Recombination-dependent mtDNA partitioning: *in vivo* role of Mhr1p to promote pairing of homologous DNA

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Yeast *mhr1-1* was isolated as a defective mutation in mitochondrial DNA (mtDNA) recombination. About half of *mhr1-1* cells lose mtDNA during growth at a higher temperature. Here, we show that *mhr1-1* exhibits a defect in the partitioning of nascent mtDNA into buds and is a base-substitution mutation in *MHR1* encoding a mitochondrial matrix protein. We found that the Mhr1 protein (Mhr1p) has activity to pair single-stranded DNA and homologous double-stranded DNA to form heteroduplex joints *in vitro*, and that *mhr1-1* causes the loss of this activity, indicating its role in homologous mtDNA recombination. While the majority of the mtDNA in the mother cells consists of head-to-tail concatemers, more than half of the mtDNA in the buds exists as genome-sized monomers. The *mhr1-1* Δ *ce1* double mutant cells do not maintain any mtDNA, indicating the strict dependence of mtDNA maintenance on recombination functions. These results suggest a mechanism for mtDNA inheritance similar to that operating in the replication and packaging of phage DNA.

Keywords: concatemer/heteroduplex joints/homologous recombination/rolling circle replication/*Saccharomyces cerevisiae*

Introduction

Evidence for recombination in the germ line is lacking in mammalian mitochondria because of strict maternal inheritance. Recently, some *in vivo* evidence for the presence of recombination was obtained from mammalian somatic cultured cells and human heart muscle (Tang *et al.*, 2000; Kajander *et al.*, 2001). Many organisms, including the yeast *Saccharomyces cerevisiae*, exhibit mitochondrial DNA (mtDNA) recombination during sexual reproduction. Although mtDNA recombination has been characterized in *S.cerevisiae* (Dujon, 1981), the mechanisms and the functions of mtDNA recombination are not well understood.

Homologous recombination is initiated by double-stranded breaks. The termini of the breaks are processed into single-stranded tails, which interact with homologous double-stranded DNA to form heteroduplex joints (Szostak *et al.*, 1983). Heteroduplex joints that consist of an intermolecular double helix are a general intermediate

of homologous DNA recombination (Holliday, 1964). Biochemical studies on the RecA protein (RecAp) of *Escherichia coli*, a prototype of the RecA/Rad51 family proteins, revealed that the heteroduplex is formed through two successive steps (McEntee *et al.*, 1979; Shibata *et al.*, 1979; Cox and Lehman, 1981; Kahn *et al.*, 1981; Shinohara *et al.*, 1993; Sung, 1994): homologous pairing and branch migration. Homologous pairing is the formation of a core heteroduplex joint from double-stranded DNA and homologous single-stranded DNA, and branch migration is the unidirectional, but slow, extension of the heteroduplex joint. It has been shown that RecAp and the Rad51 protein (Rad51p) must bind ATP to promote homologous pairing, and that ATP hydrolysis is necessary for branch migration (Shibata *et al.*, 2001).

Some homologous recombination systems do not require RecA/Rad51 family proteins. In these systems, homologous pairing is probably carried out by ATP-independent proteins. Examples of proteins that promote the ATP-independent pairing of single-stranded DNA and homologous double-stranded DNA *in vitro* are the RecT protein (RecTp; Noirot and Kolodner, 1998) and the RecO protein (RecOp; Luisideluca, 1995) of *E.coli*, and the human Rad52 protein (HsRad52p; Kagawa *et al.*, 2001). *In vivo*, RecTp promotes RecA-independent plasmid recombination in *E.coli* (Noirot and Kolodner, 1998), and Rad52p promotes Rad51p-independent mitotic recombination between a pair of small homologous sequences in *S.cerevisiae* nuclei (Bai and Symington, 1996; Ivanov *et al.*, 1996). Homologous recombination of *E.coli* phage λ requires the functions of two genes, *red α* and *red β* , but is independent of the host RecAp or its homologs (Enquist and Skalka, 1973). The β protein encoded by *red β* is a functional homolog of RecTp (Muyrers *et al.*, 2000).

Mammalian mtDNA exists in a circular form with a genome size (for a review, see Clayton and Smith, 1975), but the major species of *S.cerevisiae* mtDNA are linear head-to-tail multimers of genomic unit DNA with variable sizes (concatemers; Maleszka *et al.*, 1991; Bendich, 1996). Concatemers can be formed through rolling circle DNA replication or homologous DNA recombination. Rolling circle DNA replication is initiated through recombination-dependent mechanisms in some DNA replication systems, such as bacteriophage λ at the late stage of infection (Enquist and Skalka, 1973), plasmids (Silberstein *et al.*, 1990) and even the chromosome in SOS-induced *E.coli* cells (Asai *et al.*, 1993). In the later stages of λ infection, the DNA replication switches from a theta (θ) mode to a rolling circle (σ) mode, and this switch requires the proteins encoded by the *red α* (λ exonuclease) and *red β* (β protein) genes required for homologous DNA recombination. In phage T4 of *E.coli*, concatemers are formed through homologous DNA recombination, which depends

Fig. 1. The role of Mhr1p in the transmission of nascent mtDNA. (A) Confocal images of BrdU-labeled nascent mtDNA within *MHR1* and *mhr1-1* cells at 37°C. Nascent (newly synthesized) mtDNA within *MHR1* (left column) or *mhr1-1* cells (right column), arrested at G₁ phase by α -factor, was specifically labeled by BrdU at 30°C. After the removal of BrdU and α -factor, the cells were allowed to divide synchronously at 37°C for 1.5 h in YPD medium. Collected cells were fixed, and the BrdU-labeled mtDNA was detected by using fluorescein-conjugated monoclonal anti-BrdU F(ab')₂ fragments with a confocal scanning laser microscope. Panels in the top two rows are confocal images of BrdU-labeled nascent mtDNA (visible as dots); panels in the bottom two rows are cells under visible light. Bar, 10 μ m. (B) Lack of nascent mtDNA in the buds of *mhr1-1* cells. Cells were from the same samples used in (A) (top panel: micrographs under visible light). Buds and mother cells were separated, and each part was purified (panels in the second row: micrograph under visible light). Whole-cell lysates of *MHR1* and *mhr1-1* buds or mother cells (from top to bottom in the panels in the second row from the bottom: ~0.5, 1 and 1.5 μ g in proteins to each dot for bud, ~0.8, 1.6 and 2.4 μ g in protein to each dot for mother cell) were applied to an N⁺ membrane for immunoblotting using anti-BrdU serum to detect BrdU-labeled nascent mtDNA. The amounts of protein are represented by a mitochondrial protein, porin, in the panels in the bottom row.

on at least two T4-encoded proteins, UvsXp and endonuclease VII, a recombination junction (Holliday junction)-resolving endonuclease encoded by *gene 49* (Mosig, 1998).

The *S.cerevisiae mhr1-1* mutant is the first mutant defective in wild-type (ρ^+) mtDNA recombination and in mtDNA repair (Ling *et al.*, 1995, 2000). The *mhr1-1* mutation was mapped to a single locus on a nuclear chromosome. This mutation shows extensive vegetative petite production during cultivation at a higher temperature or after UV irradiation of the cells. About half of such petite cells were found to be ρ^0 (Ling *et al.*, 1995). This suggests that Mhr1p by itself or an Mhr1p-dependent recombination function plays a role in the maintenance of mtDNA. We have studied the *in vivo* and *in vitro* functions of Mhr1p to look for the connections between mtDNA recombination and mtDNA maintenance, and found that Mhr1p has activity to form a heteroduplex joint *in vitro*. This finding provides a clue towards understanding the basis of the apparently diverse phenotypes of *mhr1-1*.

Results

The mhr1-1 mutation causes a defect in the partitioning of nascent mtDNA into buds

To characterize the process of mtDNA loss at the restrictive temperature, we investigated the behavior of nascent (newly replicated) mtDNA. The nascent mtDNA of haploid cells was specifically labeled with 5-bromo-2'-deoxyuridine (BrdU) at 30°C by arresting the cells at G₁ with mating factor α (α -factor; Nunnari *et al.*, 1997). After the α -factor and BrdU were removed, the cells grew synchronously at 37°C. The labeled nascent mtDNA was bound by a BrdU-labeled DNA-specific antibody and was visualized by confocal scanning laser microscopy. The BrdU-labeled nascent mtDNA was unevenly distributed in the peripheral cytoplasm (Figure 1A). No signal was detected in ρ^0 cells (data not shown). The pattern of the nascent mtDNA distribution in the mother cells was very similar between *mhr1-1* and *MHR1* cells (Figure 1A). In budding (dividing) *MHR1* cells, the nascent mtDNA was detected in both the mother cell and the bud in most cases

(74%, $n = 57$), but the majority of budding *mhr1-1* cells (77%, $n = 64$) lacked nascent mtDNA in their buds (Figure 1A). The defect in the partitioning was also shown by a quantitative comparison of the bud mtDNA content between *mhr1-1* and *MHR1* (Figure 1B), and DAPI staining experiments using the same samples (Supplementary figure 1 available at *The EMBO Journal* Online).

The intracellular distribution of the mitochondrial reticulum in budding cells revealed by fluorescent images of mtHSP70 was not altered by *mhr1-1*, indicating that the partitioning of the mitochondrial reticulum to the growing buds is independent of the *MHR1* function (data not shown).

Thus, we conclude that the partitioning of nascent mtDNA to buds is independent of the partitioning of the mitochondrial reticulum into buds and that Mhr1p plays a role in a specific mitochondrial system for the partitioning of mtDNA into buds. This defect can explain the production of ρ^0 cells in the *mhr1-1* culture at 37°C.

The *mhr1-1* mutation is a single base substitution resulting in a single amino acid replacement within Mhr1p

As a step to identify the molecular functions of *MHR1*, we cloned a 0.94 kbp *ClaI*–*MluI* DNA fragment (in pRS416CM) containing a single open reading frame (ORF) that complements the deficiencies of *mhr1-1* cells in mtDNA recombination and the maintenance of functional mtDNA at a higher temperature (FL67-1423 Δ ura3; Table I). The 678 bp ORF (DDBJ/EMBL/GenBank accession No. AB016430) encodes a protein of 26.9 kDa and is identical to YDR296w. All corresponding ORFs of the three clones independently obtained by PCR of DNA isolated from *mhr1-1* cells share a single base pair substitution of A for G at nucleotide 515, causing the substitution of Asp for Gly172. Thus, the ORF is a nuclear gene, *MHR1*, and the *mhr1-1* mutation is the single base substitution.

***MHR1* encodes a protein (Mhr1p) that is localized in the mitochondrial matrix**

PSORT and PSORTII (<http://psort.nibb.ac.jp:8800/index.html>) analyses predicted that Mhr1p is localized in the mitochondrial matrix. Immunoblotting experiments using rabbit antiserum against recombinant Mhr1p expressed in *E.coli* showed that a 27 kDa protein is the only protein from the mitochondrial fraction that interacts with this antiserum (Figure 2A) and that the protein is absent from *mhr1*-null cells (Figure 2A). These results indicate that the 27 kDa protein is encoded by *MHR1*. We detected Mhr1p in both the inner membrane fraction and the matrix fraction, as is the case for mtHSP70, by examining fractionated mitochondrial components (Figure 2B).

The polypeptides recognized by the anti-Mhr1p serum, in the Mhr1p preparation from *E.coli* and yeast mitochondria, had the same mobility on SDS–PAGE, and corresponded to a mol. wt of ~26.7 kDa (Figure 2C). This suggests that no leader sequence with a significant size had been removed from the mitochondrial Mhr1p.

We constructed the gene for an Mhr1–green fluorescent protein (GFP) fusion protein that is fully functional, since its overproduction causes the same peculiar phenotype as

that of *MHR1* overexpression (F.Ling, unpublished observation). Microscopic observations indicated that the Mhr1–GFP fusion protein expressed in the cells is exclusively associated with mitochondria (Figure 2D).

Mhr1p promotes the pairing of single-stranded DNA and homologous double-stranded DNA *in vitro*

We purified the overproduced Mhr1p and *mhr1-1p* from *E.coli* cells to give a single band on SDS–polyacrylamide gels (Figure 3A). The amino acid sequence of the N-terminus matches the predicted N-terminal 17 amino acid sequence starting with Met of the *MHR1* ORF. We did not detect endo- or exonuclease or topoisomerase activities in the Mhr1p preparations (data not shown).

By assaying for the DNA recombination activities of Mhr1p, we found that Mhr1p can promote the pairing of linear single-stranded DNA (a 50mer oligo-DNA) and homologous superhelical closed circular double-stranded DNA to form heteroduplex joints, as follows (Figure 3B and C): the homologous pairing signal is dependent on the homologous sequences of double-stranded DNA and single-stranded DNA, and is so in a sequence independent way, i.e. two unrelated DNAs (pGsat4 and *E.coli* phage ϕ X174) can be substrates only if the double-stranded DNA and the single-stranded DNA share homology (Figure 3B). The protein-free products of the Mhr1p-promoted reaction migrate at the same velocity on gels as do heteroduplex joints formed by RecAp (McEntee *et al.*, 1979; Shibata *et al.*, 1979). In addition, they dissociate after the release of the topological constraint by cleavage of the double-stranded DNA at a site outside the region of homology (Figure 3B, lane 8; Radding *et al.*, 1977). The extents of the reaction depend on the presence of Mhr1p (Figure 3C) and on the time of incubation with Mhr1p (data not shown), and are similar to those obtained with RecAp (Figure 3B). Homologous pairing reaches an optimum at a medium protein concentration, but seems to be inhibited at a high protein concentration (Figure 3C), as is the case with Rad52p (Kagawa *et al.*, 2001). Unlike RecAp and Rad51p (McEntee *et al.*, 1979; Shibata *et al.*, 1979; Sung, 1994), but like RecTp (Noirot and Kolodner, 1998), RecOp (Luisideluca, 1995) and Rad52p (Kagawa *et al.*, 2001), Mhr1p neither requires nor is stimulated by ATP or any other high energy co-factor to promote homologous pairing (Figure 3B).

The *mhr1-1* mutant protein (*mhr1-1p*) is defective in homologous pairing (Figure 3B and C), i.e. at any concentration tested, or even when 16-fold more *mhr1-1p* protein was added, no signal for the pairing reaction was detected (Figure 3C). Since homologous pairing is an important step for homologous recombination, and since the single substitution of Asp for Gly172 results in the simultaneous loss of homologous pairing activity *in vitro* and Mhr1p functions *in vivo*, including mtDNA recombination, repair, partitioning and maintenance (this study; Ling *et al.*, 1995, 2000), it is very likely that Mhr1p plays a role in the initiation of mtDNA homologous recombination, and that the homologous pairing activity of Mhr1p is also important for its other *in vivo* functions.

Agarose gel electrophoresis of DNA–protein complexes revealed that Mhr1p was able to bind to single-stranded DNA, as well as linear or closed circular double-stranded

Table I. Yeast strains

Strain	Nuclear genotype	Mitochondrial genotype	Source or reference
IL166-187	α <i>his1 trp1 can1 MHR1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	Stock culture of our laboratory
FL67	α <i>his1 trp1 can1 mhr1-1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	Ling <i>et al.</i> (1995)
FL67 ρ^0	α <i>his1 trp1 can1 mhr1-1</i>	ρ^0	Ling <i>et al.</i> (1995)
FL67-1423	α <i>his1 trp1 can1 mhr1-1</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	Ling <i>et al.</i> (1995)
FL67-1423 Δura3	α <i>his1 trp1 $\Delta \text{ura3 can1 mhr1-1}$</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	<i>ura3</i> derivative of FL67-1423
FL67-1423 Δura3 (pRS416CM)	α <i>his1 trp1 $\Delta \text{ura3 can1 mhr1-1}$ pRS416CM (MHR1, URA3)</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	Transformant of FL67-1423 Δura3
FL67-1423 Δura3 (pRS416)	α <i>his1 trp1 $\Delta \text{ura3 can1 mhr1-1}$ pRS416(URA3)</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	Transformant of FL67-1423 Δura3
FL67-2c	a <i>leu2 trp1 can1 mhr1-1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	Ling <i>et al.</i> (1995)
IL166-6b	a <i>leu2 ura3 trp1 can1 MHR1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	Ling <i>et al.</i> (1995)
IL166-6b-mhr1::LEU2	a <i>leu2 ura3 trp1 can1 mhr1::LEU2</i>	ρ^-	This study
YKN1423	a <i>leu2 ura3 met3 MHR1</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
FL67-2c-cce1::LEU2 ρ^0	a <i>leu2 trp1 can1 mhr1-cce1::LEU2 1</i>	ρ^0	This study
IL166-6b-cce1::LEU2	a <i>leu2 ura3 trp1 can1 cce1::LEU2MHR1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
YKN1423-cce1::LEU2	α <i>leu2 ura3 met3 cce1::LEU2 MHR1</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
YKN1423/YES2	α <i>leu2 ura3 met3 MHR1 YES2(URA3)</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
YKN1423/YESMHR1	α <i>leu2 ura3 met3 MHR1 YESMHR1(MHR1,URA3)</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
AFS98	a <i>ade2 leu2 his3 ura3 trp1 can1::GPD-HSVTK MHR1</i>	ρ^+	Nunnari <i>et al.</i> (1997)
AFS98 ρ^0	a <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	ρ^0	Derived from AFS98 by treatment with ethidium bromide
AFS98-4a	a <i>ade2 leu2 his3 ura3 trp1 can1::GPD-HSVTK mhr1-1</i>	ρ^+	Segregant from FL67 $\rho^0 \times$ AFS98
W303a	a <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	ρ^+	Nunnari <i>et al.</i> (1997)
W303a ρ^0	a <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	ρ^0	Derived from W303a by treatment with ethidium bromide
W303a-187	a <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303 α	α <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	ρ^+	Nunnari <i>et al.</i> (1997)
W303 $\alpha \rho^0$	α <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	ρ^0	Derived from W303 α by treatment with ethidium bromide
W303 α -1423	α <i>ade2 leu2 his3 ura3 trp1 can1 MHR1</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
W303 α /YESMHR1	α <i>ade2 leu2 ura3 his3 trp1 can1 YESMHR1(MHR1,URA3)</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
W303a/YES2	a <i>ade2 leu2 his3 ura3 trp1 can1 MHR1 YES2(URA3)</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303a/YESMHR1	a <i>ade2 leu2 his3 ura3 trp1 can1 YESMHR1(MHR1,URA3)</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303aGalMHR1	a <i>ade2 leu2 his3 ura3 can 1 GalMHR1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303aGalCCE1	a <i>ade2 leu2 his3 ura3 can1 MHR1 GalCCE1</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303aGalABF2	a <i>ade2 leu2 his3 ura3 can1 MHR1 GalABF2</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
W303 α GalCCE1	α <i>ade2 leu2 ura3 trp1 can1 MHR1GalCCE1</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
W303 α GalABF2	α <i>ade2 leu2 ura3 trp1 can1 MHR1GalABF2</i>	$\rho^+ \omega^+ \Delta \text{ens2 Oli}_2^R$	This study
W303a/YESMHR1-GFP	a <i>ade2 leu2 his3 ura3 trp1 can1 YESMHR1-GFP (MHR1-GFP,URA3)</i>	$\rho^+ \omega^+ \text{ens2 Chl}_{321}^R$	This study
pTY24	a <i>ade2 leu2 ura3 MHR1</i>	ρ^- pMK2 in mitochondria	Thorsness <i>et al.</i> (1990)

DNA (Supplementary figure 2), suggesting that Mhr1p has a structure-independent DNA-binding activity. The *mhr1-1* mutation causes an extensive loss of DNA-binding activity, even at 30°C, indicating that the cause of the deficiency in homologous pairing by *mhr1-1* is defective DNA binding (Supplementary figure 2).

A genome size monomer is a major species of mtDNA in buds

The majority of mtDNA in *S.cerevisiae* is found as concatemers with variable sizes consisting of multiple genome units (Maleszka *et al.*, 1991; Bendich, 1996). As expected from the previous studies, most of the mtDNA remained in the well of the gel after pulsed-field gel electrophoresis (PFGE) (Figure 4A; Bendich, 1996). It has been reported that the yeast mtDNA that remains in the well after PFGE is in linear forms larger than the genome size (concatemers) and contains a circular form as a minor group (Bendich, 1996). Compared with the *MHR1* cells, the *mhr1-1* cells contain a slightly larger fraction of the monomeric form of mtDNA (Supplementary figure 3).

A surprising result was obtained when we analyzed the forms of mtDNA in growing buds of *MHR1* cells using the same technique. Budding *MHR1* cells in culture were synchronized by an α -factor arrest-and-release protocol. The buds and mother cells were separated and purified (Ling *et al.*, 1995), and their DNA was prepared for PFGE. As in case of the whole-cell mtDNA, the majority of the mtDNA isolated from the *MHR1* mother cells remained in the well after PFGE (Figure 4A). However, about half of the mtDNA from the buds of *MHR1* cells behaves as a monomeric form with a genome size (~80 kbp). Thus, there is a difference in the size distribution of the mtDNA between mother cells and buds: while concatemers are the major species and monomers are the minor species in mother cells, the monomeric form is the major species in buds.

To confirm the above result with mitochondria in a very different physiological state and to obtain further information about the structures of mtDNA in buds and mother cells, we employed standard gel electrophoresis to avoid a limitation of the analysis by PFGE (circular DNA is trapped in the well; Bendich, 1996) and examined budding

cells that had artificial ρ^- mitochondrial DNA {pTY24: *MHR1* [ρ^- maintaining *coli* plasmid pMK2 DNA (unit size 9.3 kbp) in mitochondria]} (Thorsness and Fox, 1990). We purified buds and mother cells from dividing cells in a culture synchronized by an α -factor arrest-and-release procedure and their DNA was extracted, electrophoresed and analyzed by Southern hybridization using a probe containing the sequence of pMK2 (pUC119). The majority of the mitochondrial plasmid DNA from mother cells and whole cells was detected as a smeared band and species trapped in wells, and these preparations contained nicked circular monomers as a minor species (Figure 4B). In contrast, a nicked circular monomer is the major species of mitochondrial plasmid DNA from the buds, and its signal intensity is of a comparable level to that of concatemers (Figure 4B). Very little DNA from the buds was trapped in the well, whereas more from the mother cells was trapped (Figure 4B). After treatment of the DNA preparations from mother cells with the restriction endonuclease *SalI*, which cleaves pMK2 DNA once, the smeared bands disappeared, and a linear monomer and significant amounts of fragments with various sizes smaller than the linear monomer appeared (Figure 4B), suggesting that the smeared bands consist of concatemers. Thus, in the ρ^- budding cells, the fraction of monomer in the whole mtDNA is much larger in buds than the mother cells, as is the case for ρ cells.

The *SalI* treatment of DNA from mother cells eliminates the DNA species trapped in the wells and reveals the presence of a species that migrates more slowly than the nicked circular monomer (Figure 4B). This slower moving species was identified to include branched molecules such as X-shaped molecules (Holliday junction), Y-shaped molecules and double Y-shaped molecules, as judged by two-dimensional gel electrophoresis (data not shown). Thus, the mtDNA trapped in the well seems to be a DNA

network connected by such junctions. This slower migrating species was not detected in the mtDNA from buds (Figure 4B).

Fig. 2. Mitochondrial localization of Mhr1p. **(A)** Presence of Mhr1p in mitochondria. Denatured proteins (3 μ g) from the indicated fractions were separated by SDS-PAGE and immunoblotted with rabbit anti-Mhr1p serum as a probe. Lane 1, whole-cell extracts prepared from the *mhr1::LEU2* mutant (IL166-6b-*mhr1::LEU2*); lanes 2 and 4, whole-cell extracts prepared from *MHR1* cells (IL166-6b); lane 5, cytoplasmic fraction (without mitochondria) prepared from *MHR1* cells; lane 6, mitochondrial fractions prepared from *MHR1* cells. **(B)** Presence of Mhr1p in the mitochondrial matrix. Denatured proteins (4 μ g each) prepared from the mitochondrial matrix space (MatSp), inner membrane (IM), intermembrane space (ItMSp) and outer membrane (OM) were subjected to SDS-PAGE and probed with an anti-Mhr1p serum and antisera raised against mitochondrial marker proteins: porin (outer membrane), cytochrome *b*₂ (intermembrane space), the Tim23/Mas6 protein (inner membrane) and mtHSP70/Ens1p (mitochondrial matrix). Of the marker proteins, only HSP70 was detected in more than one fraction, the matrix and inner membrane fractions. **(C)** Comparison of sizes of Mhr1p expressed in *E. coli* and that obtained from mitochondria. Purified Mhr1p (without a His tag) expressed in *E. coli* (0.1 μ g in lane 1) and a protein fraction prepared from purified mitochondria (1 μ g in lane 2) were denatured and subjected to SDS-PAGE, and an immunoblotting analysis was carried out using the anti-Mhr1p serum. **(D)** Mitochondrial localization of the Mhr1-GFP fusion protein. Cells (W303a) containing the pYESMHR1GFP plasmid were grown in a raffinose medium to an early log phase. Living cells were observed by fluorescence microscopy after staining with DAPI and MitoTracker. Top left, Mhr1-GFP (green); top right, mitochondrial reticulum as indicated by MitoTracker; bottom left, cellular DNA after DAPI staining; bottom right, visible light.

Fig. 3. Pairing of single-stranded DNA with homologous double-stranded DNA promoted by purified Mhr1p, and the absence of this activity in mhr1-1p. (A) SDS-PAGE of purified proteins. Mhr1p (~5.1 μ g) or mhr1-1p (~5.8 μ g) was subjected to 15% SDS-PAGE after purification. Proteins were stained with Coomassie Blue. (B) Mhr1p promotes homologous pairing. The indicated 32 P-labeled single-stranded oligo-DNA (pG, SAT-1 containing a sequence from pGsat4; ϕ X, ϕ X-1 containing a sequence from ϕ X174) was incubated with Mhr1p or mhr1-1p for 5 min, and then closed circular double-stranded pGsat4 or ϕ X174 RF I DNA was added, followed by an incubation for 10 min in the presence of 2.0 μ M Mhr1p. After the removal of the proteins, the products were analyzed by agarose gel electrophoresis. Homo and hetero indicate homologous and heterologous combinations of double-stranded DNA and single-stranded DNA, respectively. Lanes 1 and 4–8, Mhr1p; lanes 2 and 3, without Mhr1p or RecAp; lanes 13 and 14, mhr1-1p. Lane 1 includes 1.3 mM ATP. Lanes 9–12, *E. coli* RecA (5 μ M) with 13 mM Mg^{2+} plus (lanes 9 and 10) or minus (lanes 11 and 12) ATP (incubation time, 5 min). Lane 8, after heteroduplex joints were formed from SAT-1 single-stranded oligo-DNA and pGsat4 double-stranded DNA, Mhr1p was inactivated at 75°C for 5 min, and the products were treated at 37°C for 30 min with the restriction endonuclease *NheI*, which cuts pGsat4 at a single site outside the sequence homologous to SAT-1. (C) Homologous pairing is mediated by Mhr1p, but not by mhr1-1p. The 32 P-labeled single-stranded oligo-DNA was SAT-1. After homologous (pGsat4, lanes 1–6 and 9–14) or heterologous (ϕ X174, lanes 7, 8, 15 and 16) double-stranded DNA was added, the reaction mixture was incubated for 10 min. Lanes 2–8, Mhr1p; lanes 10–16, mhr1-1p. Protein amounts: lanes 1 and 9, 0 μ M; lanes 2 and 10, 0.49 μ M; lanes 3, 7, 11 and 15, 0.98 μ M; lanes 4 and 12, 2.0 μ M; lanes 5 and 13, 3.9 μ M; lanes 6, 8, 14 and 16, 7.8 μ M.

***mhr1-1 cce1*-null double mutant cells do not maintain any mtDNA**

Concatemers are formed through recombination-dependent pathways as obligatory intermediates for the

duplication of various phages and plasmids. If concatemers are the obligatory intermediate of mtDNA partitioning into buds, then mtDNA maintenance could depend strictly on mtDNA recombination functions. Since some *mhr1-1*

Fig. 4. Genomic-size mtDNA is a major species in the buds of dividing *MHR1* cells. (A) PFGE profiles of mtDNA in the bud, the mother cell and the whole cell. *MHR1* cells were grown synchronously and were harvested under the conditions described in Figure 1A, except that BrdU was omitted. The buds were separated from the mother cells, and each part was purified, as shown in Figure 1B. Then, the total cellular DNA was prepared and subjected to PFGE. Right panel: the detection of signals from mtDNA, using ^{32}P -labeled mtDNA as a probe. Left panel: the detection of DNA under UV light after ethidium bromide staining. λ ladder DNA (concatemers) and λ DNA digested with *Hind*III were used as size markers. (B) Standard gel electrophoresis profiles of ρ^- mtDNA in the bud, the mother cell and the whole cell. The *MHR1* cells (PTY24) with pMK2 plasmid DNA in the mitochondria were grown in YPD medium at 30°C to 2×10^7 cells/ml. Cell growth was arrested at G₁ by α -factor for 2 h at 30°C, and the cells then allowed to grow synchronously in YPD medium for 1.5 h at 30°C by the removal of α -factor. The buds were separated from the mother cells, and each part was purified. Whole DNA was isolated and subjected to electrophoresis through a 0.5% agarose gel at 1.0 V/cm in TAE buffer (40 mM Tris-acetate, 10 mM EDTA pH 8.0) at room temperature. Right panel: the detection of signals from mitochondrial pMK2 DNA, using ^{32}P -labeled pUC119 DNA (which shares sequence with pMK2 DNA) as a probe. Left panel: the detection of DNA under UV light after ethidium bromide staining. The size marker was a 5 kb ladder obtained from Bio-Rad. The signals in the area indicated by an asterisk are those from Y-shaped molecules, double Y-shaped molecules and X-shaped molecules. Note that a precise determination of the amounts of mtDNA in the preparations is not possible, and the total amounts of DNA vary among the lanes. Thus, compare the relative amounts between the forms of DNA within each lane. The region indicated as C is concatemers.

cells grown at 37°C or cells with disrupted *mhr1* still maintain ρ^- mtDNA, and we observed residual partitioning of nascent mtDNA in the *mhr1-1* cells at 37°C (Figure 1; Ling *et al.*, 1995), we searched for the presence of another recombination-dependent system to maintain mtDNA. *CCE1/MGT1* encodes a recombination junction-resolving endonuclease (Cce1p). Although *cce1* mutants exhibit a normal level of mtDNA recombination, Cce1p is likely to function in a mtDNA recombination system (Kleff *et al.*, 1992; Ezekiel and Zassenhaus, 1993). In cells with functional ρ^+ mtDNA, *cce1*-null cells show only a mild phenotype for the maintenance of mtDNA (except for hypersuppressiveness, see below; Zweifel and Fangman, 1991; F.Ling, unpublished observation).

We constructed a *mhr1-1 cce1::LEU2* (*cce1*-null) double mutant by disrupting the *CCE1* gene within an *mhr1-1* mutant. All haploid *mhr1-1 cce1*-null double mutants were unable to grow in glycerol medium, suggesting that they were ρ^- or ρ^0 . Then, we crossed the *mhr1-1 cce1*-null double mutant haploids with *MHR1 CCE1* haploids to obtain ρ^+ diploid cells, which were

allowed to sporulate. All of the outgrowing cells on YPD medium at 30°C in the *mhr1-1 cce1*-null double mutant clones derived from each tetrad were devoid of mtDNA, as shown in Figure 5A. A quantitative Southern hybridization analysis indicated that the signals for mtDNA (normalized to that of the chromosomal gene, *URA3*) in the *mhr1-1 cce1*-null clones were much smaller than those in *MHR1* cells, *mhr1-1* cells or *cce1*-null cells, and were the background level exhibited by ρ^0 cells (Figure 5B). These results indicate that *mhr1 cce1*-null double mutants are unable to maintain mtDNA (i.e. ρ^0 phenotype) and, thus, that the maintenance of mtDNA strictly depends on either of the recombination-related genes, *MHR1* or *CCE1*.

Discussion

Molecular functions of Mhr1p

In this study, we found that Mhr1p has activity to pair linear single-stranded DNA with homologous closed circular double-stranded DNA, as shown in an assay of homologous pairing. This assay ('D-loop assay') was used

for the first demonstration of homologous pairing by RecA *in vitro* (Shibata *et al.*, 1979). The *in vivo* defects in mtDNA recombination and repair, and the *in vitro* defects in the homologous pairing caused by *mhr1-1*, suggest that Mhr1p-promoted homologous pairing is important for mtDNA recombination and repair *in vivo*, as is the case for RecAp.

Unlike with RecAp, the homologous pairing promoted by Mhr1p neither requires nor is stimulated by ATP, and in this sense it resembles the pairing of homologous DNA molecules promoted by RecTp (Noirot and Kolodner, 1998), RecOp (Luisdeluca, 1995) and Rad52p (Kagawa *et al.*, 2001). The homologous pairing by ATP-independent proteins may not be surprising, since ATP hydrolysis is not required for homologous pairing by RecAp. ATP-independent proteins might not promote branch migration efficiently by themselves, and instead the core heteroduplex joints formed by homologous pairing are stabilized by the topological stress in the superhelical double-stranded DNA substrates (Holloman *et al.*, 1975). *In vivo*, double-stranded DNA may experience topological stress, or other proteins, such as DNA helicase, may facilitate branch migration and stabilize heteroduplex joints.

The dependence of mtDNA partitioning on homologous recombination functions

The major species of mtDNA in mother cells are concatemers, which can be either intermediates of the replication/partitioning of mtDNA or byproducts. We found that the fraction of the monomeric form with the single genome size (probably nicked circular DNA) among the mtDNA in buds is much larger than that in either p^+ or p^- mother cells. In addition to this finding, the biochemical functions of Mhr1p (ATP-independent pairing of homologous DNA molecules) and Cce1p (resolution of recombination junctions), and the dependence of mtDNA maintenance and partitioning on recombination functions, reveal the resemblance between mtDNA replication and partitioning, and *E. coli* phage DNA replication and maturation. The packaging and maturation of the DNA of *E. coli* phages such as λ , T4 and T7 require a concatemer as an intermediate, and the concatemer is cleaved into monomers upon packaging into phage particles. The concatemers are formed by rolling circle replication in phage λ and through a crossing-over type of homologous DNA recombination in phage T4. The concatemer formation in phage T4 requires a recombination junction (Holliday junction)-resolving endonuclease as an essential component (Mosig, 1998). In phage λ infection or induction, the phage DNA replicates initially in a theta (θ) mode, and then the replication is switched to a rolling circle (σ) mode. The initiation of rolling circle replication depends on the β protein. The β protein also initiates rolling circle replication of a plasmid DNA that originally replicates in a theta mode (Silberstein *et al.*, 1990). Note that this pathway does not require a recombination junction-resolving enzyme. The β protein is a functional and structural homolog of RecTp and Rad52p (Passy *et al.*, 1999; Muirers *et al.*, 2000). Mhr1p could functionally be a member of this protein family, because of its ATP-independent homologous pairing activity (this study) and its ability to form a ring structure by itself, as observed by electron microscopy (S. Ikawa, personal communication).

Fig. 5. mtDNA is absent in cells carrying the *mhr1-1 cce1*-null double mutation. (A) DAPI staining of whole cellular DNA. Cells from a colony on a YPD plate were cultivated in YPD medium at 30°C, and the cells then subjected to DAPI staining. The large bright spot in each cell is DNA in the nucleus, and the small spots are mtDNA. Top left, *MHR1 CCE1*; top right, *mhr1-1 CCE1*; bottom left, *MHR1 cce1*-null; bottom right, *mhr1-1 cce1*-null double mutant. (B) Quantitative Southern hybridization analysis of the variation in mtDNA per unit number of cells. The amount of mtDNA relative to the nuclear *URA3* copy was calculated as described previously (Ling *et al.*, 1995), i.e. signals obtained with a mtDNA probe were normalized by signals obtained with a nuclear *URA3* gene probe in a Southern hybridization analysis.

Thus, if we assume that mtDNA is replicated and partitioned by a system analogous to the phage systems, then the phenotype of the *mhr1-1* and *mhr1-1 cce1*-null double mutants described here and previously (Ling *et al.*, 1995, 2000), and the difference in the mtDNA size distribution in mother cells and buds, can be explained by the molecular functions of Mhr1p found in this study. A model based on this assumption is illustrated in Figure 6. In this model, we assume that the concatemer is the essential intermediate for mtDNA partitioning into buds, and transmission occurs with the cleavage of the concatemer into monomers by a mechanism similar to

that operating in phage packaging (Figure 6). Like *E.coli* phages, we further assume that the concatemers are formed through either recombination-dependent rolling circle mtDNA replication or a crossing-over type of recombination (Figure 6). mtDNA may replicate by a θ mode at first, but soon, a recombination pathway is initiated by a double-stranded break (DNA is linearized) followed by processing into single-stranded tails. The Mhr1p-mediated interaction of either tail of the linearized DNA with a homologous circular double-stranded DNA molecule initiates rolling circle replication.

The interaction of both ends of the linearized DNA with a homologous circular double-stranded DNA would initiate a crossing-over type of homologous recombination to generate a dimer or multimer, through the function of Cce1p, as the double-stranded break repair process (Szostak *et al.*, 1983). The crossing over should be promoted by a different homologous pairing protein than Mhr1p. The presence of another mitochondrial homologous pairing protein is suggested by the phenotype of *mhr1-1* cells, i.e. *mhr1-1* causes a strong deficiency in the gene conversion type of mtDNA recombination, but this mutation exhibits some of the crossing-over type of mtDNA recombination (Ling *et al.*, 1995).

The recombination-dependent rolling circle DNA replication can be initiated by the synthesis of a leading strand from a 3'-OH terminus invading into a double-stranded template DNA and might not require RNA synthesis at the replication origin. On the other hand, *S.cerevisiae* mtDNA has a conserved sequence, called *replori*, which shares features with the replication origin of mammalian mtDNA (Van Dyck and Clayton, 1998), and evidence exists for RNA-primed DNA replication in yeast (Graves *et al.*, 1998; MacAlpine *et al.*, 2001), suggesting the contribution of θ mode replication to mtDNA maintenance. The fact that the ρ^- type mtDNA is maintained even in the absence of the mitochondrial RNA polymerase indicates that yeast mitochondria also have an RNA priming-independent mtDNA replication system (Van Dyck and Clayton, 1998).

Possible roles of homologous recombination in mammalian mtDNA inheritance

Since the majority of mtDNA exists as a monomeric circle in mammals and in a concatemeric linear form in yeasts and plants, it is possible that the basic mechanisms of mitochondrial genetic inheritance are different between mammals and yeasts or plants (e.g. Lecrenier and Foury, 2000). However, yeast mitochondria and mammalian mitochondria still share various common features, as discussed.

Recent observations suggest a possible mammalian machinery for mtDNA recombination (Introduction). Proteins with an ATP-independent pairing activity on homologous DNA molecules were found in a wide variety of organisms, from prokaryotes (e.g. RecTp, RecOp), yeast (Rad52) and its mitochondria (Mhr1p), to human (HsRad52). It is very likely that mammalian mitochondria have a functional homolog of Mhr1p. Thus, our findings may be applicable beyond yeast and provide insights into the phenomenon of mitochondrial inheritance in other organisms as well.

Fig. 6. Model of the functions of Mhr1p and its role in the transmission of nascent mtDNA. As is the case for phage DNA packaging, concatemers are essential intermediates for partitioning of mtDNA into buds, and are processed into genomic-size monomers (probably a circular form) during the partition. Concatemers are formed by either of two pathways: Mhr1p-initiating rolling circle replication, as in phage λ , or Cce1-dependent crossing over, as in phage T4.

Materials and methods

Yeast strains and media

Yeast strains are listed in Table I or as described previously (Ling *et al.*, 1995). Raffinose medium consists of 2% raffinose, 0.7% yeast nitrogen base and supplementary amino acids.

Cloning of MHR1 and mhr1-1

A yeast genomic library constructed in the vector YCp50 (Rose *et al.*, 1987) was used to transform FL67-1423 Δ ura3 (*mhr1-1*); Ura⁺ and temperature-resistant transformants were selected (Ito *et al.*, 1983). The 0.74 kbp *mhr1-1* allele was amplified by PCR using total cellular DNA from *mhr1-1* cells as the template, with primers containing the sequences from positions -29 to 0 (5'-upstream region) and +683 to +709 (3'-downstream region) of *MHR1*.

Disruption of MHR1 and CCE1

A 2.8 kbp *Bgl*II *LEU2* fragment from YEp13 was ligated into the unique *Nco*I site of the 0.94 kbp *MHR1* gene. IL166-6b cells were transformed by the linear DNA fragment containing the disrupted *mhr1* (*mhr1::LEU2*) and *LEU2* gene. The *Leu*⁺ transformants were selected as IL166-6b-*mhr1::LEU2* disruptants.

The DNA fragment starting from position 1 of the *CCE1* gene to position 1062 in the 3'-downstream region was amplified by PCR. A 2.2 kbp *Hpa*I *LEU2* fragment was ligated to the unique *Spe*I site of the 1.06 kbp *CCE1* gene. The linear DNA fragment containing the *cce1::LEU2* (*cce1*-null) gene was introduced into the *MHR1* cells (YKN1423) and the *mhr1-1* mutant (FL67-2c), to construct a *cce1*-null single mutant and a *mhr1-1 cce1*-null double mutant, respectively. The

presence of the disrupted gene in all disruptants was confirmed by a Southern hybridization analysis using an *MHR1* or *CCE1* probe.

To ensure that *mhr1-1 cce1*-null double mutant spores with mtDNA were obtained, the diploid formed by mating FL672c-*cce1::LEU2* (a *mhr1-1 cce1*-null *trp1 leu2* p^o) and YKN1423 [*α*.*MHR1 CCE1 leu2 met3 ura3* (p⁺)] was allowed to form asci. Each spore was germinated and allowed to form a colony on a YPD plate at 30°C, and then the colonies on the plate were replica plated onto two YPGly plates, with one incubated at 30°C and the other at 37°C. The colonies containing cells carrying *mhr1-1 CCE1* formed normal colonies on YPGly plates at 30°C, but not at 37°C. The colonies of cells carrying the *cce1*-null gene were selected based on the results of a Southern hybridization using a ³²P-labeled *CCE1* probe on DNA isolated from cells grown on the YPD plates.

Construction of the GFP-tagged *Mhr1p*

A DNA fragment containing the *MHR1* ORF (without the terminator) was introduced into pYES2 at the *KpnI*–*Bam*HI site. Then, a *Bam*HI–*Xba*I DNA fragment harboring the GFP gene was excised from the vector pVT100U–GFP2 (provided by J.Nunnari, University of California, Davis) and was introduced into the *Bam*HI–*Xba*I site (pYESMHR1GFP). Following incubation with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and 0.5 µg/ml MitoTracker, the unfixed cells with the plasmid were observed with an Olympus IX70 microscope.

Construction of *Mhr1p*- and *mhr1-1p*-overproducing plasmids

The *MHR1* and *mhr1-1* ORFs were ligated into the expression vector pET14b (Novagen) to produce a His₆-tagged fusion protein (pET14b-*MHR1* and pET14 *mhr1-1*, respectively) and were introduced into *E.coli* BL21 (DE3) pLyS host cells (Stratagene) by transformation. Proteins expressed in *E.coli* were detected by immunoblot analysis (Towbin *et al.*, 1979).

Labeling of nascent mtDNA and microscopic observation of the behavior of labeled and unlabeled mtDNA

AFS98 (*MHR1*) or AFS98-4a (*mhr1-1*) cells were grown in YPGly medium to log phase at 23°C from 1×10^5 to $\sim 1 \times 10^7$ cells/ml. The nascent mtDNA was labeled with BrdU under cell cycle arrest at G₁ by 15 nM α -factor, as described previously (Nunnari *et al.*, 1997), except for the period of incubation with BrdU (30 min). The arrest of cells (75% of the population) at G₁ was confirmed by light microscopy.

After the removal of BrdU and α -factor, the cells were incubated at 37°C for 1.5 h with shaking. The cells were collected, fixed with 3.7% formaldehyde and then treated as described previously (Nunnari *et al.*, 1997) for the localization of BrdU-labeled DNA within cells by using fluorescein-conjugated monoclonal anti-BrdU F(ab')₂ fragments (Boehringer Mannheim). Images were collected with an Olympus BX60 microscope equipped with a real-time, spinning-disk confocal scanning unit (Yokogawa CSU10) and were processed on a Macintosh computer using the IPLab Spectrum software (Scanalytics).

For the detection of whole cellular DNA, the fixed cells were stained with 1 µg/ml DAPI and observed with an Olympus IX70 microscope.

The separation of buds and mother cells, and the quantitation of nascent mtDNA

To compare the amount of BrdU-labeled DNA in *mhr1-1* and *MHR1* buds and mother cells, and to examine the mtDNA structures in buds and mother cells, the buds were separated from the mother cells using a previously published procedure (Ling *et al.*, 1995), except that the sonication of zymolyase-treated cells was omitted. Total DNA from *mhr1-1* and *MHR1* mother cells was prepared from the separated buds and mother cells, which had been adjusted previously to have a fixed amount of the mitochondrial protein porin.

For the quantitation, the same volume of DNA solution from each sample was applied to an N⁺ membrane (Amersham), and the DNA was fixed to the membrane by UV cross-linking. BrdU-labeled DNA was detected through immunoblotting using anti-BrdU serum (Sigma) as described previously (Towbin *et al.*, 1979).

Total bud DNA and total mother cell DNA for PFGE were prepared as described previously (Jacobs *et al.*, 1996).

Subfractionation of mitochondria

Mitochondria were prepared from IL166-6b cells by the fractionation of whole *MHR1* cell extracts, according to a previously published procedure (Daum *et al.*, 1982). Mitochondrial intermembrane space and matrix fractions were prepared (Daum *et al.*, 1982). Submitochondrial fractions, including outer and inner membranes, were further separated on linear

sucrose gradients (Pon *et al.*, 1989). Immunoblotting was carried out as described previously (Towbin *et al.*, 1979).

Purification of *Mhr1p* and *mhr1-1p*

Escherichia coli strains carrying pET14bMHR1 or pET14 *mhr1-1* were cultured in LB medium containing ampicillin at 37°C. An initial culture of 5×10^6 cells/ml was grown to 1.5×10^8 cells/ml and then cultured at 16°C for another 4 h in the presence of 1 mM isopropyl-β-D(-)-thiogalactopyranoside (IPTG). *Mhr1p* and *mhr1-1p* were purified using the HisTrap™ system (Pharmacia Biotech). The His-tag sequence was removed by the use of a Cleavage Capture kit (Novagen). According to the procedure described above, ~0.8–1.0 mg of each purified protein was obtained from a 300 ml culture.

Assay for homologous pairing ('D-loop' assay)

The standard reaction mixture (21 µl) for the assay consists of 15.6 mM Tris–HCl pH 7.5, 1.8 mM dithiothreitol, 1 mM MgCl₂ and 88 µg of bovine serum albumin per milliliter. After the ³²P-labeled single-stranded DNA (1.0 mM in nucleotides) was incubated with *Mhr1p* in the reaction mixture (20 µl) for 5 min at 37°C, 15 µM (in nucleotides) double-stranded DNA was added, and the incubation was continued at 37°C for the indicated time. For the reaction with RecAp, 1.3 mM ATP and MgCl₂ (final 13 mM) were added together with double-stranded DNA. After the proteins were removed, the products were examined by agarose gel electrophoresis. After electrophoresis, the gel was dried and exposed to an imaging plate, which was analyzed with a Fuji BAS2000 image analyzer.

The preparation of superhelical double-stranded pGsat4 and φX174 RF I (replicative form I) DNA, with care to avoid denaturation, the preparation of single-stranded DNA (50mer oligonucleotides; SAT1 and φX-1), the 5'-end ³²P-labeling of the single-stranded DNA and the details of the assay have been described previously (Kagawa *et al.*, 2001). SAT1 and φX-1 contain sequences from pGsat4 and φX174, respectively.

PFGE

PFGE was performed using the CHEF-DR III (Bio-Rad) apparatus in 0.5% low melting agarose (FMC) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8.0) at 4°C, with a pulse time of 50 s for 20 h and 100 s for 2 h. Total cellular DNA for PFGE was prepared according to a previously published procedure (Jacobs *et al.*, 1996).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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