁺ colonies versus time (in generations) was plotted on semilog

paper, and the rates of loss of the ring chromosome were determined from the slopes of the straight lines (31).

The loss rates determined by these three procedures were similar. The data in Table 2 demonstrate that the colony isolation procedure and the liquid culture procedure yielded similar loss rates for a number of derivatives of the 61-kb ring chromosome. The loss rate of the 61-kb ring chromosome derivative in PF6-5B (Table 2) was also measured by the time course procedure and found to be 0.025 losses per cell per generation. This observation demonstrates that results from the time course procedure are comparable to those from the colony isolation procedure and the liquid culture procedure.

RESULTS

Construction and characterization of *ARS* deletions. To delete individual *ARS* elements from chromosome III, we constructed plasmids carrying chromosome III segments from which 0.5 kb (*ARS307*) or 1.7 kb (*ARS309*) of DNA carrying the *ARS* element had been deleted (*ARS* deletion plasmids) as described in Materials and Methods (see Fig. 2). The plasmids were tested for *ARS* activity by the high-frequency transformation assay (25), and both were *Ars*⁻.

For construction of chromosomal *ARS* deletions, yeast strains were transformed with an *ARS* deletion plasmid that had been cut within yeast sequences to direct integration, and *Ura*⁺ transformants were selected. In these integrants, the vector is flanked on one side by the wild-type *ARS* element and on the other by the *ARS* deletion. Recombination between the duplicated flanking sequences excises the integrated plasmid, leaving behind either the wild-type *ARS* element or the *ARS* deletion.

To identify cells which had lost the plasmid, several single colonies of an appropriate integrant were patched on plates containing 5-fluoroorotic acid, and two single *Ura*⁻ colonies were picked from each patch. Total genomic DNA was prepared from several *Ura*⁻ popouts from each integrant, and Southern analysis was used to determine which retained the wild-type sequences and which retained the *ARS* deletion sequences.

Because intergenic regions on yeast chromosomes are very small, the deletions that removed *ARS* elements were also expected to remove portions of adjacent open reading frames, an expectation that was confirmed by analysis of transcripts on chromosome III (47) and open reading frames predicted by the DNA sequence of the chromosome (30) (Fig. 2). To determine whether the *ARS* deletions had dominant deleterious effects in a diploid, each deletion was constructed in strain YNN216. Neither deletion caused a change in the growth rate. The heterozygous diploid was then sporulated, and tetrads were dissected to determine spore viability and growth rate. The *ARS307* deletion was found to reduce the growth rate, with deletion-containing spores requiring several more days to produce visible colonies than wild-type spores in the same tetrad. This slow-growth phenotype must be caused by either the deletion of the *YCL4W* open reading frame or a frameshift mutation in the third amino acid from the carboxyl terminus of the *YCL5W* open reading frame (Fig. 2A). Symington and Petes (41) have noted that a frameshift mutation caused by filling in an *Xba*I site within *YCL5W* resulted in slow growth. The spores carrying the *ARS309* deletion, which also deletes most of the *YCR9C* open reading frame and about 30% of the *YCR10C* open reading frame (Fig. 2B), had a normal growth rate.

FIG. 2. Restriction maps of *ARS307* and *ARS309* regions and location of *ARS* deletions. (A) The 8.4-kb C2G *Bam*HI fragment containing *ARS307* and *CEN3*. The *ARS307* deletion plasmid was constructed with the fragments indicated by black bars (see Materials and Methods). A *Cla*I linker was added to the *Eco*RI site that defines the left end of *ARS307* for construction of the deletion plasmid. To direct integration, the deletion plasmid was linearized with *Bgl*II at the site marked with an asterisk. For analysis of transformants carrying the integrated plasmid and the popouts derived from them, genomic DNA was digested with *Eco*RI and probed with the probe indicated by the stippled bars below the map. The wild-type *ARS* element was identified by the presence of a 3.4-kb fragment, and the *ARS307* deletion was identified by the presence of a 2.9-kb fragment. In all strains marked with *SUP11-1*, the *SUP11-1* fragment was inserted into the *Bgl*II site marked with an asterisk. *ARS308* is coincident with *CEN3*. (B) A portion of the J11D *Bam*HI fragment containing *ARS309*. The *ARS309* deletion was constructed with the fragments indicated by black bars. To direct integration, the deletion plasmid was linearized by digestion at the *Kpn*I site indicated by the asterisk. For analysis of transformants carrying the integrated plasmid and the popouts derived from them, genomic DNA was digested with *Bgl*II and probed with the probe indicated by the stippled bar below the map. The presence of the wild-type *ARS* element was signalled by the presence of a 3.2-kb fragment, and the *ARS* deletion was signalled by a 1.6-kb fragment. This figure shows the correct position of *ARS309* in the J11D region of chromosome III. This *ARS* element was placed approximately 1 kb to the left of its correct position in Fig. 3 of reference 28. In both panels, open reading frames predicted by the chromosome III DNA sequence (30) are indicated by arrows above the map. The unfilled arrows represent open reading frames affected by the *ARS* deletions. The tick marks on the restriction maps are at 500-bp intervals.

To determine whether deletion of these *ARS* elements also removed the corresponding replication origin, 2-D gel electrophoresis was performed on the wild-type and deletion strains. We have shown previously that both the *ARS307* deletion described here and some point mutations in the *ARS307* consensus sequence abolish chromosomal origin function (7, 11). The data in Fig. 3 show that the *ARS309*

FIG. 3. Analysis of *ARS309* replication intermediates. Genomic DNA isolated from strain CF4-16B (A) or CF4 Δ 309 (C) was digested with *Bam*HI and *Eco*RV (Fig. 2B), separated on a 2-D agarose gel, blotted to Nytran, and probed with the *Hind*III fragment indicated in Fig. 2B (B and D). Diagrams of the patterns shown in panels A and C, respectively. The arcs are labeled to show the replication intermediates that migrate in them, and the sizes of the intense spots containing nonreplicating, monomer-length fragments identified by the probe are indicated. The 4.9-kb fragment contains *ARS309*, and the 3.2-kb fragment contains the deletion of *ARS309* diagrammed in Fig. 2B. The intense hybridization extending downward and to the right from the monomer spots is due to partially degraded DNA fragments in the preparation. This signal is prominent because overexposure of this region is necessary to visualize the arcs of replication intermediates. The streaks that extend leftward from the monomer spots and the streaks above the monomer spot in the upper part of the blots are caused by trailing of monomer-length fragments during the first- and second-dimension electrophoresis, respectively.

deletion removes the *ARS309*-associated replication origin from chromosome III. Figure 3A and B show the 2-D agarose gel analysis of the replication intermediates of the 4.9-kb *Bam*HI-*Eco*RV fragments containing *ARS309* in strain CF4-16B. This strain is disomic for chromosome III, and the presence of only a single spot of nonreplicating fragment demonstrates that the *Bam*HI-*Eco*RV fragment identified by the probe is the same size in both copies of chromosome III. If *ARS309* is active as an origin in this strain, then replication forks are expected to move away from *ARS309* in both directions and replication intermediates are expected to contain replication bubbles until one of the replication forks moves out of the fragment, which causes a transition to a Y-shaped replication intermediate. Since bubble-containing replication intermediates and Y-shaped replication intermediates fall on different arcs (3), the 2-D gel pattern is expected to be discontinuous, with early replication intermediates falling on a bubble arc and late replication intermediates falling on a Y arc (Fig. 3B). The pattern shown in Fig. 3A is consistent with this expectation, with the intense signal at the lower left end of the Y arc representing the transition from bubble-containing inter-

mediates to Y-shaped intermediates. Our previous analysis of overlapping restriction fragments (10) demonstrated that the replication origin maps to *ARS309* and that the light complete Y arc is the result of breakage of bubble-containing replication intermediates at one fork. Figure 3C and D shows the analysis of CF4 Δ 309, which is heterozygous for the *ARS309* deletion. The transition from bubble arc to Y arc characteristic of an active replication origin is readily apparent in the replication intermediates arising from the wild-type *ARS309*-containing fragment (4.9 kb). However, the replication intermediates of the *ARS* deletion fragment (3.2 kb) are all Y shaped, demonstrating that the *ARS* deletion fragment is replicated from an external origin and therefore that deleting *ARS309* removes a chromosomal replication origin.

Effects of *ARS* deletions in the 61-kb ring derivative of chromosome III. The 61-kb ring chromosome III (Fig. 1B) provides a well-defined starting point for studying the contribution of multiple *ARS* elements to chromosome stability. It is part of the 200-kb region of chromosome III that has been cloned and analyzed for *ARS* elements (28). In addition, a replication map has been prepared by analyzing the replication structures present in overlapping restriction fragments covering the entire ring chromosome with 2-D gel electrophoresis (10). Each of the three *ARS* elements on the ring chromosome functions as an origin of replication.

To determine whether deleting one or more replication origins from the 61-kb ring chromosome III affected its stability, we focused on the two highly active origins, *ARS307* and *ARS309*, both of which initiate replication more than 90% of the time (10). Haploid strains carrying the 61-kb ring chromosome in addition to a full-length copy of chromosome III were constructed so that loss of the ring chromosome would not affect the viability of the cell under nonselective conditions. This strain was transformed with the appropriate *ARS* deletion plasmid, and transformants were screened to determine which copy of chromosome III contained the integrated plasmid by cosegregation of the plasmid *URA3* gene with *LEU2* or *SUP11-1* on the marked ring chromosome. *Ura*⁻ popouts were selected and screened as described in Materials and Methods to identify ring chromosomes carrying the *ARS* deletion. Wild-type popouts were used as isogenic controls. The loss of the ring chromosome was monitored genetically by the identification of segregants that had lost markers on the ring chromosome, either *LEU2* or *SUP11-1*.

Table 2 summarizes the loss rates determined by fluctuation analysis of the wild-type ring chromosome and derivatives of the ring chromosome from which one or more *ARS* elements had been deleted. Examination of the data demonstrates that deletion of *ARS307* caused a 2- to 2.5-fold increase in loss rate and that deletion of *ARS309* caused a 2.5- to 4.5-fold increase in the loss rate of the ring chromosome. These increases in the rate of loss of the chromosome occurred irrespective of whether loss rates were measured during growth in liquid cultures or on plates, and they were also consistent when chromosome loss was scored by the appearance of *Leu*⁻ segregants or the appearance of red colonies in *SUP11-1*-marked strains.

Deletion of both *ARS307* and *ARS309* (strains 11w' and PF6-5B-21a; Table 2) caused a 60- to 200-fold increase in the rate of loss of the ring chromosome. Under selective conditions, these strains grew very slowly, with doubling times of greater than 10 h. They rapidly accumulated revertants that maintained the ring chromosome more stably, as manifested by a steady decrease in doubling time and the appearance of

fast-growing colonies when single cells were plated. Therefore, the measured loss rates are minimum loss rates and are likely to be higher. One class of faster-growing revertants was found to carry ring chromosomes in which either *ARS307* or *ARS309* had been restored, presumably by gene conversion from the full-length chromosome III (data not shown).

Comparison of the actual rates of chromosome loss reveals that the *SUP11-1*-marked chromosomes are lost at a rate two- to fivefold higher than that of the corresponding wild-type chromosomes. The reason for this higher loss rate is unknown. The strains carrying the *SUP11-1*-marked chromosome are not isogenic with the others, and it is possible that genetic background contributes to the different rates of loss. However, in a disomic strain that carries two full copies of chromosome III, one of which is marked with *SUP11-1* (see Fig. 4), the *SUP11-1*-marked chromosome is lost at a rate three- to fourfold higher than that of the unmarked chromosome (data not shown). This observation suggests that the presence of *SUP11-1* influences the loss rate directly. Nevertheless, the relative increases in loss rate caused by deleting one or both highly active origins in both sets of strains were consistent.

These results demonstrate that at least one highly active origin is required for the stable maintenance of the ring chromosome. The third *ARS* element on the ring chromosome, *ARS308*, is active as a chromosomal origin in only 10 to 20% of cell divisions. If *ARS308* is the only sequence in the ring chromosome capable of origin function after the deletion of *ARS307* and *ARS309*, then the double deletion ring would be expected to be lost at a rate of 0.8 to 0.9 losses per cell per generation. Since the measured loss rates are minimum estimates and only fivefold lower than expected, it seems unlikely that cryptic origins are activated upon deletion of the highly active ones.

The increase in loss rate caused by deletion of a single highly active origin can be explained in at least two ways. First, the rate of replication fork movement may be inadequate to consistently replicate the whole ring chromosome from only one highly active origin during the S phase, and the loss events would reflect failures to completely replicate the chromosome. In this case, replication forks would move more slowly than average through this portion of chromosome III. Alternatively, the highly active origins may not initiate replication in every S phase, and the increased rates of loss would reflect failure of the remaining origins on the ring chromosome to initiate replication. To distinguish between these two hypotheses, we deleted *ARS307* and *ARS309* from a full-length copy of chromosome III. If the fork rate is inadequate, this chromosome should also be lost at an increased rate as *ARS* elements are removed. In contrast, if the efficiency of initiation of replication is inadequate in the ring chromosome, deletion of *ARS307* and *ARS309* from the full-length chromosome should not affect its stability, because neighboring *ARS* elements would compensate for those removed.

Effect of *ARS* deletions on the stability of a full-length copy of chromosome III. To examine the effect of deleting single or multiple *ARS* elements from full-length chromosome III, strain CF4-16B, a haploid strain disomic for chromosome III, was used (Fig. 4). *ARS* deletions were made in the *SUP11-1*-marked chromosome, whose loss could be detected by the appearance of red colonies. *SUP11-1* can be lost three ways: (i) by loss of the marked chromosome, (ii) by mitotic recombination between *SUP11-1* and *CEN3* followed by segregation of the recombinant chromatid lacking

FIG. 4. The chromosome III disome in strain CF4-16B: schematic diagram of the two copies of chromosome III. *his4-280* and *his4-290* are complementing alleles. Loss of either allele results in a His⁻ phenotype.

SUP11-1 to the same pole as the nonrecombinant chromatid lacking *SUP11-1*, or (iii) by gene conversion. These events can be distinguished by assaying red colonies for their abilities to grow without histidine and for their mating abilities. Chromosome loss produces red colonies that are His⁻ and mating proficient, usually mating type a. Mitotic recombination events give rise to red colonies that are His⁻ and nonmating. Gene conversion results in red colonies that are His⁺ and nonmating. As expected, the loss of *SUP11-1* by mitotic recombination or gene conversion was infrequent, and most (80 to 99%) red colonies resulted from chromosome loss.

Table 3 summarizes the loss rates determined for the full-length chromosome III from which *ARS307*, *ARS309*, or both had been deleted. As expected from its larger size, the loss rate of the full-length chromosome III is several-hundredfold lower than that of the 61-kb ring chromosome (for a review, see reference 25). All three deletion derivatives were at least as stable as the wild-type chromosome. Although the rate of loss of the wild-type chromosome III from strain CF4-16B appears to be significantly greater than the rate of loss of the double *ARS* deletion chromosome from strain CF4Δ307-37, further experiments with additional strains suggest that the values reported in Table 3 are the extremes of variation seen for normal loss rates and that the apparent trend in loss rates is not significant (6).

In strain CF4Δ307-37, from which *ARS307* and *ARS309* have been deleted, the active flanking origins are *ARS306* (7, 48) and *ARS310* (6). These results demonstrate that the 100-kb region between *ARS306* and *ARS310* (28) can be efficiently replicated by the forks that move from them toward the centromere. Since the amount of DNA replicated by the forks emanating from *ARS306* and *ARS310* (50 kb) is larger than the amount of DNA replicated by the two forks from a single active origin on the 61-kb ring chromosome (30 kb), these data suggest that the increase in the loss rate caused by deleting a single origin from the 61-kb ring chromosome results from inefficient initiation at the remaining highly active origin.

DISCUSSION

The results presented here demonstrate that each of the highly active replication origins on the 61-kb ring chromo-

TABLE 3. Mitotic loss rates of full-length chromosome III derivatives

Strain	<i>ARS</i> element(s) deleted	Loss rate (losses/cell/generation) ^a
CF4-16B	None	$6.34 \times 10^{-5} \pm 1.93 \times 10^{-5}$
CF4Δ307	<i>ARS307</i>	$4.88 \times 10^{-5} \pm 2.83 \times 10^{-5}$
CF4Δ309	<i>ARS309</i>	$4.27 \times 10^{-5} \pm 1.92 \times 10^{-5}$
CF4Δ307-37	<i>ARS307</i> and <i>ARS309</i>	$2.10 \times 10^{-5} \pm 1.48 \times 10^{-5}$

^a Loss rates ($\pm$ standard deviation) determined by fluctuation analysis (colony isolation procedure) as described in Materials and Methods.

some contributes to its stability. Deletion of both highly active origins results in a chromosome that is lost in at least 20% of cell divisions. This very high loss rate is close to that expected if *ARS308* is the only sequence left on the ring chromosome that is capable of origin function and if there are no cryptic origins in the ring chromosome capable of ensuring its replication in the absence of the origins normally used. Moreover, extending our previous observation that deleting *ARS307* did not increase the activity of *ARS308* (10), the high loss rate of the double deletion ring demonstrates that even in the absence of both highly active origins, *ARS308* is 30- to 70-fold less active than either *ARS307* or *ARS309*.

Since deleting *ARS307* and *ARS309* from the full-length chromosome had no measurable effect on its stability, it is likely that the decrease in the stability of the ring chromosome caused by deleting either *ARS307* or *ARS309* is the result of the failure of the remaining origin to initiate replication in a small fraction of cells. The absolute loss rates of the ring chromosome in strains 33-4 and 3-1 (2×10^{-3} and 4×10^{-3}) suggest that *ARS307* and *ARS309* initiate replication in more than 99% of the population. This confirms and extends our previous observation, based on replication fork direction analysis, that these two *ARS* elements are active as replication origins in at least 90% of the population (10).

The mitotic loss rate of the 61-kb ring chromosome reported here is similar to those reported previously for other small derivatives of chromosome III (39) and artificial circular minichromosomes (14, 24). The mitotic stability of small circular chromosomes appears to increase as size increases, to a plateau of approximately 4×10^{-3} losses per division, while linear chromosomes of comparable size are less stable but continue to increase in stability as length increases, to a rate of 10^{-5} to 10^{-6} losses per division for naturally occurring chromosomes. The circular minichromosomes previously studied contained small amounts of yeast DNA and multiple copies of bacteriophage λ chromosomes, which do not contain an *ARS* element. The observation that bacteriophage λ DNA is not packaged into normal yeast chromatin (1) suggested that some of the instability of small circular minichromosomes could be due to faulty chromatin structure. Our finding that a natural ring chromosome carrying only yeast DNA is lost at a similar rate demonstrates that the instability of small circular minichromosomes cannot be attributed to the presence of foreign DNA.

We unexpectedly found that the *SUP11-1*-marked copies of chromosome III are lost at a higher rate than chromosome III in a *Sup11⁺* background. Louis and Haber (22) have noted that the presence of *SUP11-1* induces meiosis I nondisjunction of yeast chromosomes, an effect that was independent of the chromosomal location of *SUP11-1*. Our preliminary observation that *SUP11-1* may act in *cis* on the copy of chromosome III that carries it suggests that the effect we have observed is different from the *trans* effect previously described.

Strains in which both highly active origins were deleted from the 61-kb ring chromosome grew very slowly under conditions requiring the ring but rapidly accumulated revertants which grew faster. Some of these revertants were gene convertants in which an *ARS* deletion on the ring was replaced by the functional copy carried on the wild-type chromosome. It is possible that characterization of additional revertants will reveal interesting *trans*-acting mutations that allow more stable maintenance of the ring or new replication origins created by mutations of ring chromosome sequences. Kipling and Kearsley (20) have reported the

creation of *ARS* elements by mutation of bacteriophage DNA sequences.

Although replication of full-length chromosome III appears unaffected by the removal of highly active replication origins from 100 kb of its central region, this distance is within the range that should be replicated efficiently by a pair of converging replication forks. These results demonstrate that not every active origin is essential for normal stability of the full-length chromosome. However, it is possible that in the strain carrying *ARS* deletions in the full-length chromosome, a mitotic checkpoint, such as the one identified by *rad9* mutations, delays the cell cycle until replication is completed (12, 45). In this case, the high fidelity of chromosome transmission would be dependent on the *RAD9* gene product. In the case of strains carrying *ARS* deletions in the 61-kb ring chromosome, the *RAD9* system would not recognize chromosomes which have not initiated replication at all. Such chromosomes would simply be lost to one of the daughter cells. It will be interesting to determine whether the *ARS* deletion chromosome is less stable in *rad9* mutants and also to determine how many origins can be deleted before chromosome stability is compromised.

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