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A DNA Polymerase Mutation That Suppresses the Segregation Bias of an *ARS* Plasmid in *Saccharomyces cerevisiae*

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Yeast autonomously replicating sequence (*ARS*) plasmids exhibit an unusual segregation pattern during mitosis. While the nucleus divides equally into mother and daughter cells, all copies of the *ARS* plasmid will often remain in the mother cell. A screen was designed to isolate mutations that suppress this segregation bias. A plasmid with a weak *ARS* (*wARS*) that displayed an extremely high segregation bias was constructed. When cells were grown under selection for the *wARS* plasmid, the resulting colonies grew slowly and had abnormal morphology. A spontaneous recessive mutation that restored normal colony morphology was identified. This mutation suppressed plasmid segregation bias, as indicated by the increased stability of the *wARS* plasmid in the mutant cells even though the plasmid was present at a lower copy number. An *ARS1* plasmid was also more stable in mutant cells than in wild-type cells. The wild-type allele for this mutant gene was cloned and identified as *POLδ* (*CDC2*). This gene encodes DNA polymerase δ, which is essential for DNA replication. These results indicate that DNA polymerase δ plays some role in causing the segregation bias of *ARS* plasmids.

The autonomously replicating sequence (*ARS*) element is defined by its effect on a plasmid transformed into the yeast *Saccharomyces cerevisiae*. The *ARS* element converts an integrating plasmid, a plasmid that must integrate into the chromosome in order to be stably inherited, into an autonomously replicating plasmid, a plasmid that can persist in the cell as an extrachromosomal element (28). *ARS* elements have always been suspected to be origins of replication, and there is now direct evidence that replication initiates at some *ARS* elements when they are present either on a plasmid or in a chromosome (reviewed in references 6 and 18).

One property of *ARS* elements that is difficult to explain in view of their role as origins of replication is the maternal segregation bias of *ARS* plasmids. During mitosis, it is common for all of the circular *ARS* plasmids, often over 30 copies per cell, to remain in the mother cell. As a result, *ARS* plasmids are lost from one of the resulting cells at the rate of about 40% per division (17). This segregation bias may be even stronger in some weak *ARS* plasmids, which are extremely unstable yet present in higher copy number per cell containing the plasmid than are standard *ARS* plasmids (4). This segregation bias is suppressed by the addition of a *CEN* element, containing the centromere sequence, to the *ARS* plasmid. The stability of the *CEN-ARS* plasmid increases dramatically as a result of the segregation machinery of the mitotic spindle, even though the copy number falls to one (reviewed in reference 18).

Murray and Szostak (17) have discussed possible explanations for the segregation bias of *ARS* plasmids. Segregation bias is not simply a result of unequal partitioning of nuclear volume between mother and daughter cell. When viewed with both light and electron microscopes, the nucleus divides approximately equally between mother and daughter. They propose that the circular *ARS* plasmid becomes associated with some site or compartment in the nucleus which is destined to segregate predominantly to the mother cell and that the attachment of the *ARS* elements to these fixed nuclear sites occurs during DNA replication.

This work describes the isolation of a mutation which affects the segregation bias of an *ARS* plasmid. Starting with a weak *ARS* plasmid that is present in high copy number, a spontaneous mutation that increased the stability of the plasmid was isolated. The mutation suppresses segregation bias, since the *ARS* plasmid has a lower copy number in mutant than in wild-type cells. The mutation was shown to be in *POLδ* (*CDC2*), a gene which encodes a DNA polymerase (26). This result is consistent with the idea that DNA replication is involved in the segregation bias of *ARS* plasmids.

MATERIALS AND METHODS

Genetic methods, media, and strains. Standard laboratory protocols for handling yeast strains were used (25). For nonselective growth, YPDA (1% yeast extract, 2% peptone, 2% dextrose, 0.003% adenine sulfate) was used. Selective medium contained 2% dextrose, 0.17% yeast nitrogen base without ammonium sulfate, 0.5% ammonium sulfate, and all of the supplements required for growth. Strains are listed in Table 1. SH31 (*MAT***a**) was transformed with plasmid YCp50::HO (10) to switch mating type, the resulting diploid was sporulated, and SH63 (*MATα*) was isolated by micromanipulation. SH73, SH74, SH75, and SH95 were from the same tetrad. SH92 was constructed by backcrossing K382-19D (*MATα can1 cyh2 hom3*) four times into the SH31 strain background. SH130 and SH131 were constructed from SH129 by inserting *POLδ* from pSH16 in place of *polδ-1*, using the two-step transplacement method (31).

Plasmids. YIp5 has the yeast *URA3* gene inserted into pBR322. YRp7 has the 1.453-kb *TRP1-ARS1 Eco*RI fragment inserted into the *Eco*RI site of pBR322, and YRp17 has the same *Eco*RI fragment inserted into the *Eco*RI site of

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TABLE 1. Yeast strains

Strain(s)	Relevant genotype	Source
SH31	*MAT***a** *POLδ trp1Δ ade2-101° ura3-52*	M. Fasulo
SH56	*MAT***a** *polδ-p trp1Δ ade2-101° ura3-52*	Original *polδ-p* mutant strain
SH63	*MATα POLδ trp1Δ ade2-101° ura3-52*	SH31 with *MAT***a** changed to *MATα*
SH75	*MAT***a** *POLδ trp1Δ ade2-101° ura3-52*	Progeny of SH56 × SH63
SH95	*MATα POLδ trp1Δ ade2-101° ura3-52*	Progeny of SH56 × SH63
SH73	*MAT***a** *polδ-p trp1Δ ade2-101° ura3-52*	Progeny of SH56 × SH63
SH74	*MATα polδ-p trp1Δ ade2-101° ura3-52*	Progeny of SH56 × SH63
SH92	*MAT***a** *polδ-p can1ʳ cyh2ʳ hom3 ura3*	Derived from SH31
SH97	*MAT***a** *polδ-p::(POLδ URA3) trp1Δ ura3-52*	pSH16 integrated into SH73
K65-3D	*MAT***a***/MATα HO/HO ura3-52/ura3-52*	S. Klapholtz
SH100	*MAT***a** *cdc9 ura3-52*	S. Houtteman
SH106, SH107, SH119	*MATα polδ-p::(POLδ URA3) trp1Δ ura3-52*	Three progeny of SH97 × SH95
SH112	*MAT***a** *ste7 ura3-52*	S. Houtteman
H82-5-1, H82-8-4	*MAT***a** *polδ-1, MATα polδ-1*	J. R. Pringle
SH120, SH121	*MAT***a** *polδ-1 ura3-1*	Derived from H82-8-4
SH129	*MATα polδ-1 can1ʳ cyh2ʳ trp1Δ ura3-1*	Derived from H82-5-1
SH130, SH131	*MAT***a** *POLδ can1ʳ cyh2ʳ trp1Δ ura3-1*	Replacements of *polδ-1* in SH129 with *POLδ*

YIp5 (28). YCp50 has the yeast *CEN4* and *ARS1* regions added to YIp5 (23). The weak *ARS* (*wARS*) plasmid was constructed by first inserting the 827-bp *Eco*RI-*Pst*I fragment containing *TRP1* into pBR322 in place of the small pBR322 *Eco*RI-*Pst*I fragment. There is no known *ARS* activity on these DNA fragments, and no transformants were ever recovered when this construct was introduced into strain SH31. This 4.4-kb plasmid was cut at the *Eco*RI site and ligated with random *Eco*RI fragments of *Drosophila melanogaster* genomic DNA. The resulting pool of plasmids was introduced into yeast strain SH31 and grown selectively in liquid medium lacking tryptophan. Slow-growing transformants were isolated, and the 7.4-kb plasmid recovered from the slowest-growing one, which had a doubling time of 13.5 h, was named the *wARS* plasmid. The *CEN4-wARS* plasmid was constructed by inserting a 2.0-kb *Eco*RI-*Bgl*II fragment from YCp19 (27), after changing the *Bgl*II site to an *Eco*RI site, into an *Eco*RI site of the *wARS* plasmid.

To clone the wild-type allele of *polδ-p*, a plasmid library similar to that constructed by Rose et al. (23) was made by partially digesting DNA from SH75 with *Sau*3A and inserting the fragments into the *Bam*HI site of YCp50. The hybrid plasmids were introduced into *Escherichia coli* to obtain 16,000 independent plasmids. From the analysis of 40 plasmids from the library, 83% of the plasmids contained inserts, and the average insert size was 9.0 kb.

Plasmids pSH8 to pSH13 were deletions of restriction fragments from the original plasmid, pSH7 (see Fig. 4A). Plasmid pSH8 deleted a *Cla*I fragment, and plasmids pSH9, pSH10, pSH11, pSH12, and pSH13 deleted *Bam*HI fragments. Plasmids pSH14, pSH15, pSH18, pSH19, and pSH26 contained various fragments from pSH7 inserted into YCp50. Plasmid pSH14 contained a 2.7-kb *Bam*HI fragment, and pSH15 contained a 6.2-kb *Cla*I fragment. Plasmids pSH18 and pSH19 were derivatives of pSH15 in which *Bam*HI fragments were removed. pSH26 was derived from pSH18 by deletion from the *Hpa*I site in the insert, after addition of *Sal*I linkers, to the *Sal*I site in YCp50. pSH16 contained the same 6.2-kb *Cla*I fragment as did pSH15, but the plasmid vector was YIp5. Plasmid pSH28, used to disrupt *POLδ*, was constructed by inserting a *Bgl*II fragment containing the *URA3* gene into the *Bgl*II site of pSH16 and moving the *Cla*I fragment into pBR322. The plasmid used to make the probe for blot hybridization of the pulsed-field gel was constructed by adding *Hin*dIII linkers to the *Hpa*I end of the 0.47-kb *Hpa*I-*Bam*HI central fragment (see Fig. 4C) and inserting it into the polylinker region of pSPT19 (Pharmacia no. 27-3515-01).

Plasmid copy number. The *wARS* plasmid was introduced into wild-type diploid SH75/SH95 and mutant diploid SH73/SH74, and transformants were recovered on plates containing synthetic medium lacking tryptophan. A 6-ml culture of synthetic medium lacking tryptophan was inoculated with the *wARS*-containing strains at 10^4 cells per ml and grown to stationary phase. The fraction of cells which contained the plasmid was measured by comparing the CFU on synthetic medium lacking tryptophan and on complete medium. The ratio of plasmid DNA to genomic DNA in the culture was determined as follows. DNA preparations from each culture were digested with *Cla*I, subjected to electrophoresis through 0.7% agarose, blotted to a nitrocellulose filter, and hybridized to a probe made by nick translation of YIp5. The filter was washed and exposed on Kodak XAR5 film. The *URA3* DNA on the probe hybridized to a genomic specific band of 5 kb, and the pBR322 portion of the probe hybridized to a plasmid-specific band of 7.4 kb. The appropriate regions of the filter were cut out, placed in 0.2 ml of Econofluor (New England Nuclear), and counted in a scintillation counter. Background counts, obtained by cutting and counting a blank area of the filter, were subtracted from the total counts. The samples were counted for 1 to 4 h, until there was a total of at least 400 counts above background.

The copy number per cell containing the plasmid was estimated by combining the following factors: the ratio of the plasmid counts per minute to the genomic counts per minute, two *URA3* genes per diploid genome, the different lengths of hybridization with the probe (YIp5 has 1.1 kb in common with the genomic *URA3* gene and 3.6 kb in common with the *wARS* plasmid pBR322 sequences), and the fraction of cells with plasmid (mitotic stability). A sample calculation for a *polδ-p* culture was (5.68 cpm/27.5 cpm × 2 × 1.1/3.6)/0.025 (mitotic stability) = 5.0. The copy number was determined for at least four cultures, and the sample mean and standard error were calculated. The absolute values of the copy numbers are likely to be affected by a systematic error because they depend on the relative size of the probe networks that form during hybridization at the plasmid and genomic bands (30) and the genomic hybridization occurs on

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two fragments (24). Since the bands were formed by hybridization of different DNA fragments, the extents of network formation might be different. However, the relative copy number between mutant and wild type will not be affected because this possible systematic error will cancel out.

Chromosome stability. Chromosome stability was measured by using the method of Hartwell and Smith (8). Diploids were grown in liquid YPDA and then spread on plates containing synthetic medium. The wild-type strains were incubated for about 1.5 days, until the colony diameter was 1.2 mm (about 10^6 cells). The mutant strains were incubated for about 1 day, until the colony diameter was 0.4 mm (about 5×10^4 cells). Mutant colonies were harvested earlier than wild-type colonies because mutant colonies produced an inconveniently large number of events when allowed to grow for as long as were wild-type colonies. Twenty colonies were removed from the plate, and each was suspended in 1 ml of water and briefly sonicated. To obtain the average cell number for the mutant and wild-type strains, a 10-μl sample from each suspension was pooled, the cells were counted in a hemacytometer, and a dilution was plated on complete medium. The remaining sample from each of the 20 colonies was plated on canavanine medium, incubated for 4 to 6 days, and replicated to synthetic medium lacking methionine. The numbers of Can^r Met^- and Can^r Met^+ colonies for each culture were counted. The rate of the appearance of these colonies and the standard error of this rate were determined by the method of the median (14).

***POLδ* cloning.** The wild-type allele of *polδ-p* was cloned by transforming the genomic library into the mutant diploid SH92/SH74 and screening the resulting transformants for the low rates of chromosome loss and mitotic recombination characteristic of the wild type. The transformants were patched onto medium selecting for the *URA3* marker on the plasmid. The plates were replicated onto canavanine and cycloheximide media after 3 to 4 days of growth. Thirty transformants, one positive control (wild-type diploid SH92/SH95) and one negative control (mutant diploid SH92/SH74), were included on each plate. The frequency of drug-resistant papillae (paps) that appeared within the area of the patch reflected the frequency of chromosome loss and mitotic recombination. About 2,000 patches were screened, one of which showed the low, or wild-type, pap formation frequency on both drug plates. When the cells from this patch were cured of the plasmid and retested, the pap formation frequency reverted to the high frequency of the mutant strain. The plasmid in this yeast transformant was recovered by transformation into *E. coli* and selection for the *amp* gene carried on the plasmid. This plasmid recovered from *E. coli*, pSH7, complemented the mutant phenotype when retransformed into the yeast tester strain.

DNA transformations. Two methods were used to transform *E. coli*, the $CaCl_2$ method (15) and electroporation with the Gene Pulser (Bio-Rad) (5). Yeast cells were transformed by the spheroplast method (11) or by the Gene Pulser method (5). The electroporation conditions for yeast cells were the same as for *E. coli*, with these modifications. Yeast cells were grown to stationary phase in YPDA. They were washed and resuspended in 1/20 volume of 10% glycerol. After the electroporation pulse, 1 ml of YPDA was added, and the cells were incubated for 1 h at 30°C prior to plating on selective medium.

Isolation of DNA from *E. coli* and *S. cerevisiae*. The alkaline lysis method was used for the rapid isolation of plasmid DNA from *E. coli* (15). For large-scale DNA isolation, the alkaline lysis method was scaled up, and the closed circular plasmids were purified on an ethidium bromide-CsCl gradient (15). A spheroplast method was used for rapid isolation of yeast DNA (31). Large-scale yeast DNA preparations were purified on Hoechst dye-CsCl gradients (20). A blot of yeast chromosomes separated by pulsed-field electrophoresis (7) was provided by Yogesh Pattel and Michael McClelland.

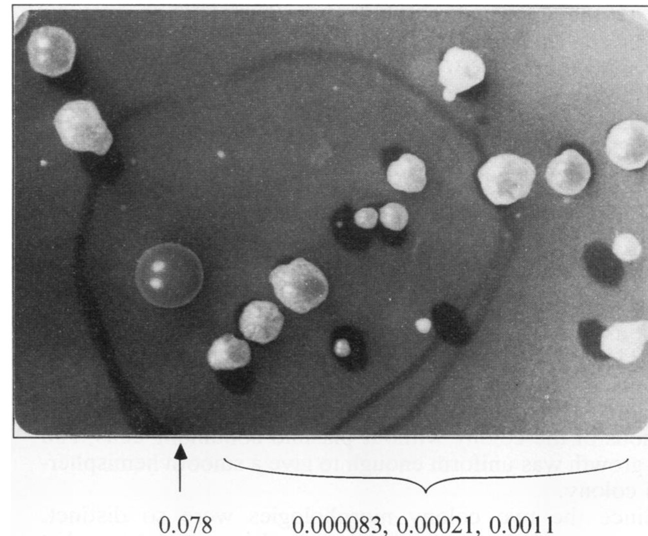

FIG. 1. Colonies growing under selection for a weak *ARS* plasmid. Yeast strain SH31(*trp1*) was transformed with the *wARS* plasmid, which contains the *TRP1* gene, and the resulting transformants were plated onto synthetic medium lacking tryptophan and grown for 4 days. The fractions shown below the photograph are the plasmid mitotic stabilities determined for the plasmids in the smooth, red colony and in three of the rough, mottled colonies. The plasmid stability in the smooth, red colony is much higher than those in the rough, mottled colonies.

RESULTS

Mutant isolation. The isolation of the mutant depended on the characteristics of the *wARS* plasmid. The *wARS* plasmid could not become stabilized by homologous integration into the host chromosome because the plasmid shared no homology with the host chromosomes. The selectable marker on the plasmid, *TRP1*, had been deleted from the host genome, and the *ARS* element on the plasmid was isolated from *D. melanogaster* DNA. This *wARS* plasmid gives a transformant with a doubling time of 13.5 h on selective medium, in comparison with a doubling time of 4 h for the YRp17 plasmid, which contains *ARS1*.

When strains with the *wARS* plasmid or the YRp17 plasmid were plated on selective medium, only the cells containing YRp17 grew into colonies with normal morphology. These colonies appeared in 2 days and were red with smooth margins. The red color is normal for *ade2* strains, which synthesize a red pigment when the adenine in the medium is exhausted (22). The mitotic stability (the fraction of cells in the colony that contained the plasmid) was about 0.01. In contrast, the cells containing the *wARS* plasmid grew into abnormal colonies on selective medium (Fig. 1). The colonies took 4 days to appear and were colored white with a red mottling and had rough, irregular margins. The average mitotic stability of the *wARS* plasmid was 0.0005.

The low mitotic stability of the *wARS* plasmid may have

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caused the abnormal morphology in the following way. As the *wARS*-containing colony grew, large areas lost the plasmid with the *TRP1* gene, became starved for tryptophan, and stopped growing. These areas remained white even though the *ade2* mutation is present because the cells stopped growing before the adenine in the medium was exhausted. Conversely, when a more stable plasmid was present, there was enough tryptophan synthesized to keep the colony growing until the adenine was depleted, and the red pigment developed. The irregular colony shape also could have been caused by the lack of plasmid-containing cells. The colony may have grown a little more slowly in regions lacking plasmid-containing cells, so eventually the retarded growth in that area would have caused the concave nibbles in the normally smooth margin of the colony. When a more stable plasmid was present, there were no large regions of the colony without plasmid-containing cells, and the growth was uniform enough to give a smooth hemispherical colony.

Since the two colony morphologies were so distinct, colony morphology was used to isolate a mutation that stabilized the *wARS* plasmid. The cells containing the *wARS* plasmid were plated on selective media and incubated for 4 days, and normal colonies were sought from among the abnormal colonies. About 10,000 abnormal colonies were screened, and 5 with normal morphology were isolated (Fig. 1). By determining the mitotic stability, it was verified that the colonies with normal morphology had more stable plasmids. In every case, the mitotic stability of the normal colonies was about 0.01, while that of the abnormal colonies was about 0.0005. To ensure that the mutation was not on the plasmid, all five isolates were cured of their plasmids and the *wARS* plasmid was reintroduced into the cells. In three of the five variants, the reintroduced *wARS* plasmid was stabilized. The other two variants were not studied further.

The three remaining variants were named SH54, SH55, and SH56. Analyses of crosses involving SH54 and SH55 suggested that they had become diploids which were homozygous at the mating-type loci. When SH54 and SH55 were mated to a haploid strain, both crosses yielded strains that sporulated to give a low frequency of viable spores (34 and 32%), which is typical of a triploid. When SH54 and SH55 were mated to a diploid homozygous for the opposite mating type, both crosses yielded strains that sporulated to give a high frequency of viable spores (72 and 88%), which is typical of a tetraploid. Thus, SH54 and SH55 appear to be diploids, and these strains were not analyzed further.

Mutant strain SH56 was mated to SH63, a wild-type strain of opposite mating type that was otherwise isogenic. The *wARS* plasmid was introduced into this diploid, and the plasmid showed the low stability characteristic of the wild type. Therefore, the mutation is recessive. When the diploid was sporulated, there was a high frequency of viable spores, and the phenotype of stabilizing the *wARS* plasmid segregated 2:2 in the four tetrads analyzed (Fig. 2). The mutation in this strain was the subject of all further experiments reported here.

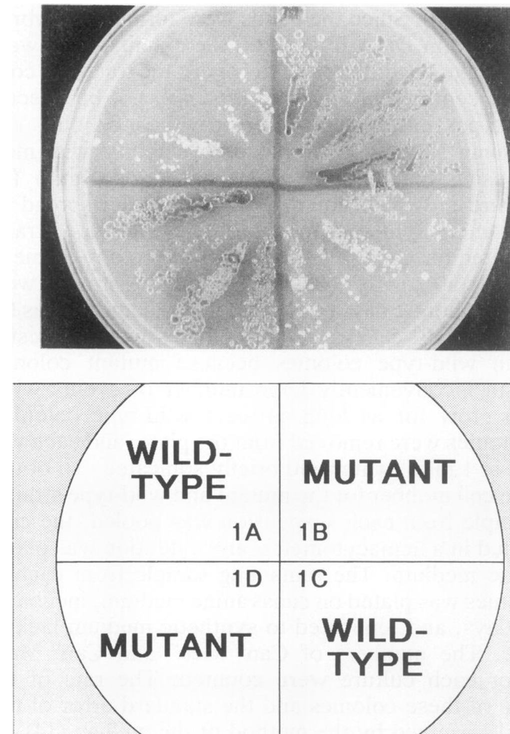

FIG. 2. Segregation pattern of the mutation affecting plasmid stability. SH56 (*MAT***a** *polδ-p*) was crossed to SH63 (*MATα POLδ*), a strain which was otherwise isogenic to SH56. The resulting diploid was sporulated, and the four spores from a single tetrad were cultured and transformed with the *wARS* plasmid. Independent transformants from each of the four spore cultures were streaked onto one quarter of a plate of selective medium and incubated for 4 days at 30°C. The plate shows that the mutant colony morphology segregated 2:2 in this tetrad.

Plasmid copy number is lower in mutant cells than in wild-type cells. There were two general mechanisms by which the *wARS* plasmid could be stabilized: an increase in replication or a more efficient partitioning into both mitotic products. If the stabilization is due to increased replication, then the average copy number should increase. If the stabilization is due to better partitioning alone, then the copy number should decrease. Copy number per cell containing the plasmid was estimated by measuring two features of a given culture. First, the amount of DNA in a plasmid-specific band was measured relative to the amount of DNA in a genomic-specific band by hybridization of a gel transfer. A representative hybridization is shown in Fig. 3. Second, the fraction of cells in the culture that contained the plasmid was determined by comparing the number of colonies that grew on selective and nonselective media. The copy number of the *wARS* plasmid in wild-type cells was 320 ± 100, while the copy number in the mutant cells decreased to only 4.6 ± 1. This 70-fold reduction in copy number in the mutant cells indicated that more efficient partitioning was responsible for the increased stability of the *wARS* plasmid in the mutant cells.

A second experiment also indicates that the mutation affects segregation of the *wARS* plasmid rather than its replication. A fragment containing *CEN4* was added to the *wARS* plasmid. Since the centromere now provides a segregation mechanism, this *CEN4-wARS* plasmid should have similar stability in the wild-type and mutant strains if the mutation affects only the segregation bias of the *wARS* plasmid. The stability of the *CEN4-wARS* plasmid in the wild-type strain SH95/SH75 is $(5.0 \pm 1.0) \times 10^{-2}$, while its stability in the mutant strain SH73/SH74 is $(6.6 \pm 2.3) \times 10^{-2}$. This similar stability of the *CEN4-wARS* plasmid is further evidence that the mutation affects the segregation bias of the *wARS* plasmid.

A strong *ARS* plasmid is stabilized in mutant cells. The

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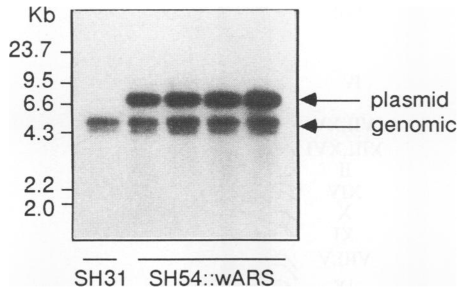

FIG. 3. Hybridization of a gel transfer in the determination of plasmid copy number. Yeast DNA digested with *Cla*I was fractionated on an agarose gel and blotted to nitrocellulose. The blot was hybridized with a YIp5 probe, which will hybridize to the yeast genomic DNA through *URA3* sequences and to the *wARS* plasmid through pBR322 sequences. The corresponding bands are labeled genomic and plasmid on the right. The ratio of the plasmid to genomic hybridization signal was used in the calculation of copy number for the *wARS* plasmid. The lengths of the fragments in the size standard are shown on the left. The genomic band is a doublet because the *ura3-52* allele in these yeast strains is due to the insertion of a Ty transposable element which contains a *Cla*I site (24). SH54 is one of the mutant strains derived from SH31 which stabilized the *wARS* plasmid. The four lanes of SH54::*wARS* are four independent cultures of SH54 containing the *wARS* plasmid grown under selective conditions.

mitotic stabilities of the *wARS* plasmid and of a standard *ARS* plasmid, YRp7 (*ARS1*), were measured in wild-type (SH75/SH95) and mutant (SH73/SH74) diploids. For the *wARS* plasmid under selective conditions, $(5.1 \pm 0.1) \times 10^{-4}$ of wild-type cells and $(2.7 \pm 0.1) \times 10^{-2}$ of mutant cells had the plasmid, a 50-fold increase. For the YRp7 plasmid, 0.11 ± 0.09 of the wild-type cells had the plasmid while 0.48 ± 0.06 of the mutant cells had the plasmid. Therefore, the mutant also stabilized a plasmid containing *ARS1*, a strong *ARS*.

Decreased chromosome stability in mutant cells. Chromosome stability was measured to determine whether the mutation affects chromosomes as well as plasmids. Stability of chromosome V was measured in diploids heterozygous at the *CAN1* locus, where resistance to canavanine is recessive (8). Diploids that lose the chromosome carrying *CAN1*^{s} express *can1*^{r} and grow on canavanine medium. Canavanine resistance could also result from a mitotic recombination event causing all the markers which were centromere distal to the crossover to become homozygous. These recombination events were distinguished from chromosome loss with the aid of a marker, *hom3*, on the opposite arm of the chromosome from the *can1*^{r} allele. The *HOM3* allele on the other chromosome arm will be lost only when the drug resistance is caused by chromosome loss.

Loss rates were measured by applying the method of the median to 20 independent cultures (see Materials and Methods). The wild type (SH92/SH95) had a chromosome loss rate of $(1.3 \pm 0.2) \times 10^{-5}$ and mitotic recombination level of $(3.1 \pm 0.4) \times 10^{-5}$, which are similar to the rates measured by Hartwell and Smith (8). The mutant (SH92/SH74) had a loss rate of $(8.8 \pm 1.8) \times 10^{-5}$, which was seven times higher than the wild-type rate. Thus, in contrast to the stabilization of the *wARS* plasmid in the mutant strain, chromosome V is less stable in the mutant strain. The mitotic recombination rate was also elevated in the mutant. The rate was $(16 \pm 3) \times 10^{-5}$ in the mutant and $(3.1 \pm 0.4) \times 10^{-5}$ in the wild type. Qualitatively similar results were obtained by following the

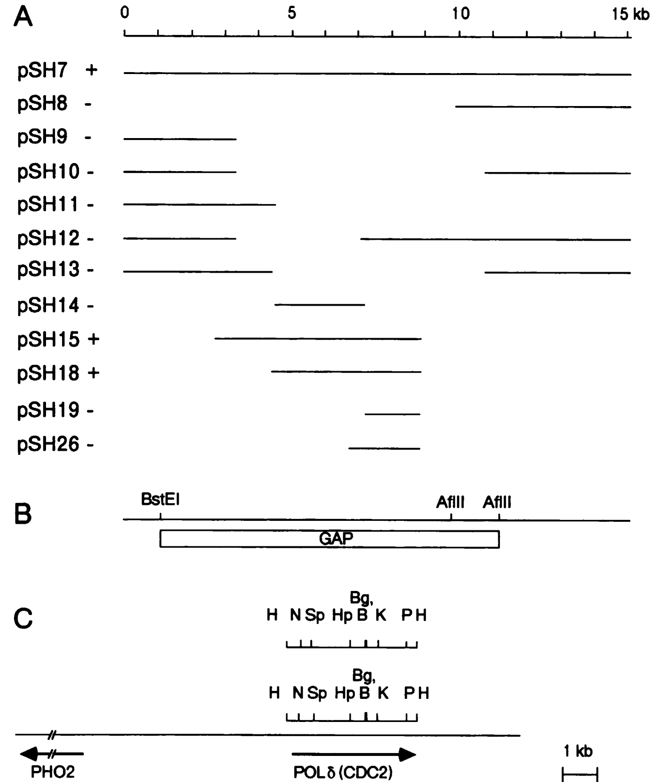

FIG. 4. Physical characterization of cloned DNA. (A) Plasmids pSH8 to pSH26 were derived from pSH7, which carries a 15-kb insert that complements the chromosome stability/mitotic recombination phenotype of the mutant diploid SH92/SH74. The subclones derived from pSH7 were tested for the ability to complement the mutant phenotype for chromosome stability. +, complementation; −, no complementation. The subclone analysis limits the complementing activity to a 4.3-kb region. (B) A map of the *Bst*EI and *Afl*II restriction sites shows the location of the gap which was repaired in wild-type and mutant strains. (C) A restriction map of the 3.9-kb *Hin*dIII fragment from the cloned DNA is shown above a map of the 3.9-kb *Hin*dIII fragment from the published DNA sequence of *POL*δ (*CDC2*) (2). Transcription data from the *POL*δ (*CDC2*) region are also shown (2). The slight differences between the restriction maps are within the experimental error expected when fragment sizes are determined by electrophoresis. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; P, *Pst*I; Sp, *Spe*I.

recessive *cyh2*^{r} marker on chromosome VII. In diploids heterozygous at the *CYH2* locus, approximately 10-fold more cells resistant to cycloheximide arose in the mutant strains than in the wild type. These results indicated that the mutation also affects the behavior of chromosome VII and that it therefore most likely affects the stability and mitotic recombination of all chromosomes.

Cloning of the wild-type gene. As described in Materials and Methods, the wild-type gene was cloned by making a yeast genomic library from a wild-type strain, transforming this library into a mutant diploid, and screening for a plasmid which reduced the high mutant rates of chromosomal loss and mitotic recombination to the rates characteristic of the wild type. The plasmid pSH7 isolated from this screen was reintroduced into the mutant diploid, in which it again reduced the rates of chromosome loss and mitotic recombination to wild-type levels. The pSH7 plasmid contained a

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15-kb insert, and the complementing activity was located in a 4.3-kb segment (Fig. 4A).

The phenotype originally associated with the mutation was an increase in mitotic stability of the *wARS* plasmid. Complementation of this phenotype by the insert in pSH7 could not be determined while the insert was present in plasmids; the *wARS* plasmid and the pSH7 plasmid both contain pBR322 sequences. If the two plasmids are present in the same cell, the *wARS* plasmid would recombine with the pSH7 plasmid and be stabilized.

To test for complementation of the mutant phenotype for plasmid stability, the cloned DNA was transferred from the plasmid into the yeast chromosome by using the two-step transplacement method (31). A 6.2-kb *Cla*I fragment containing the region complementing the phenotype of chromosome loss was subcloned into the yeast integrating vector YIp5 carrying the *URA3* gene to form plasmid pSH16. This plasmid was integrated into the yeast genome of strain SH73 by selecting for Ura^+ transformants and then excised from the yeast genome in strains SH106 and SH107 by selecting for Ura^- revertants with 5-fluoro-orotic acid (1). Twenty-seven independent Ura^- isolates were obtained. Depending on where the integration and excision events occurred, different portions of the 6.2-kb *Cla*I fragment from the cloned DNA will be found in the 27 isolates. Therefore, some isolates should have the mutation and some should not. The wild-type phenotype for chromosome loss and mitotic recombination appeared in 10 of the 27 strains, and in all 10 cases the wild-type phenotype for plasmid stability also appeared. The mutant phenotype for chromosome loss and mitotic recombination, which was present in the strain prior to the transformation, was still present in the other 17 Ura^- isolates. The mutant phenotype for plasmid stability also remained in these cells. Thus, both mutant phenotypes (plasmid stability and high rates of chromosome loss and mitotic recombination) were complemented by the cloned DNA, and the two mutant phenotypes are caused by the same mutation.

To verify that the clone was the wild-type allele of the mutant gene and not an unlinked suppressor, two other experiments were done to show that the cloned DNA and the mutation are located at the same locus. In the first experiment, a cross was carried out in which one parent carried the mutation along with the cloned DNA marked with *URA3* (SH97) and the other parent was wild type (SH95). (In both parents, the indigenous *URA3* locus contained auxotrophic alleles so that there would be no interference.) If the cloned DNA contains a suppressor gene, then the nonsuppressing allele will often be found in the Ura^- progeny of the cross. Half of the Ura^- progeny will get the unlinked mutant gene and show the mutant phenotype. However, if the cloned DNA is for the mutant gene, then the mutation will be tightly linked to *URA3* and all Ura^- progeny will have the wild-type phenotype. Twelve tetrads were analyzed, and all of the 24 Ura^- products showed the wild-type phenotype for plasmid stability. Thus, the clone and the mutation were linked and, with 50% confidence, were within 3 centimorgans.

For the second experiment, the gap repair method (19) was used. A gapped plasmid was made by removing a 10-kb region of pSH7 that contained the complementing activity (Fig. 4B). This gapped plasmid was transformed into mutant strain SH73 and into wild-type strain SH75 where the missing plasmid DNA was replaced by homologous recombination with the chromosomal DNA. The plasmids recovered from both strains by transformation of *E. coli* were tested for the ability to complement the mutant phenotype for chromosome loss and mitotic recombination. The plasmids recovered from the wild-type strain complemented the mutant phenotype, while plasmids recovered from the mutant strain did not. This result indicated that the mutation is located in the 10-kb gap.

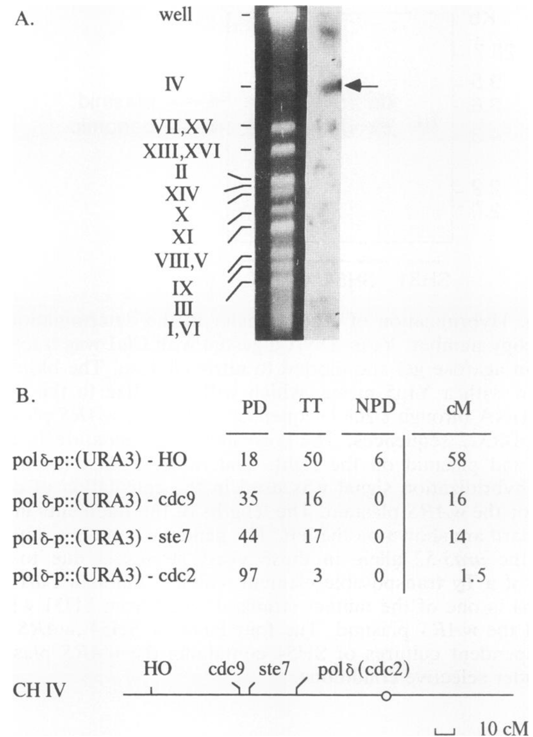

FIG. 5. Evidence that the cloned DNA is in the *POL*δ (*CDC2*) region of chromosome IV. A pulsed-field gel of yeast chromosomes was blotted to a nylon filter and hybridized with a probe made from the cloned DNA. (A) On the left is the pulsed-field gel stained with ethidium bromide viewed under UV. The chromosome(s) associated with each band is indicated (3). On the right is an autoradiogram of the blot hybridization. (B) *URA3* was integrated into the genome at the site of the cloned DNA. Strains containing the integrated *URA3* gene (SH106, SH107, and SH119) were mated to tester strains (K65-3D, SH100, SH112, SH120, and SH121). The segregation pattern of Ura^+ was monitored in the resulting tetrads with respect to the genetic markers on chromosome IV. At the bottom is a genetic map showing the relevant loci (16). PD, parental ditype; TT, tetratype; NPD, nonparental ditype; cM, centimorgans.

The cloned DNA encodes the *POL*δ (*CDC2*) gene. To map the cloned DNA to a yeast chromosome, chromosomal DNA was separated on a pulsed-field gel, transferred to a nylon membrane, and hybridized to a radioactive probe made from the cloned DNA. The probe hybridized to chromosome IV (Fig. 5A). On the basis of this result, a series of crosses was carried out between strains carrying the mutation and strains carrying markers for chromosome IV. Because the phenotypes of the mutation were difficult to assay, a strain with *URA3* inserted into the chromosome at the mutant locus was used to monitor the segregation of the mutation. In all of the crosses, homozygous *ura3* alleles were present at the normal *URA3* locus. These crosses revealed that the inserted *URA3* gene was very closely linked to the gene encoding DNA polymerase δ (Fig. 5B) and that the mutation could be an allele of *POL*δ.

Further evidence strongly supported the conclusion that the mutation is in the *POL*δ (*CDC2*) gene. The published

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*POL*δ DNA sequence shows that the coding region is contained within a 3.9-kb *Hin*dIII fragment (2). In the current study, the smallest subclone, SH18, that complemented the mutation contained a 3.9-kb *Hin*dIII fragment. A restriction map of the *Hin*dIII fragment from the current study was compared with a restriction map generated from the DNA sequence of *POL*δ gene (2). These two maps are identical (Fig. 4C). Furthermore, the pSH14 and pSH19 subclones subdivide the minimal subclone pSH18 into two parts, with the separation point being in the *POL*δ open reading frame. Neither of these subclones complemented the mutation (Fig. 4A), indicating that an intact *POL*δ gene is required for complementation.

A complementation experiment with a transplacement of *POL*δ provides additional evidence that the mutation is in the *POL*δ gene. A fragment containing the *URA3* gene was inserted into the *Bgl*II site (Fig. 4C) where it interrupts the open reading frame of the *POL*δ. This transplacement eliminates the function of the *POL*δ gene, since diploids heterozygous for this transplacement give no more than two viable spores per ascus and no viable spores are Ura$^+$. When the *pol*δ::*URA3* fragment is transplaced into strain SH75/SH92 heterozygous for the mutation, it should sometimes change the phenotype for plasmid stability of this diploid from wild type to mutant if the mutation is in the *POL*δ gene. This change in phenotype would happen when the wild-type *POL*δ allele is transplaced, leaving the mutant *pol*δ as the only functional allele. In contrast, if the mutation is not in the *POL*δ gene, then the transplacement should not change the phenotype. It was found that 3 of 19 transplacements changed to the mutant phenotype both for *wARS* plasmid stability and for rates of chromosome loss and mitotic recombination. This change from wild-type to mutant phenotype by the *pol*δ::*URA3* transplacement confirms that the mutation is in the *POL*δ gene, and the mutation was named *pol*δ partitioning (*pol*δ*-p*).

Other alleles of *POL*δ are temperature-sensitive lethal alleles and arrest at the large unbudded stage of the cell cycle (21). The *pol*δ*-p* allele did not appear to be temperature sensitive on solid medium, but a slight effect of temperature on growth may not have been detected. A comparison was made between the growth rates in liquid medium of a *pol*δ*-p* strain and of a *POL*δ strain otherwise isogenic. The strains were grown at 30 and 37°C. No significant differences in growth rates were seen at either temperature, although *pol*δ*-p* cells entered stationary phase slightly earlier than did wild-type cells at 37°C (data not shown). Therefore, unlike other alleles of *POL*δ, *pol*δ*-p* is not significantly temperature sensitive for growth.

Another allele of *POL*δ stabilizes the *wARS* plasmid. To determine whether stabilization of the *wARS* plasmid is a phenotype specific for the *pol*δ*-p* allele, another allele of *POL*δ was tested. A *pol*δ*-1* (*cdc2-1*) strain, SH129, and two *POL*δ strains, SH130 and SH131, were constructed so that the strains were isogenic at all but the *POL*δ locus. The *wARS* plasmid was introduced into both, and the mitotic stability was measured at 30°C. The plasmid stability in the wild-type strains SH129 and SH131 was $(6.1 \pm 0.35) \times 10^{-3}$, and plasmid stability in the *pol*δ*-1* strain SH129 was $(2.1 \pm 0.5) \times 10^{-2}$. Thus, the *pol*δ*-1* strain stabilized the *wARS* plasmid 3.4-fold. This is a small but significant difference ($P < 0.05$). Thus, *ARS* plasmid stabilization is not unique to the *pol*δ*-p* allele.

Plasmid stability was 12 times less in the wild-type strain SH75, from which the mutation was isolated, than in the wild-type strains SH130 and SH131. Thus, *wARS* plasmid stability varied 12-fold between these two strain backgrounds. In the original SH75 strain background, there was a 50-fold difference in stability of the *wARS* plasmid between *pol*δ*-p* and *POL*δ strains. In the SH130 and SH131 background, only a 3.4-fold difference was seen between *pol*δ*-1* and *POL*δ strains. It is not known whether the difference in the magnitude of the effect is due to the different mutant alleles or to the different strain backgrounds.

DISCUSSION

A mutation in the *POL*δ gene stabilizes *ARS* plasmids. Since the plasmid copy number per cell containing the plasmid decreases 70-fold, the *pol*δ*-p* mutation stabilizes the *wARS* plasmid by reducing the segregation bias of *ARS* plasmids. In contrast to the increase in stability seen for *ARS* plasmids, the *pol*δ*-p* mutation decreases the stability of chromosome V and also increases mitotic recombination on this chromosome.

These results have two important implications about the mechanism of segregation bias for circular *ARS* plasmids. First, they support the idea that the actual event of DNA replication is intimately involved in segregation bias, since the mutation reducing segregation bias is in a gene required for DNA replication. Second, the mechanism of segregation bias is likely to be important in the functioning of normal chromosomes. It is possible to argue that segregation bias is simply an accidental result of the *ARS* element being out of its normal place in the chromosome and thus has little physiological relevance. However, a mutation selected only for suppression of segregation bias also increases the rate of loss and mitotic recombination for a normal chromosome. A number of temperature-sensitive mutations which cause higher rates of chromosome loss and mitotic recombination at a semipermissive temperature are thought to have partially defective DNA synthesis (8, 9). These results suggest that processes responsible for the segregation bias of *ARS* plasmids are also necessary for fully efficient DNA replication on a normal chromosome.

Two other studies (12, 29) have identified mutations which increase the stability of a yeast plasmid, but these mutations affect a different property of the *ARS* and would not be expected to include mutations with the characteristics of the *pol*δ*-p* mutation. Both studies used a plasmid containing a *CEN* element, but because of a weak *ARS*, the plasmid was unstable in comparison with a *CEN* plasmid with a strong *ARS*. Since the *CEN* element provides a segregation mechanism, the instability of the plasmid is not caused by segregation bias, and mutations increasing stability of these plasmids act by improving replication ability of the weak *ARS*. This approach contrasts with the mutant selection done in our study, in which no *CEN* was present and plasmid instability was due to segregation bias.

One class of mutations affect segregation when the plasmid contains the *HMRE ARS* from the silencer of mating type information on the right arm of chromosome III (13). In wild-type strains, plasmids with the *HMRE ARS* are comparatively stable because they segregate randomly. Mutations in the *SIR2*, *SIR3*, or *SIR4* which eliminate silencer function also disable the specific mechanisms responsible for random segregation so that the *HMRE ARS* plasmid is now unstable as a result of segregation bias. These *sir* mutations therefore affect only the segregation and stability of plasmids containing the *HMRE ARS* and do not significantly affect other *ARS* plasmids.

The segregation bias typically seen for *ARS* plasmids

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presumably depends on attachment of the plasmid to a site which segregates preferentially to the mother cell (17), but this segregation site has not been identified. A mutation decreasing segregation bias of *ARS* plasmids could affect either the segregation site or the attachment of the *ARS* plasmid to the segregation site. One simple model is that the *POL*δ protein, perhaps as part of a replisome, is the site that segregates asymmetrically. Although there is at present no direct evidence for asymmetrical segregation of the *POL*δ protein, in terms of this model the *ARS* plasmid remains tightly bound to the *POL*δ protein and therefore segregates asymmetrically in the *POL*δ strain. However, the *pol*δ-*p* mutation might weaken the attachment of the *ARS* plasmid so that the released plasmid now segregates randomly with little or no bias. The alternative model is that some alteration in DNA replication in the *pol*δ-*p* strain prevents stable attachment of the *ARS* plasmid to a segregation site which is separate from the *POL*δ protein itself. In terms of this model, other mutants affecting DNA replication, such as *cdc9*, which encodes DNA ligase (8, 9), might also stabilize an *ARS* plasmid. Isolation and characterization of additional mutations reducing the segregation bias of *ARS* plasmids should provide further insight into the role of DNA replication in segregation bias and help identify the attachment site necessary for segregation bias.

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