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A nuclear mutation defective in mitochondrial recombination in yeast

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Homologous recombination (crossing over and gene conversion) is generally essential for heritage and DNA repair, and occasionally causes DNA aberrations, in nuclei of eukaryotes. However, little is known about the roles of homologous recombination in the inheritance and stability of mitochondrial DNA which is continuously damaged by reactive oxygen species, by-products of respiration. Here, we report the first example of a nuclear recessive mutation which suggests an essential role for homologous recombination in the stable inheritance of mitochondrial DNA. For the detection of this class of mutants, we devised a novel procedure, 'mitochondrial crossing in haploid', which has enabled us to examine many mutant clones. Using this procedure, we examined mutants of *Saccharomyces cerevisiae* that showed an elevated UV induction of respiration-deficient mutations. We obtained a mutant that was defective in both the ω-intron homing and Endo.*Sce*I-induced homologous gene conversion. We found that the mutant cells are temperature sensitive in the maintenance of mitochondrial DNA. A tetrad analysis indicated that elevated UV induction of respiration-deficient mutations, recombination deficiency and temperature sensitivity are all caused by a single nuclear mutation (*mhr1*) on chromosome XII. The pleiotropic characteristics of the mutant suggest an essential role for the *MHR1* gene in DNA repair, recombination and the maintenance of DNA in mitochondria.

Keywords: DNA maintenance/DNA repair/gene conversion/*mhr1*/*Saccharomyces cerevisiae*

Introduction

Mitochondria contain high levels of reactive oxygen species as by-products of oxidative respiration. Reactive oxygen species continuously damage mitochondrial DNA oxidatively, and cause mitochondrial DNA aberrations. Mitochondria should have systems to repair DNA damage and to suppress DNA aberrations for the maintenance of its DNA and organelle functions. Homologous recombination is essential for DNA repair in the nuclear DNA of eukaryotes, as well as in bacteria and viruses (for a review relating to yeast see Haynes and Kunz, 1981). Thus, it is very likely that homologous recombination could play an important and general role in mitochondrial DNA repair.

Homologous recombination, if it works irregularly, induces DNA aberrations at a pair of short homologous sequences, such as deletions caused by unequal crossing over between a pair of short direct repeats. Deletions formed between a pair of such repeats in mitochondrial DNA are often associated with the mitochondrial diseases (Holt *et al.*, 1988) and ageing (Cortopassi and Arnheim, 1990; Hayakawa *et al.*, 1991). A recessive nuclear allele, *nd* (natural death) of *Neurospora crassa*, causes higher levels of DNA rearrangement in mitochondria; this was proposed to occur through unequal crossing over mediated by hyperactive homologous recombination (Bertrand *et al.*, 1993).

Little is known about the role of homologous recombination in the inheritance and stability of mitochondrial DNA. After the mating of yeast haploid cells, a very high frequency of homologous recombination is detected in mitochondria, but the significance of this recombination is unclear. In mammals, mitochondrial genes exhibit maternal inheritance, and homologous recombination cannot be detected. Thus, considering the available genetic information (for a review see Dujon, 1981) and the conservation of genes relating to DNA repair and recombination between yeast and mammals, yeast is the best model system by which to study the roles of mitochondrial homologous recombination.

At present, there are few descriptions of mutations that cause a deficiency in homologous recombination in mitochondria from any organism. *pif1* of budding yeast *Saccharomyces cerevisiae* could be a sole mutation somehow relating to recombination in mitochondria (Foury and Kolodynski, 1983). However, *pif1* cells exhibit a recombination deficiency in the integration of a group of markers derived from ρ^- mitochondrial DNA into ρ^+ mitochondrial DNA, but they are proficient in mitochondrial homologous recombination (Foury and Kolodynski, 1983).

Genetic recombination is classified as either gene conversion or crossing over. In the nuclei of eukaryotes both classes of recombination closely relate to each other (Holliday, 1964). A multi-site-specific endonuclease, Endo.*Sce*I of *S.cerevisiae*, introduces double-stranded breaks at sites which are well defined but widely distributed in mitochondrial DNA. We have shown that Endo.*Sce*I is an initiator of mitochondrial homologous gene conversion in *S.cerevisiae in vivo* (Nakagawa *et al.*, 1992). Yeast meiotic homologous gene conversion in nuclei is also initiated by double-stranded breaks at many well-defined sites (Cao *et al.*, 1990; Sun *et al.*, 1991; Zenvirth *et al.*, 1992).

Endo.*Sce*I is a heterodimer encoded by a non-essential

Table I. Yeast strains

Strains	Nuclear genotype	[Mitochondrial genotype]	Source
IL166-187	α *his1 trp1 can1 MHR1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	stock culture from our laboratory
JC7-55R5	**a** *leu2 gal1 kar1-1 MHR1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	stock culture from our laboratory
OP11c-55R5	**a** *leu2 ura3 trp1 MHR1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	cytoductant from OP11Cρ^0×5045-55R5 crossing
CG378ρ^0	**a** *ade5 leu2 ura3 trp1 can1 MHR1*	[ρ^0]	stock culture from our laboratory
5045-55R5	α *his4-Δ15 kar1-1 MHR1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	stock culture from our laboratory
5010	α *his4-Δ15 kar1-1 MHR1*	[ρ^+ ω^+ *ENS2* Ery^R Ant^R]	stock culture from our laboratory
5011	α *his4-Δ15 kar1-1 MHR1*	[ρ^+ ω^+ *ens2* Ery^R Ant^R]	stock culture from our laboratory
JC7-1423	**a** *leu1 gal2 kar1-1 MHR1*	[ρ^+ ω^+ Δ*ens2* $Oli_2{}^R$]	stock culture from our laboratory
FL67	α *his1 trp1 can1 mhr1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	derived from IL166-187 by treatment with EMS
FL67ρ^0	α *his1 trp1 can1 mhr1*	[ρ^0]	derived from FL67 by treatment with ethidium bromide
FL67-55R5	α *his1 trp1 can1 mhr1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	cytoductant from FL67ρ^0×JC7-55R5 crossing
FL67-1423	α *his1 trp1 can1 mhr1*	[ρ^+ ω^+ Δ*ens2* $Oli_2{}^R$]	cytoductant from FL67ρ^0×JC7-1423 crossing
FL67-2c	**a** *leu2 trp1 can1 mhr1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	segregant from FL67×CG378ρ^0
FL67-2cρ^0	**a** *leu2 trp1 can1 mhr1*	[ρ^0]	derived from FL67-2c by treatment with ethidium bromide
FL672c-55R5	**a** *leu2 trp1 can1 mhr1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	cytoductant from FL67-2cρ^0×5045-55R5 crossing
FL67-ura3	α *his1 trp1 ura3 can1 mhr1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	*ura3* derivative of FL67
FL672c-5010	**a** *leu2 trp1 can1 mhr1*	[ρ^+ ω^+ *ENS2* Ery^R Ant^R]	cytoductant from FL67-2cρ^0×5010 crossing
FL672c-5011	**a** *leu2 trp1 can1 mhr1*	[ρ^+ ω^+ *ens2* Ery^R Ant^R]	cytoductant from FL67-2cρ^0×5011 crossing
FL67-1423-ura3	α *his1 trp1 ura3 can1 mhr1*	[ρ^+ ω^+ Δ*ens2* $Oli_2{}^R$]	*ura3* derivative of FL67-1423
IL166-6b	**a** *leu2 ura3 trp1 can1 MHR1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	segregant from FL67×CG378ρ^0
IL166-6bρ^0	**a** *leu2 ura3 trp1 can1 MHR1*	[ρ^0]	derived from IL166-6b by treatment with ethidium bromide
IL1666b-55R5	**a** *leu2 ura3 trp1 can1 MHR1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	cytoductant from IL166-6bρ^0×5045-55R5 crossing
IL1666b-5010	**a** *leu2 ura3 trp1 can1 MHR1*	[ρ^+ ω^+ *ENS2* Ery^R Ant^R]	cytoductant from IL166-6bρ^0×5010 crossing
IL1666b-5011	**a** *leu2 ura3 trp1 can1 MHR1*	[ρ^+ ω^+ *ens2* Ery^R Ant^R]	cytoductant from IL166-6bρ^0×5011 crossing
UV11	α *his1 trp1 can1 MHR1*	[ρ^+ ω^+ $Chl_{321}{}^R$]	derived from IL166-187 by treatment with EMS
UV11ρ^0	α *his1 trp1 can1 MHR1*	[ρ^0]	derived from UV11 by treatment with ethidium bromide
UV11-55R5	α *his1 trp1 can1 MHR1*	[ρ^+ ω^- *ens2* $Oli_1{}^R$]	cytoductant from UV11ρ^0×JC7-55R5 crossing
C82-1857	**a** *ilv5-1 asp5 ura4 met1 arg1 gal2*		Yeast Genetic Stock Center (University of California, Berkeley)
FL67-82	α *ilv5-1 ura4 asp5 arg1 met1 his1 trp1 mhr1*		segregant from FL67×C82-1857
A364A-321	**a** *cdc25-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*		Yeast Genetic Stock Center (University of California, Berkeley)
YMR420	**a** *cdc42-1 ura3 leu2 his3 trp1*		segregant from DJTD2-16A×YPH500 (Matsui and Tohe, 1992)

mitochondrial gene, *ENS2* (Nakagawa *et al.*, 1991), and an essential nuclear gene, *ENS1* (identical to *SSC1*; Morishima *et al.*, 1990). Yeast mitochondria appear to have more than one species of such endonucleases (for a review see Shibata *et al.*, 1995). In an attempt to understand the roles and mechanisms of mitochondrial homologous recombination, we started to isolate nuclear recessive mutations that caused a deficiency in Endo.*Sce*I-initiated mitochondrial homologous gene conversion because most of the mitochondrial proteins were encoded on nuclear chromosomes (for a review see Dujon, 1981).

Examining the many clones for nuclear recessive mutations on mitochondrial homologous recombination is extremely laborious. Previous procedures have required (i) the construction of both **a** and α derivatives that have the same nuclear genotype as each mutagenized haploid cell, (ii) the introduction of differently marked mitochondria into either the **a** or α derivative through the isolation of a ρ^0 derivative and cytoduction experiments, and (iii) mating experiments of the **a** and α derivatives to measure the recombination frequencies of the mitochondrial markers.

As a solution to this problem, we designed a new approach in which, without the construction of derivatives, mitochondrial DNAs with different genetic markers were allowed to recombine in cells with haploid nuclei derived solely from mutagenized clones. This procedure enabled us to identify *mhr1*, the first mutation in this class. The characterization of an *mhr1* mutant suggests a new role for recombination.

Results

Isolation of mutants for elevated UV induction of respiration-deficient mutations

Because recombination-deficient mutations are generally a class of DNA repair-deficient mutations (see Introduction), we searched for mitochondrial homologous recombination-deficient mutants among mitochondrial, UV-damaged, DNA repair-deficient mutants which would cause elevated levels of UV-induced DNA aberrations, including deletion formation. We first screened mutants showing an elevated UV induction of respiration-deficient mutations. A major class of respiration-deficient mutation

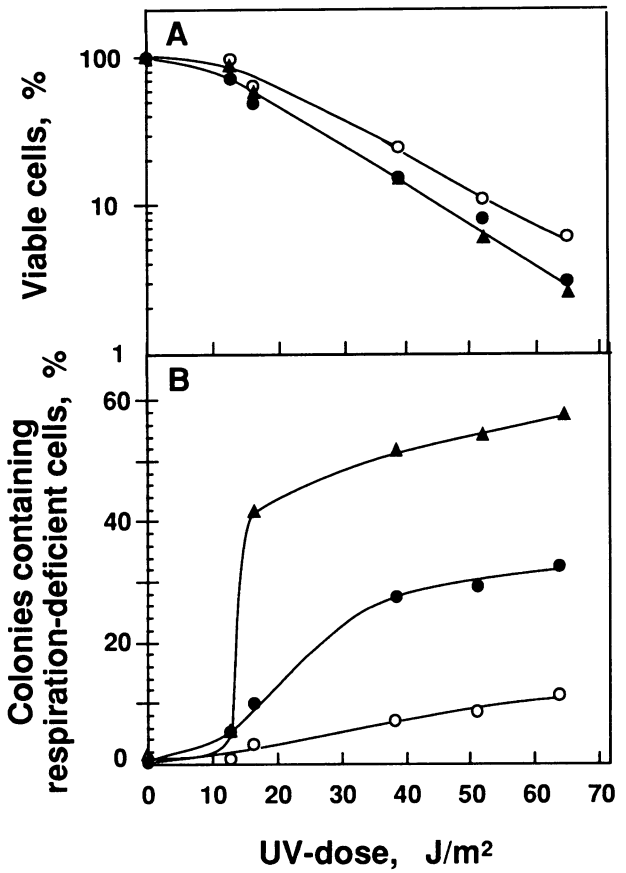


Fig. 1. Effects of UV irradiation on the production of respiration-deficient cells in newly isolated mutants of *S.cerevisiae*. (**A**) Cell viabilities after irradiation by various doses of UV light (254 nm). Cells grown for 48 h at 30°C in a glycerol medium (YPGly) were spread on YPD plates (~300 cells/plate) and irradiated under a UV lamp at room temperature. (**B**) Production of respiration-deficient cells after UV irradiation. Colonies containing respiration-deficient mutants were detected using the TTC overlay technique. The fractions of the colonies containing respiration-deficient mutants among the total colonies were plotted against UV dosage. Respiration-deficient colonies in the non-irradiated populations after growth on a YPD plate: 0.7%, the parental strain; 1.0%, FL67; 1.5%, UV11. (▲) FL67 (*mhr1*); (●) UV11; (○) the parental strain (IL166-187).

is ρ^-, which is a deletion in mitochondrial DNA (Dujon, 1981). We obtained two such mutants (FL67 and UV11) among 2×10^4 clones of ethylmethane sulfonate (EMS)-treated mitochondria-recipient cells (IL166-187; see Table I). Both mutants showed no difference from the wild-type cells in their sensitivity towards (i) cell killing by UV irradiation (Figure 1A) and (ii) induction of respiration-deficient mutation without irradiation (Figure 1B). After UV (254 nm) irradiation at 38 J/m^2, FL67 and UV11 produced colonies containing respiration-deficient mutants at a 7.6- and 4.0-fold higher frequency, respectively, than the parental strain (Figure 1B). We used these mutants in the tests for significance of our new procedure to detect nuclear recessive mutations deficient in mitochondrial homologous recombination.

'Mitochondrial crossing in haploid': a procedure to detect nuclear recessive mutations causing a deficiency in mitochondrial recombination

As outlined in Figure 2, mitochondria with a set of genetic markers of a mitochondria-recipient haploid cell and those

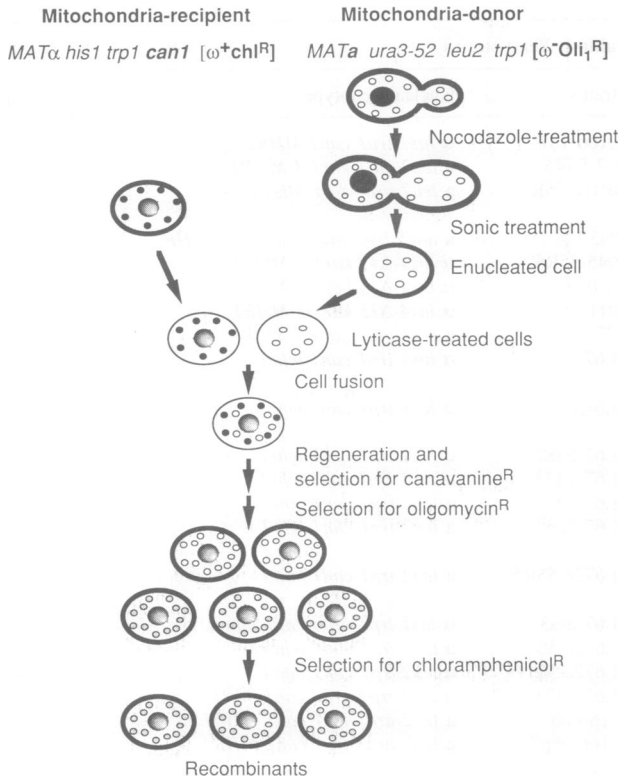


Fig. 2. Mitochondrial crossing in haploid. After cultivation of ω^- Oli_1^R (oligomycin-resistant mutation at the *Oli1* locus) mitochondria-donor (OP11c-55R5) cells in a medium containing nocodazole, enucleated cells derived from buds were separated from the mother cells. Mitochondria-recipient cells that contain ω^+ Chl^R (chloramphenicol resistance) mitochondria have a nuclear recessive mutation, *can1*, that confers canavanine resistance to the cells. Chl^R is tightly linked with the ω^+ allele and used for the detection of the ω^+ allele. The enucleated cells and the recipient cells were mixed, treated with lyticase to remove cell wall (to form spheroplasts), and then fused with each other. The treated cells were cultured in a medium containing canavanine to remove both the mother cells of the ω^- mitochondria donor and the diploid cells formed by the fusion of the donor (**a**) and recipient (α) spheroplasts. During this cultivation, mitochondria in each cell became homoplasmic. Then the cells were cultured in a medium containing oligomycin to select the fused cells that have haploid nuclei derived from the recipient cells and the Oli_1^R mitochondrial marker derived from the enucleated cells. Next, cells containing Chl^R Oli_1^R recombinant mitochondria, resulting from the polar gene conversion of ω^- to ω^+, were selected by cultivation on a solid medium containing both oligomycin and chloramphenicol.

with another set of markers derived from an enucleated mitochondria-donor cell were crossed by cell fusion and allowed to recombine in the presence of the single haploid nucleus derived solely from the recipient. Thus, nuclear recessive mutations in the recipient cells can be tested by the phenotype on mitochondrial recombination. We prepared enucleated mitochondria-donor cells by treatment with nocodazole, which inhibited the translocation of a nucleus into a newly formed bud from the mother cell (Jacobs *et al.*, 1988).

As a preliminary test for the deficiency of mitochondrial homologous recombination, we measured a polar gene conversion at the ω locus (called 'ω-intron homing') of mitochondrial DNA. Conversion at this locus shows an extreme polarity, i.e. almost 100% of the ω^- allele (21S ribosomal RNA gene without an intron) is replaced by the ω^+ allele (21S ribosomal RNA gene containing ω-intron; Bos *et al.*, 1978) in crossing involving both alleles

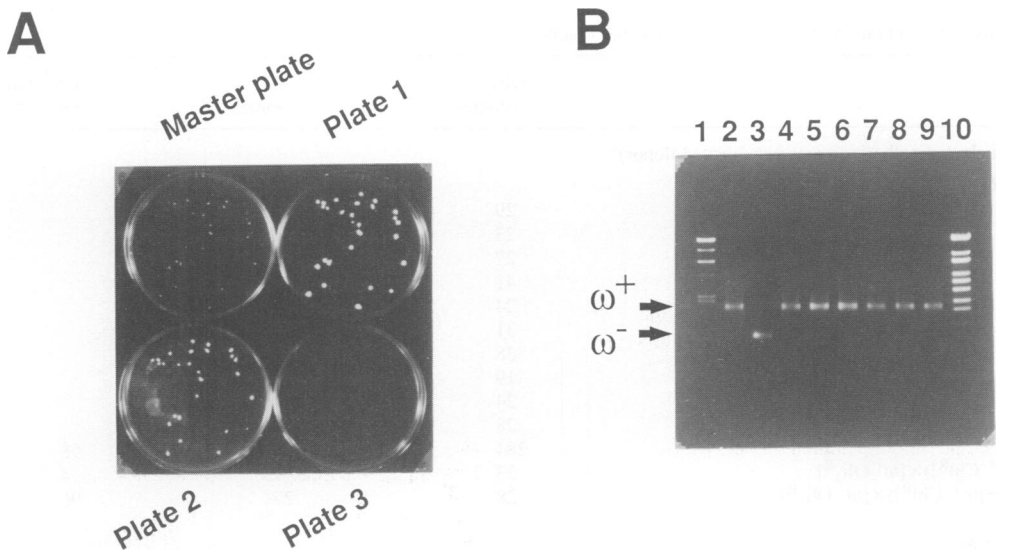


Fig. 3. Polar gene conversion at the ω locus (ω-intron homing) after the fusion of haploid cells with enucleated cells. (**A**) Mitochondria-recipient cells of IL166-187 (*can1* [ω^+ Chl^R]) were fused with enucleated cells containing ω^- $Oli_1{}^R$ mitochondria. Cells containing haploid nuclei derived from recipient cells were selected by growth in the presence of canavanine (crossing 1(1) in Table II). Then the cells that received the $Oli_1{}^R$ mitochondrial marker from the enucleated cells were selected on a YPGly plate containing oligomycin by incubation at 30°C for 4 days. The plate was used as a master plate. The colonies on the master plate were replicated onto (i) a YPGly plate containing chloramphenicol and oligomycin to select cells containing $Oli_1{}^R$ Chl^R (ω^+) recombinant mitochondria (plate 1), (ii) an SD plate supplemented with histidine, tryptophan and canavanine to select cells containing haploid nuclei derived from the recipient cells (plate 2), and (iii) an SD plate supplemented with tryptophan to select diploid cells formed by fusion of the recipient cells and the mitochondria-donor cells. Note that all colonies on the master plate contain the recombinant mitochondria and haploid nuclei derived from the recipient cells, and none are diploid. (**B**) Six colonies were randomly selected from plate 1, and whole-cell DNA was prepared from cells from each colony. Next, the DNA fragments containing the ω locus of each sample were amplified by PCR. The amplified DNA was electrophoresed (lanes 4–9) through a 0.7% agarose gel slab with a Tris–acetate buffer (Sharp *et al.*, 1973). Lane 2, DNA fragments amplified from DNA of the mitochondria-recipient ω^+ cells (IL166-187); lane 3, DNA fragments amplified from DNA of the mitochondria-donor ω^- cells (PO11c-55R5); lane 1, λ DNA digested by *Hin*dIII for size markers (23.0, 9.4, 6.6, 4.4, 2.3, 2.0, 0.564 and 0.125 kbp); lane 10, λ DNA digested by *Eco*T14R for size markers (19.0, 7.7, 6.2, 4.3, 3.5, 2.7, 1.9, 1.5, 0.925, 0.421 and 0.074 kbp). Arrowheads with ω^+ and ω^- indicate the positions of the ω^+ and ω^- DNA fragments, respectively. The gel was stained by ethidium bromide after electrophoresis, and DNA bands on the gel were photographed under UV irradiation at 254 nm.

(Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Zinn and Butow, 1985). The ω-intron homing is classified into a type of site-specific gene conversion, but we assumed that at least a part of those mechanisms which produced recombinants were common between homologous gene conversion and ω-intron homing (see Discussion about the validity of the assumption).

As shown by the procedure in Figure 2, we selected fused cells containing both a haploid nucleus derived from the recipient and mitochondria with the $Oli_1{}^R$ marker derived from the enucleated cells on plates containing oligomycin. We used the plates as master plates (Figure 3A). Colonies formed on the master plate were replicated onto a YPGly plate containing both oligomycin and chloramphenicol (plate 1) to select ω^+ $Oli_1{}^R$ recombinants which resulted from the polar gene conversion of ω^- to ω^+. Chl^R is linked tightly with the ω^+ allele and was used for the detection of the ω^+ allele. Almost all colonies on the master plate formed new colonies on plate 1, indicating an extreme polarity in the conversion at the ω locus (Figure 3A). We confirmed that the nuclei of the cells in the colonies were derived solely from the recipient cells, i.e. all colonies on the master plate grew on a plate containing canavanine (plate 2) but not on a plate that supported the growth of only diploid cells that would be formed by the fusion of the donor and recipient spheroplasts (plate 3). The yield of cells that received mitochondria with $Oli_1{}^R$ marker and the yield of cells having the recombinant mitochondria were not changed significantly by a 5-fold variation in the mixing ratio of the recipient and enucleated cells [Table II, crossings 1-(5–9)].

As shown in Figure 3B, we confirmed the presence of the ω-intron in all of the randomly selected Chl^R $Oli_1{}^R$ mitochondria by using the DNA polymerase chain reaction (PCR). Thus, this procedure reproduced the typical strong polar gene conversion at the ω locus by crossing the ω^+ and ω^- cells.

Effects of a mitochondrial recombination-deficient mutant (mhr1) on polar gene conversion at the ω locus (ω-intron homing)

Using the above newly developed procedure, we examined the effects of mutations in FL67 and UV11 cells on mitochondrial recombination. Mutant cells containing ω^+ Chl^R mitochondria were fused with enucleated cells containing ω^- $Oli_1{}^R$ mitochondria. A number of Chl^R (ω^+) $Oli_1{}^R$ recombinants were counted. As shown in Table II, while UV11 produced the wild-type level of the recombinants (crossing 3 versus crossing 1), FL67 exhibited a clear deficiency in the recombination (crossing 2). This apparent deficiency in recombination is not caused by the extensive decrease of mitochondrial DNA in the mutant cells (Figure 7H). As described below, FL67 is defective in homologous gene conversion. A tetrad analysis of FL67 mated with a wild-type haploid indicated that this deficiency in gene conversion was caused by a single mutation on a nuclear chromosome. We named

Table II. Effect of the *mhr1* mutation on gene conversion at the ω locus

Crossing	Oli_1^R colonies	Chl^R colonies	Chl^R/Oli_1^R recombinants (%)
Cell fusion with enucleated cells (recipient×enucleated donor)[a]			
1 wild type (*MHR1* [ω^+ Chl^R])×[ω^- Oli_1^R]			
(1)	29	29	
(2)	23	23	
(3)	27	27	
(4)	41	40	
(5)	24	24	
(6)	31	30	
(7)	38	37	
(8)	19	18	
(9)	24	23	
(10)	28	28	
Total	284	279	98
2 FL67 (*mhr1* [ω^+ Chl^R])×[ω^- Oli_1^R]	23	1	4
3 UV11 (*unknown* [ω^+ Chl^R])×[ω^- Oli_1^R]	28	27	96
Normal crossing (α×**a**)[b]			
4 *MHR1* [ω^+ Chl^R]×*MHR1* [ω^- Oli_1^R]	703	690	98.2
5 *mhr1* [ω^- Oli_1^R]×*mhr1* [ω^+ Chl^R]	825	312	37.8
6 *mhr1* [ω^- Oli_1^R] × *MHR1* [ω^+ Chl^R]	287	273	95.1
7 UV11 [ω^- Oli_1^R]×UV11 [ω^+ Chl^R]	423	418	98.8

Square brackets indicate mitochondrial genotype or marker

[a]After the fusion of mutant or wild-type cells with enucleated cells of OP11c-55R5, cells with a nucleus derived from the ω^+ cells were selected. Then the colonies grown on YPGly plates containing oligomycin were replicated onto YPGly plates containing chloramphenicol. In crossings 1(10), 2 and 3 we used the same preparation of enucleated cells. In crossings 1-(5–9) we used the same preparations but the ratios of mitochondria-recipient cells to enucleated cells were 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. In the rest of the crossings, the ratio was 1:3, as described in Materials and methods. Recipient strains: crossing 1, IL166-187; crossing 2, FL67; crossing 3, UV11.

[b]Normal crossing of **a** cells and α cells containing differently marked mitochondria. Strains used in each crossing (α×**a**) were: crossing 4, IL166-187×IL1666b-55R5; crossing 5, FL67-55R5×FL67-2c; crossing 6, FL67-55R5×IL166-6b; crossing 7, UV11-55R5×UV11-1a. UV11-1a was an **a** derivative from a tetrad analysis of UV11×CG378ρ^0.

Table III. Effects of Endo.*Sce*I and the *MHR1* gene on mitochondrial recombination

Crossing	**a** parent	α parent	Ery/Ant/Oli marker								
			RRS	RSR	SRR	RRR	SSS	RSS	SRS	SSR	Total
1	*MHR1* [Ery^R Ant^R *ENS2*]	*mhr1* [Oli^R Δ*ens2*]	27	12	8	4	166	22	29	132	400
2	*MHR1* [Ery^R Ant^R *ens2*]	*mhr1* [Oli^R Δ*ens2*]	17	20	9	8	11	6	12	217	300
3	*mhr1* [Ery^R Ant^R *ENS2*]	*mhr1* [Oli^R Δ*ens2*]	22	11	6	13	34	8	6	200	300
4	*mhr1* [Ery^R Ant^R *ens2*]	*mhr1* [Oli^R Δ*ens2*]	22	20	8	9	15	4	6	216	300

a parent: crossing 1, IL1666b-5010; crossing 2, IL1666b-5011; crossing 3, FL672c-5010; crossing 4, FL672c-5011. α parent, FL67-1423. *ENS2*, a mitochondrial gene that encodes a subunit of Endo.*Sce*I; *ens2*, inactive allele; Δ*ens2*, deletion of *ENS2* gene.

the mutation *mhr1*, for **m**itochondrial **h**omologous **r**ecombination.

To examine the effects of the *mhr1* mutation on ω-intron homing upon normal crossing, we constructed α-*mhr1* cells containing ω^- Oli_1^R mitochondria (FL67-55R5; Table I). We crossed FL67-55R5 with **a**-*mhr1* [ω^+ Chl^R] cells (alleles or markers indicated within '[ ]' indicate those of mitochondrial DNA), selected Oli_1^R diploid, and then counted Chl^R Oli_1^R recombinants among them. Whereas the crossing of *MHR1/MHR1* had a recombination frequency of 98% (Table II, crossings 4 and 7), the recombination frequency of the crossing of *mhr1/mhr1* was reduced to 38% (Table II, crossing 5). When an *mhr1* mutant was crossed with an *MHR1* strain, the recombination frequency was of the wild-type level (Table II, crossing 6 versus crossing 4). These results suggest that the mutation causes a loss of function required for the homing. The extent of deficiency in the polar gene conversion caused by the *mhr1* mutation was less in the normal crossing (Table II, crossing 5) than in the mitochondrial crossing by the fusion with enucleated cells (Table II, crossing 2). This will be discussed later (see Discussion).

mhr1 mutation causes a deficiency in Endo.*Sce*I-induced gene conversion

Next we examined the effects of the *mhr1* mutation on Endo.*Sce*I-induced mitochondrial homologous gene conversion. We crossed an *MHR1* or *mhr1* derivative of **a** cells containing Endo.*Sce*I-proficient (*ENS2*; Endo.*Sce*I^+) Ery^R (erythromycin resistance) Ant^R (antimycin resistance) mitochondria with α-*mhr1* cells containing Endo.*Sce*I-deficient (Δ*ens2*) Oli_2^R (oligomycin-resistant mutation at the *Oli2* locus) mitochondria that have a very sensitive Endo.*Sce*I site in the *Oli2* gene (*Oli2* is the same as *ATP6*, and is different from the gene for Oli_1^R). Based on the analysis of classes of recombinants (Table III), we calculated the transmission ratios of Oli_2^S from the

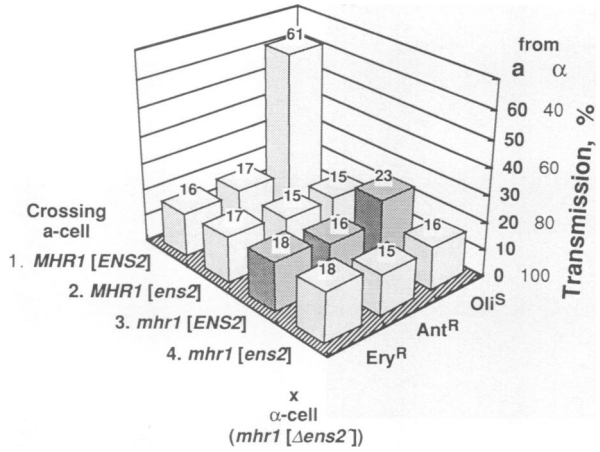


Fig. 4. Effects of the *mhr1* mutation on Endo.*Sce*I-induced homologous gene conversion. The ratios of the transmission from **a** cells to the progenies (indicated by bold letters) of Oli_2^S and those of the reference markers (Ery^R and Ant^R) were calculated from the results shown in Table III (Nakagawa *et al.*, 1992). The difference between the transmission ratio of Oli_2^S and that of Ery^R or Ant^R in crossing 1 indicates the extent of the polar gene conversion induced by Endo.*Sce*I (Nakagawa *et al.*, 1992).

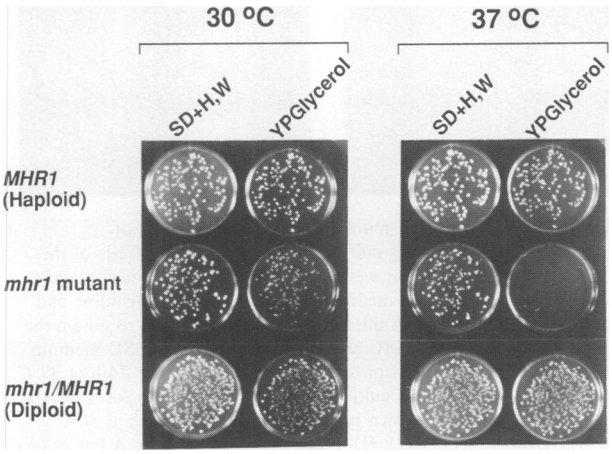


Fig. 5. Temperature-sensitive growth of the *mhr1* mutant under non-fermentable conditions. Cells from the indicated strain were grown in YPGly medium at 30°C for 3 days, diluted and spread onto a YPD plate. After incubation at 30°C for 2 days, the colonies on the plate (master plate) were replicated onto two SD plates supplemented with histidine and tryptophan, and onto two YPGly plates. A pair of the SD and YPGly plates was incubated at 37°C for 3 days and another pair was incubated at 30°C for 3 days. Strains used were as follows: wild type (haploid), IL166-187; *mhr1* mutant, FL67; *mhr1/MHR1* (diploid), diploid cells formed by the mating of FL67 and CG378.

Endo.*SceI*$^+$-**a** parents to the progenies of the crossing and those of the reference markers (Ery^R and Ant^R). The values are plotted in Figure 4.

As we reported previously, in the crossing of *MHR1*-[*ENS2*] cells and [*Δens2*] cells, polar gene conversion that caused the partial replacement of Oli_2^R from the α cells by the Oli_2^S allele from the **a** cells occurred in an Endo.*Sce*I-dependent fashion (Figure 4, crossing 1 versus crossing 2; Nakagawa *et al.*, 1992; Shibata *et al.*, 1995), i.e. the transmission of Oli_2^S from the **a** cell was 61 and 15% in the presence of *ENS2* and *Δens2*, respectively, while those of the reference markers Ery^R and Ant^R from the **a** cell were 16 and 17%, respectively. The level of transmission of Oli_2^S from the **a** cell observed here was

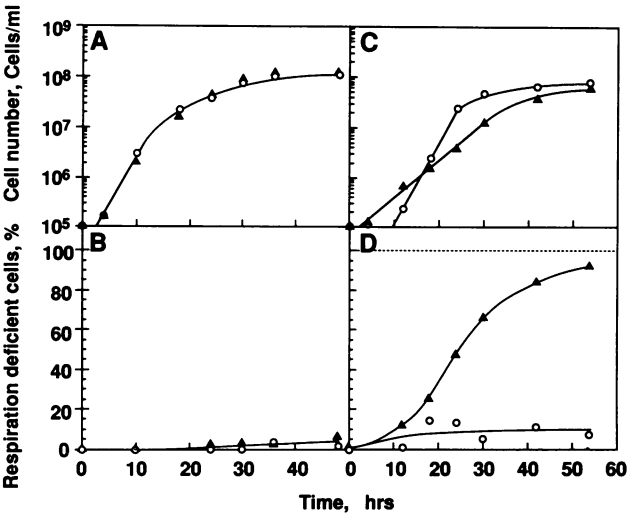


Fig. 6. Loss of respiration ability of the *mhr1* mutant during cultivation at 37°C in glucose medium. Cells of wild-type strain and *mhr1* mutant were grown in YPGly medium at 30°C for 3 days and then inoculated at a final concentration of 1.0×10^5 cells/ml into an SD medium supplemented with histidine and tryptophan. The cultures were incubated at 30 or 37°C. At the various time points, aliquots were withdrawn. The cell number in each sample was counted under a microscope and plotted (**A** and **C**). The cells from each sample were diluted and spread on YPD plates. To estimate the ratios of cells that irreversibly lost their respiration ability, sampled cells were incubated at 30°C for 4 days to form the colonies on YPD plates (master plates). The plates were replicated on YPGly plates and incubated at 30°C for 5 days. The fractions of respiration-deficient cells were calculated (**B** and **D**). (○) IL166-187 (*MHR1*); (▲) FL67 (*mhr1*).

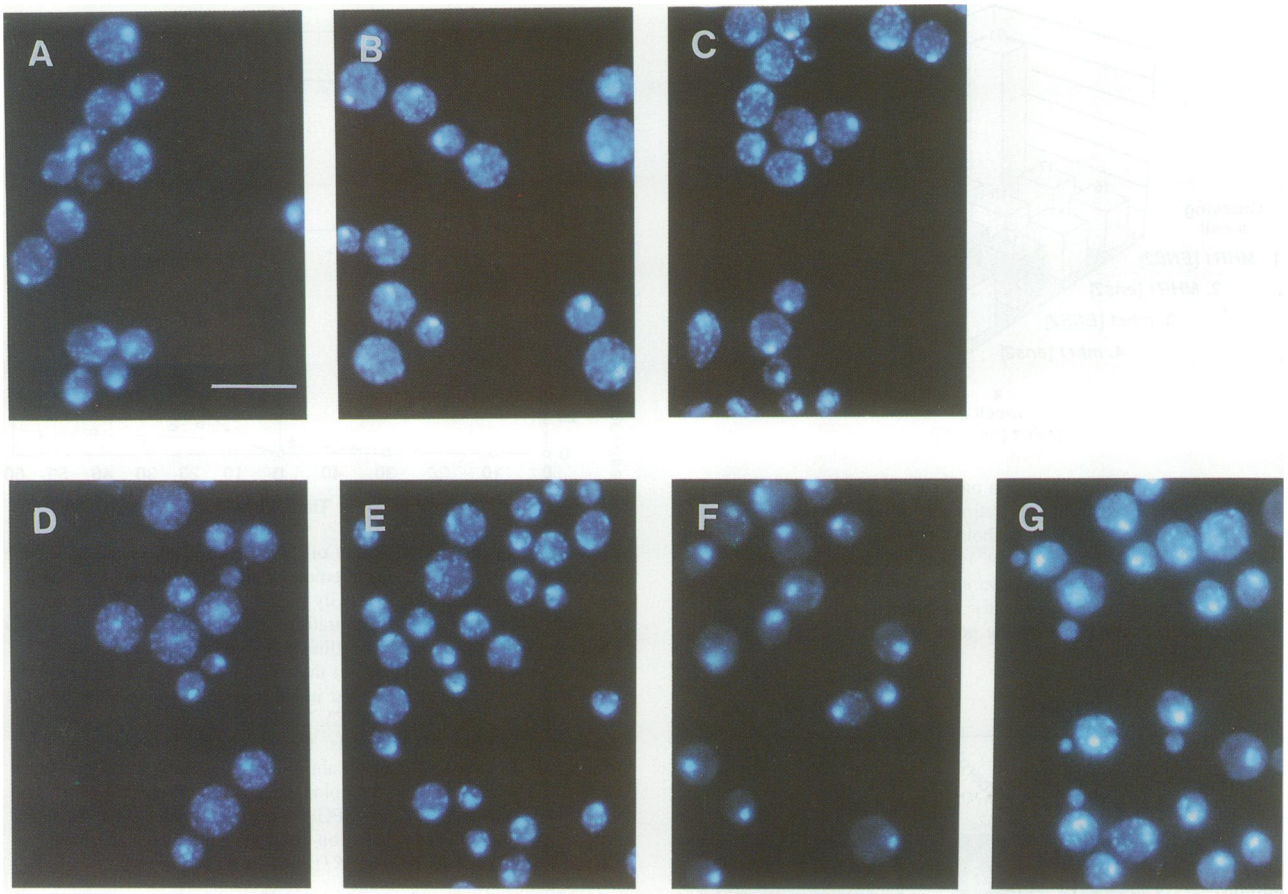
at a similar level to that observed in our previous experiment with the crossing of *MHR1* parents (Nakagawa *et al.*, 1992).

When both parents were *mhr1*, the transmission of the reference markers was the same as that in the crossing including an *MHR1* parent. However, the difference between the transmission ratio of Oli_2^S and that of either of the reference markers was much smaller than the difference in the presence of *MHR1*, indicating a clear deficiency in gene conversion (Figure 4, crossing 3 versus crossing 1). Thus, we conclude from these results that the *mhr1* mutation causes a deficiency in mitochondrial homologous gene conversion.

Unlike the case of gene conversion, we found that the *mhr1* mutation reduced only slightly intergenic recombination, i.e. the frequencies of intergenic recombination between the Ery and Ant markers in the crossing of *mhr1* × *mhr1* in the presence and absence of Endo.*Sce*I were 10 (31 recombinants in cross 3; Table III) and 13% (38 recombinants in cross 4; Table III), respectively. On the other hand, the frequencies of intergenic recombination in the crossing of *MHR1*×*mhr1* in the presence and absence of Endo.*Sce*I were 18 (71 recombinants in cross 1; Table III) and 16% (47 recombinants in cross 2; Table III), respectively.

mhr1 mutant is defective in the maintenance of mitochondrial DNA at a higher temperature

MHR1 haploid cells and *MHR1/mhr1* diploid cells were grown in a glycerol or glucose medium at 37°C (Figure 5). However, *mhr1* mutant cells (FL67) did not grow at 37°C in a non-fermentable medium (YPGly) that contained



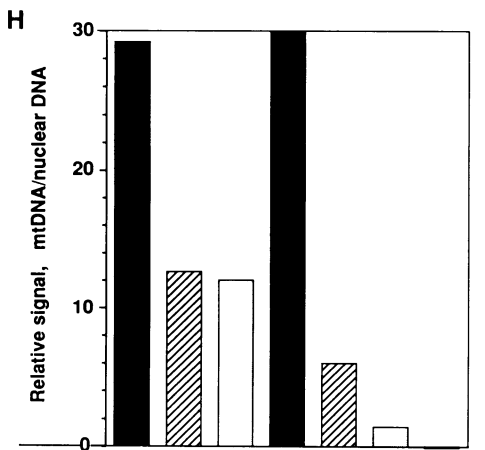


Fig. 7. Effect of the *mhr1* mutation on the maintenance of mitochondrial DNA during cultivation at 37°C. (**A–G**) Cells of the wild-type and *mhr1* mutant were stained with DAPI after cultivation in a glucose medium (SD medium supplemented with histidine and tryptophan). Cells were sampled from the cultures used to obtain the results shown in Figure 6. (C and F) Cells grown in an SD medium for 42 h at 37°C; (G) cells grown in an SD medium for 24 h at 37°C. Controls: (A and D), cells cultured in a YPGly medium for 3 days at 30°C; (B and E), cells grown in an SD medium for 36 h at 30°C. (A–C), IL166-187 (*MHR1*); (D–F and G), FL67 (*mhr1*). A bar in (A) indicates 10 μm. Note that DAPI-stained mitochondrial DNA was observed as small dots in the wild-type cells cultured at either 30 (A and B) or 37°C (C) and in *mhr1* cells grown at 30°C (D and E); such dots were not observed in most *mhr1* cells after the cultivation at 37°C (F). (**H**) Quantitative analysis of the variation of mitochondrial DNA per unit number of cells. Whole-cell DNA was extracted from cells in the same samples used in (A)–(F) and digested by restriction enzymes *Hin*dIII and *Cla*I. The fragmented DNA was separated by electrophoresis through a 0.7% agarose gel slab and transferred to a positively charged nylon membrane. To detect the mitochondrial DNA on the membrane, a ^{32}P-labeled 0.6 kbp fragment encoding part of the ω-intron was used as a probe. Radioactivities from a band of ω-intron were measured by a 2-D radioactivity counter. After the mitochondrial DNA probe was removed, the membrane was reprobed with a ^{32}P-labeled *Hin*dIII fragment containing a nuclear *URA3* gene, and radioactivities from a band of *URA3* were counted as described. As an indication of the average amount of mitochondrial DNA per unit number of cells, a signal of mitochondrial DNA was divided by a signal obtained by the *URA3* probe. Numbers in the table are net signals [arbitrary unit; a background value (0.002) was subtracted]. ρ^0, FL67-2Cρ^0.

glycerol as the sole carbon source. The cells of *mhr1* grew at 37°C when the carbon source was glucose (indicated in Figure 5 by 'SD+H, W'). Thus, the temperature sensitivity of *mhr1* cells is caused by a deficiency in mitochondrial function.

Next we cultured *mhr1* cells at 37 or 30°C in a glucose medium. Cells were sampled at various time points. After the sampled cells were allowed to form colonies on a glucose plate at 30°C, the respiration proficiency of the cells was tested by the ability of cells of the replicated

Table IV. Co-segregation of mutant phenotypes

Ascus	Ascospore phenotype			
	Spore A	Spore B	Spore C	Spore D
1	alpha ade + leu his trp ts pet rec	a + ura + + trp + + +	alpha ade ura + his trp + + +	a ade + leu his trp ts pet rec
2	alpha ade ura + + trp + + +	a ade ura leu his trp ts pet rec	a + + leu + trp ts pet rec	alpha + + + his trp + + +
3	alpha ade ura + + trp ts pet rec	a + + + + trp + + +	alpha ade ura leu his trp + + +	a + + leu his trp ts pet rec
4	alpha + ura + his trp + + +	a ade ura + + trp ts pet rec	a + + leu his trp + + +	alpha ade + leu + trp ts pet rec
5	alpha ade ura leu + trp + + +	alpha ade ura + + trp ts pet rec	a + + + his trp + + +	a + + leu his trp ts pet rec
6	alpha ade + + his trp + + +	a + ura leu + trp + + +	alpha + + + + trp ts pet rec	a ade ura leu his trp ts pet rec
7	alpha + + leu his trp ts pet rec	a + ura + + trp + + +	alpha ade + + + trp ts pet rec	a ade ura leu his trp + + +
8	a ade + leu + trp + + +	a + ura leu his trp ts pet rec	alpha ade + + his trp + + +	alpha + ura + + trp ts pet rec
9	alpha ade ura + his trp + + +	a + ura + + trp + + +	a + + leu + trp ts pet rec	alpha ade + leu his trp ts pet rec
10	a ade ura leu + trp ts pet rec	a + + + his trp + + +	alpha + + + his trp ts pet rec	alpha ade ura + + trp + + +
11	a + ura leu + trp + + +	a ade + + his trp ts pet rec	alpha + ura + his trp + + +	alpha ade + leu + trp ts pet rec
12	alpha ade + + his trp + + +	a + ura leu + trp + + +	a ade ura + his trp ts pet rec	alpha + + leu + trp ts pet rec
13	alpha + + leu + trp + + +	a ade + leu + trp ts pet rec	a ade ura + his trp + + +	alpha + ura + his trp ts pet rec
14	alpha + ura + his trp ts pet rec	a + + leu + trp + + +	alpha ade ura leu his trp + + +	a ade + + + trp ts pet rec
15	alpha ade ura leu + trp + + +	alpha ade ura + + trp ts pet rec	a + + + his trp + + +	a + + leu his trp ts pet rec
16	a + + leu + trp ts pet rec	a ade + + his trp + + +	alpha ade ura leu + trp + + +	alpha + ura + his trp ts pet rec
17	a + + leu + trp + + +	alpha ade + + his trp ts pet rec	a + ura + + trp ts pet rec	alpha ade ura leu his trp + + +
18	alpha + + leu + trp + + +	a ade + + his trp ts pet rec	a ade ura leu his trp ts pet rec	alpha + ura + + trp + + +
19	a ade ura + his trp + + +	a + + leu + trp ts pet rec	alpha + + + + trp ts pet rec	alpha ade ura leu his trp + + +
20	alpha + + + his trp + + +	a ade + leu + trp + + +	alpha + ura + his trp ts pet rec	a ade ura leu + trp ts pet rec
21	a + + leu + trp + + +	a ade ura + + trp + + +	alpha ade + leu his trp ts pet rec	alpha + ura + + trp ts pet rec
22	a ade + leu + trp + + +	a ade ura leu + trp ts pet rec	alpha + ura + his trp ts pet rec	alpha + + + his trp + + +
23	a ade + leu + trp + + +	alpha + + + + trp + + +	alpha ade ura + his trp ts pet rec	a + ura leu his trp ts pet rec
24	alpha ade ura + his trp ts pet rec	a ade + leu + trp + + +	alpha + ura + his trp + + +	a + + leu + trp ts pet rec
25	alpha ade ura + + trp + + +	a + + leu + trp ts pet rec	a + ura + his trp + + +	alpha ade + leu his trp ts pet rec
26	a + ura leu + trp ts pet rec	a ade ura + his trp ts pet rec	alpha + + leu + trp + + +	alpha + + + his trp + + +
27	alpha + ura + + trp ts pet rec	alpha + + + his trp + + +	a ade ura leu + trp + + +	a ade + leu his trp + + +
28	a + + leu + trp + + +	a ade + leu his trp ts pet rec	alpha + ura + his trp + + +	alpha ade ura + + trp ts pet rec
29	alpha + ura + + trp + + +	a + + leu + trp ts pet rec	alpha ade ura leu his trp + + +	a ade + + + trp ts pet rec
30	a + ura leu + trp + + +	a ade + + his trp + + +	alpha ade ura leu + trp ts pet rec	alpha + + + his trp ts pet rec
31	alpha ade ura + his trp ts pet rec	alpha + ura + + trp + + +	a ade + leu his trp ts pet rec	a + + leu + trp + + +

FL67 and CG378ρ^0 were mated to form diploid cells. The diploid cells were sporulated and subjected to a tetrad analysis. All spores analyzed so far are shown in this table. ade, ura, leu, his and trp indicate the requirements for adenine, uracil, leucine, histidine and tryptophan, respectively, for growth. ts, the inability of growth on a YPGly medium at 37°C but not at 30°C; pet, respiration deficiency; rec, mitochondrial gene conversion deficiency, as shown by ω-homing after normal crossing on plates.

colonies to grow on a glycerol medium at 30°C. This test showed that the number of respiration-deficient *mhr1* cells gradually increased during growth at 37°C in the glucose medium (not at 30°C), while *MHR1* cells did not at either 37 or 30°C (Figure 6). After the cultivation of *mhr1* cells at 37°C in the glucose medium for 42 h, 83% of cells showed respiration deficiency (Figure 6D). Most cells in this *mhr1* culture appeared to have lost mitochondrial DNA, as revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining (Figure 7F compared with A–E). At 24 h of incubation at 37°C in the same medium, ~45% of *mhr1* cells were respiration deficient (Figure 6D); ~50% of cells in the culture appeared to lose mitochondrial DNA, while the rest retained mitochondrial DNA (Figure 7G). These results indicate that during the cultivation of *mhr1* cells at 37°C, a loss of mitochondrial DNA heterogeneously occurred in a subpopulation of cells.

Then we quantitatively estimated the amounts of mitochondrial DNA using a Southern hybridization technique which included ^{32}P-labeled probes and a 2-D radioactivity counter. We used a ^{32}P-labeled fragment encoding part of the ω-intron as a probe to measure the amounts of mitochondrial DNA, and a ^{32}P-labeled fragment containing nuclear *URA3* gene as a probe to measure the amounts of nuclear chromosomal DNA. To obtain an indication of the average amount of mitochondrial DNA per unit number of cells, a signal of mitochondrial DNA was divided by a signal obtained by the *URA3* probe (Figure 7H). A signal derived from mitochondrial DNA was not detectable

Table V. Linkage analysis of *mhr1*

Marker tested	Chromosome[a]	PD	NPD	T	% Rec[b]	Linkage (cM)[c]
cdc25[d]	XII	37	4	24	25	38
ilv5	XII	20	7	26	38	81
asp5	XII	17	9	22	47	no
ura4	XII	15	12	24	42	no

FL67 (α *mhr1 his1 trp1*) and C82-1857 (**a** *ilv5 asp5 ura4 met1 arg1 gal2*) or A364A-321 (**a** *cdc25-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*) were crossed and then sporulated. We randomly picked up asci from the sporulation cultures. *mhr1* was detected by temperature-sensitive growth on a glycerol medium.

[a]Chromosome on which test marker was located.

[b]% Recombination = 100×(NPD + T/2)/(PD + NPD + T).

[c]Genetic distance was calculated as described by Sherman and Waken (1991). The absence of a linkage with *mhr1* was confirmed for *arg1* (chromosome XV), *met1* (chromosome XI), *his1* (chromosome V) and *trp1* (chromosome IV).

[d]The analysis was based on tests of the growth of cells derived from each spore on glucose (YPD plate) and glycerol media (YPGly plate) after incubation at 37°C for 24 h. On glucose medium, *CDC25* cells plus either *MHR1* or *mhr1* grew at 37°C, but those of *cdc25* did not. On glycerol medium, neither *CDC25 mhr1* nor *cdc25 mhr1* grew at 37°C. Conversion at the *MHR1* locus was not distinguished from Mendelian segregation in these tests, and would be included in PD or T. However, conversion at *mhr1* was five out of 90 tetrads (including those in Table IV) and would not provide a significant deviation on the calculated map distance. PD, parental ditype; NPD, non-parental ditype; T, tetratype.

in DNA from ρ^0 cells, indicating a very low level of noise in this measurement. Just after growth in a glycerol medium at 37°C, the amounts of mitochondrial DNA in cells were the same for *MHR1* and *mhr1* (Figure 7H). After 36 h of cultivation at 30°C in a glucose medium, the amount of mitochondrial DNA decreased significantly in both *MHR1* (to 43%) and *mhr1* cells (to 20%), i.e. at this time point the amount of mitochondrial DNA in *mhr1* cells was approximately half of that in *MHR1* cells. In the *MHR1* cells, the amount of mitochondrial DNA after cultivation in a glucose medium was not influenced by the temperature of cultivation between 30 and 37°C (Figure 7H). In contrast to these controls, after 42 h of cultivation in glucose medium at 37°C (respiration-proficient *mhr1* cells in the population = 17%; Figure 6D), the average amount of mitochondrial DNA in *mhr1* cells was reduced to (i) 4.7% of that before the incubation in glucose medium, (ii) 11% of the amount of DNA in *MHR1* cells after cultivation at 37°C, or (iii) 24% of the amount of DNA in *mhr1* cells after cultivation at 30°C (Figure 7H). The *mhr1* mutation is recessive with respect to the maintenance of the respiration function of mitochondria because *mhr1/MHR1* diploid formed colonies as normal on a glycerol plate at 37°C (Figure 5).

A mitochondrial gene conversion deficiency, a temperature sensitivity in the maintenance of respiration function and elevated UV induction of respiration-deficient mutations are due to a single nuclear mutation, mhr1, on chromosome XII

We carried out a tetrad analysis on 31 asci obtained from the diploid cells heterozygous at the *MHR1* locus (*MHR1/mhr1*). The results of the analysis are shown in Table IV and are discussed below. (i) ω-Intron homing tests indicated that recombination deficiency and proficiency segregated at 2:2 in most cases (30 out of 31 tetrads in this experiment). Another tetrad analysis showed a 2:2 segregation of mutant and wild-type phenotypes, as tested by the temperature sensitivity of the growth on glycerol plates (55 out of 59 tetrads). (ii) Elevated UV induction of respiration-deficient mutations (at 38 J/m^2) and the temperature sensitivity of the maintenance of respiration function were co-segregated with the recombination deficiency caused by the *mhr1* mutation in all cases (31 out of 31 tetrads). From these results, we conclude that all three mutation phenotypes are caused by a single mutation, *mhr1*, on a nuclear chromosome. The tetrad analysis also indicates that all phenotypes of the *mhr1* mutation are independent of the mating type of haploid cells (Table IV).

Another series of tetrad analyses showed that *mhr1* had a linkage with *cdc25* (38 cM) on chromosome XII and a very weak linkage with *ilv5* on the same chromosome (Table V). Since *ilv5* is located 32 cM distal to *cdc25* (Johnson *et al.*, 1987), the locus for *mhr1* is 38 cM towards the centromere from *cdc25* on chromosome XII. The nearest mutation to *mhr1* would be *cdc42*, but *cdc42* is located 54 cM distant from *cdc25* (Johnson *et al.*, 1987). In addition, both *cdc42* cells (YMR420: *MATa cdc42-1*) and *mhr1* cells (FL67-82: *MATα mhr1*) do not grow at 37°C on glycerol plates, but we observed that diploid cells formed by the mating of these mutants grow normally at 37°C on a glycerol plate. Thus, *mhr1* is different from *cdc42*, and could be a mutation in a previously unknown gene.

Discussion

In this study we have devised a new procedure that has enabled us to test many haploid clones for deficiencies in mitochondrial homologous recombination which have been caused by nuclear recessive mutations. Using this approach, we examined *S.cerevisiae* haploid cells that had mutations which caused the elevated UV induction of respiration-deficient mutations. We identified a nuclear mutation, *mhr1*, which was deficient in site-specific and homologous gene conversion in mitochondria. Finally, we found that the *mhr1* mutation exhibited a pleiotropic phenotype in the maintenance of respiration function, as well as in DNA repair and mitochondrial recombination.

A novel procedure to detect nuclear recessive mutations causing deficiencies in mitochondrial homologous recombination

In this study we assumed that at least part of the mechanism is common between ω-intron homing and homologous recombination. We monitored ω-intron homing which is a type of site-specific gene conversion. Endo.*Sce*I introduces double-stranded breaks into DNA, thus initiating mitochondrial homologous gene conversion (Nakagawa *et al.*, 1992). The initial event for ω-intron homing is also a double-stranded break induced by ω-endonuclease (called I-*Sce*I; Macreadie *et al.*, 1985; Colleaux *et al.*, 1986; Endo.*Sce*I and I-*Sce*I are different site-specific endonucleases encoded by unrelated genes). The numbers of cutting sites in mitochondrial DNA are different between these two types of recombination, i.e. >30 for Endo.*Sce*I versus only one in the ω^- allele of the 21S ribosomal RNA gene for ω-endonuclease. As for nuclear recombination in *S.cerevisiae*, both mating-type switching (a type of site-specific gene conversion; Kostriken *et al.*, 1983) and meiotic homologous gene conversion (Nicolas *et al.*, 1989; Sun *et al.*, 1989; Cao *et al.*, 1990) are initiated by a double-stranded break(s) at a defined site(s), and they require many genes in common, such as those belonging to the RAD52 family (Malone and Esposito, 1980; Ivanov *et al.*, 1994).

Factors that could result in a false recombination deficiency are an extensive decrease in the number of mitochondrial DNA in the mitochondria-recipient cells and deficiencies in the fusion of mitochondria in crossing. In this study, we confirmed by a quantitative Southern hybridization analysis (Figure 7H; 30°C, 0 h) that *mhr1* cells used in tests for recombination have the same amounts of mitochondrial DNA as the wild-type cells, while a 5-fold variation in the mixing ratio of the mitochondria-recipient cells and enucleated donor cells did not cause a significant change in the frequency of ω-intron homing (Table II). Since intergenic recombination was not changed significantly by *mhr1* mutation in the crossing between *mhr1* cells (Table III; see Results), the fusion of mitochondria seems not to be a major factor for the deficiency in mitochondrial gene conversion in *mhr1* cells.

mhr1 mutation causes a deficiency in homologous gene conversion and a site-specific polar gene conversion at the ω locus (ω-intron homing)

A deficiency caused by the *mhr1* mutation was displayed clearly in ω-intron homing in that mitochondrial crossing in haploid (Table II) and Endo.*Sce*I induced homologous gene conversion at the *Oli2* locus (Figure 4). In the normal crossing of *mhr1* and *MHR1* cells, mitochondrial gene conversion occurred at the wild-type level (Table II and Figure 4). This indicates that the *mhr1* mutation does not cause the production of an inhibitor (such as super-repressor), and that the *MHR1* gene product plays an active and essential role in double-stranded break-induced gene conversion.

mhr1 was indicated as a recessive mutation with respect to the maintenance of mitochondrial DNA (Figure 5). This and the above observations suggest that *mhr1* is also recessive in recombination.

In a normal crossing of *mhr1*×*mhr1*, the residual recombination between Oli_1^R and Chl^R (ω^+) was much higher than that in the fusion experiments (38 versus 4%; Table II, crossing 5 versus crossing 2). This could be the result of crossing over, because recombination between the Ery and Ant markers that is very likely to be crossing over was reduced only slightly by the *mhr1* mutation (Table III; see Results). A possible explanation for the residual recombination is that homologous crossing over occurs via a pathway which is independent of homologous gene conversion that was induced by a double-stranded break(s), and that *MHR1* only has a function in gene conversion. In nuclear chromosomes, double-stranded break-induced gene conversion leads to the crossing over of flanking regions (Szostak *et al.*, 1983). To confirm the above theory, we also need to examine the effects of the *mhr1* mutation on the crossing over between closely linked markers. The isolation of other mitochondrial recombination-deficient mutants will also provide us with a clearer insight into the mechanisms.

The sensitivity of cell viability towards UV irradiation is not affected by the *mhr1* mutation, indicating that *MHR1* seems not to be required for the DNA repair and recombination of nuclear chromosome. A tetrad analysis located *mhr1* at a new locus (38 cM proximal to the *cdc25* locus) on chromosome XII. On chromosome XII, *rad5* (*rev2*) (Lemontt, 1971; Johnson *et al.*, 1992) and *rec102* (Cool and Malone, 1992) were located as genes relating to DNA repair and recombination. The locus of *mhr1* is clearly different from these mutations.

mhr1 mutation exhibits a pleiotropic phenotype in DNA repair, recombination and the maintenance of DNA in mitochondria

As we had assumed, a deficiency in recombination function was shown to be a cause of elevated UV induction for respiration-deficient mutations (probably ρ^-; Figure 1 and Table IV). Mutants exhibiting an enhanced UV induction of respiration-deficient mutations are not necessarily recombination deficient. Examples of recombination-proficient and DNA repair-deficient mutations of *S.cerevisiae* are *uvsr5* (Moustacchi *et al.*, 1976), *pif1* (see Introduction; Foury and Kolodynski, 1983), some other reported mitochondrial DNA repair-deficient mutants (Foury, 1982; Backer and Foury, 1985) and UV11 (this study).

In systems with multiple genomes such as mitochondria, multiple rounds of homologous gene conversion will remove minor alleles even without selective pressure (Birky and Skavaril, 1976). Thus, is likely to act as a system to remove various kinds of both DNA lesions and mutations induced by the DNA lesions. In contrast to roles in the maintenance of DNA integrity, homologous recombination could cause gene aberrations, as described in Introduction. The *mhr1* mutation provides a first clue to allow us to study the roles of homologous gene conversion in the maintenance of mitochondrial genome integrity.

In this study we have shown that the cultivation of *mhr1* cells at 37°C results in an extensive decrease in the number of respiration-proficient cells (to 17% for 42 h) and in the average amount of mitochondrial DNA in each cell (to 11% of the wild-type level for 42 h; Figures 6D and 7F and H). The loss of mitochondrial DNA occurred heterogeneously in a subpopulation of cells (Figure 7F). Thus, the loss of mitochondrial DNA is likely to be much more extensive in respiration-deficient cells. These results suggest that the *MHR1* gene product by itself, or gene conversion depending on the *MHR1* gene, is required for the maintenance of mitochondrial DNA.

We are considering the following possibilities to explain the defective mitochondrial DNA maintenance in *mhr1* cells: (i) the accumulation of spontaneous oxidative DNA damage in the absence or a lowered level of DNA repair may cause the loss of mitochondrial DNA; (ii) the initiation and/or continuation of mitochondrial DNA replication may depend on the active *MHR1* gene; and (iii) the segregation of replicated DNA may require *MHR1* function. The cloning and characterization of the *MHR1* gene and its product will provide more information about the functions of the *MHR1* gene in mitochondrial DNA metabolism.

Materials and methods

Media and buffers

The YPD medium consisted of 1% yeast extract, 2% peptone and 2% glucose. The YPGly medium consisted of 1% yeast extract, 2% peptone, 3% glycerol and 50 mM KH_2PO_4 at pH 6.25. The SD medium consisted of 0.67% yeast nitrogen base without amino acids, 2% glucose and the required amino acids as necessary. Solid media were prepared by adding 2% agar (Difco) to the liquid media described above.

Sporulation cultures were set up at 30°C in a potassium acetate sporulation medium that consisted of 1% potassium acetate, 0.1% glucose, 1% yeast extract and 2% peptone.

KPS buffer was a 50 mM potassium phosphate buffer (pH 7.5) containing 1.2 M sorbitol.

PCR

The oligonucleotide primers used (Figure 3B) were 5′-GGCTCGAGTTT-TGATGGGGCGTCATTATC-3′ (from positions 3154 to 3182 in the 5′ exon region of the 21S ribosomal RNA gene) and 5′-GGTCTAGATT-CATTATGGTCCTTGCGTAC-3′ (from positions 4937 to 4965 in the 3′ exon region of the 21S ribosomal RNA gene). PCR was carried out using a GeneAmp™ PCR reagent kit with Ampli Taq™ DNA polymerase (Takara Shuzo Co. Ltd, Kyoto, Japan). Each set of 35 cycles of PCR consisted of denaturation at 94°C for 1.3 min, annealing at 61°C for 1 min and polymerization at 72°C for 2 min, followed by one cycle of 5 min of polymerization.

Strains

The yeast strains used in this study are listed in Table I.

Genetic crossing and sporulation

The crossing of yeast cells was achieved by (i) mixing an equal volume of a pair of suspensions of 2.0×10^7 cells/ml of haploid cells with the opposite mating types in YPD medium or (ii) cross-streaking of cells of the opposite mating types on an SD plate supplemented with the necessary amino acids. Diploid cells were isolated as colonies that grew on an appropriate selection medium and were able to sporulate on a sporulation medium. After 3 or 4 days of culture on the sporulation medium, asci were collected and analyzed by a micromanipulation method, as described by Sherman and Hicks (1991).

DAPI staining of DNA

Nuclear and mitochondrial DNAs in yeast cells were stained by DAPI, as described in Sherman *et al.* (1986). Spheroplasts were stained by the dye, as described by Miyakawa *et al.* (1984).

Isolation of the mitochondrial DNA repair-deficient mutants

The strain IL166-187 was mutagenized by treatment with 1.5% EMS. The following procedures, including the 2′,3′,5′-triphenyltetrazorium chloride (TTC) overlay technique, were carried out as described by Ogur *et al.* (1957) and Moustacchi *et al.* (1976).

A procedure for the detection of mutations deficient in mitochondrial recombination

The procedure is outlined in Figure 2. To prepare enucleated cells of an ω^- $Oli_1{}^R$ mitochondria donor, cells of OP11c-55R5 were suspended at 1.5×10^6 cells/ml in 10 ml YPGly medium supplemented with 1% dimethylsulfoxide and 15 μg/ml nocodazole (Sigma Co. Ltd) in four 50 ml Falcon tubes. After incubation at 18°C for 20 h, the cells (~2.2×10^6 cells/ml) in the four tubes were collected in one 50 ml Falcon tube by centrifugation at 1400 *g* for 5 min at 4°C. They were washed once with 20 ml KPS buffer. To separate buds without nuclei from the mother cells, the cells were resuspended in 10 ml KPS buffer and treated by sonication at 0°C for 3 min using an ultrasonic disrupter (Model UR-20GP, Tomy Seiko Co. Ltd, Tokyo, Japan); the intensity was set at 4/11. We confirmed the formation of intact enucleated cells in the suspension by fluorescent microscopy after DAPI staining and by phase-contrast microscopy. The treated suspension was centrifuged at 1400 *g* for 5 min at 4°C, and the precipitates were resuspended in 200 μl KPS buffer. The suspension was applied on a Ficoll gradient formed in a glass tube [12.5 (*D*) × 105.0 (*L*) mm] with 1 ml each of 10, 15, 20, 25 and 30% Ficoll 400 (Pharmacia Bioprocess Technology AB, Sweden). A microscopic examination after DAPI staining revealed that after centrifugation of the gradient at 150 *g* for 30 min at 30°C, enucleated cells were enriched in the 15% Ficoll fraction (to ~70% of the total cells). Therefore, the 15% Ficoll fraction was collected, diluted 2-fold with KPS buffer, and the cells were collected by centrifugation at 1400 *g* for 5 min at 30°C. After washing once with 3 ml KPS buffer, the cells were centrifuged at 1400 *g* for 5 min at 30°C and resuspended in 1 ml KPS buffer. The suspension of enucleated cells and the mother cells was mixed with mitochondria-recipient cells of IL166-187 (*can1* [ω^+ Chl^R]) or a mutant derived from this strain at a cell ratio of 3:1 (3×10^6:1 x 10^6 cells) in 100 μl KPS buffer. To the mixture, 50 U of lyticase (Boehringer-Mannheim Gmbh, Germany; 10 000 U/ml) were added and incubated at 30°C for 30 min to form spheroplasts. The spheroplasts were collected by centrifugation at 1000 *g* for 5 min at 30°C, washed twice with 1 ml KPS buffer, and collected by centrifugation at 1000 *g* for 5 min at 30°C. To fuse spheroplasts, the pellets were suspended in 250 μl 35% polyethylene glycol 4000 dissolved in KPS buffer, incubated at 30°C for 15 min, and precipitated by centrifugation at 1000 *g* for 15 min at 30°C. After washing the spheroplasts once with 1 ml KPS buffer, the treated spheroplasts were suspended in 2 ml of SD medium supplemented with the required amino acids and canavanine (1.5 μg/ml) in a glass tube [12.5 (*D*) × 105.0 (*L*) mm], and incubated at 30°C with agitation on a rotary shake at 93 r.p.m. for 3 days. Then, the culture was diluted to 100-fold and incubated for an additional 3 days under the same conditions. The regenerated cells with nuclei from the recipient *can1* cells were selected in this process. Next the cells were spread on a YPGly plate containing oligomycin (3 μg/ml) to select the cells that received the $Oli_1{}^R$ mitochondrial marker from the enucleated cells. The colonies exhibiting resistance to oligomycin ($Oli_1{}^R$) were replicated on a YPGly plate containing chloramphenicol (4 mg/ml) and oligomycin. The percentage of chloramphenicol-resistant (Chl^R) colonies among $Oli_1{}^R$ colonies was calculated as the frequency of gene conversion at the ω locus.

Tetrad analysis for temperature sensitivity of the maintenance of mitochondrial DNA, elevated UV induction of respiration-deficient mutations and mitochondrial recombination deficiency

Diploid cells formed by the mating of FL67 and CG378ρ^0 were grown on a sporulation plate to form asci. Four spores from each ascus were placed on a YPD plate. After incubation at 30°C for 4 days, mating types of colonies derived from the four spores were examined and placed on another YPD plate in the order α, α, **a**, **a**. The plate was incubated to form colonies and used as a master plate. The four colonies on the master plate were replicated on two YPGly plates. One of them was incubated at 30°C and the other at 37°C for tests for temperature sensitivity of the maintenance of mitochondrial DNA.

The four colonies on the master plate were also replicated on two YPD plates; one of them was irradiated by UV light at 38 J/m^2. The two plates were incubated at 30°C for 4 days and then overlaid with TTC to detect those containing respiration-deficient cells. Colonies derived from irradiated mutant cells showed a pink coloration, whereas irradiated wild-type cells were dark red.

Each of the α or **a** colonies was transferred to a YPGly plate and incubated to form colonies. The two α colonies were cross-replicated on a lawn of **a** cells of *mhr1* (FL672c-55R5), and the two **a** colonies were cross-replicated on a lawn of α cells of *mhr1* (FL67-55R5). After incubation for 8 h at 30°C (mating with **a** or α *mhr1* mutant), colonies of diploid cells were replicated onto a YPGly plate containing oligomycin and chloramphenicol to select cells containing $Oli_1{}^R$ Chl^R (ω^+) recombinant mitochondria (YPGly Oli and Chl) which were derived by ω-intron homing.

Estimation of the amounts of mitochondrial DNA (a quantitative Southern hybridization analysis)

The isolation of whole-cell DNA from yeast was carried out according to the method described by Philippsen *et al.* (1991). About 500 ng of the DNA sample were digested by restriction enzymes *Hin*dIII and *Cla*I, and then separated by electrophoresis through a 0.7% agarose gel slab. DNA in the gel was transferred to a positively charged nylon membrane (Amersham) by a procedure described by Southern (1975). To detect the mitochondrial DNA on the membrane, a 0.6 kbp fragment encoding a part of the ω-intron was labeled with ^{32}P using a random primer DNA labeling kit (Pharmacia Bioprocess Technology AB) and used as a probe. Then the membrane was hybridized with the mitochondrial DNA probe and radioactivities from a band of ω-intron were measured using a 2-D radioactivity counter (BAS-2000 Bio Imaging Analyzer, Fuji Film). Next the mitochondrial DNA probe was removed by boiling in 0.1% SDS solution. Then, the membrane was reprobed with a ^{32}P-labeled *Hin*dIII fragment containing nuclear *URA3* gene cloned on YEp24 vector. Radioactivities from a band corresponding to *URA3* were counted as described above. BAS-2000 exhibited a linear correlation between signals and the amounts of radioactivity in a range of four orders of magnitude. To obtain an indication of an average amount of mitochondrial DNA per unit number of cells, a signal of mitochondrial DNA was divided by a signal obtained by the *URA3* probe. This method does not detect mitochondrial DNA that has a deletion including ω-intron and thus the amount of mitochondrial DNA can be underestimated under certain conditions, such as those causing extensively large deletions.

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