An endonuclease with multiple cutting sites, Endo.SceI, initiates genetic recombination at its cutting site in yeast mitochondria

Kei-ichi Nakagawa, Nobuhiro Morishima¹ and Takehiko Shibata

Laboratory of Microbiology, RIKEN (Institute of Physical and Chemical Research), Wako-shi, Saitama 351-01, Japan

¹Corresponding author

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Endo.SceI is a mitochondrial sequence-specific endonuclease which has multiple cutting sites. In order to examine the possible role of Endo.SceI in homologous recombination, we analyzed the mode of recombination upon mating using antibiotic resistance markers on the mitochondrial genome. The segregation of a marker located very close to one of the Endo.SceI cutting sites showed a disparity (polarized segregation, i.e. gene conversion). This gene conversion depended on the presence of the functional Endo.SceI gene. *In vivo* cutting of mitochondrial DNA upon mating was detected at the cutting site in the antibiotic marker region, which also depended on the Endo.SceI activity. These results suggest that mitochondrial recombination is induced by cleavage of mitochondrial DNA by this sequence-specific endonuclease. This is the first demonstration that a sequence-specific endonuclease with multiple cutting sites induces genetic recombination.

Key words: gene conversion/homologous recombination/mitochondria/sequence-specific endonuclease/yeast

Introduction

Genetic recombination is observed in almost all kinds of organisms, including both prokaryotes and eukaryotes. This phenomenon is a consequence of the breaking and rejoining of DNA molecules (reviewed by Whitehouse, 1982). Recent advances in our understanding of the molecular genetics of the recombination process have revealed that breaks in DNA are made at well defined sites in the genome, where recombination initiation sites or recombination hot spots had been mapped genetically. In budding yeast (*Saccharomyces cerevisiae*), double-strand breaks have been detected at the initiation sites for both homologous recombination (Game *et al.*, 1989; Sun *et al.*, 1989) and site-specific gene conversion (Szostak *et al.*, 1983; Colleaux *et al.*, 1986; Xiong and Eickbush, 1988; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989).

For the site-specific gene conversion of yeast, several sequence-specific endonucleases have been identified as initiators of recombination; these cause specific double-strand scission at either the *MAT* locus in the nuclear genome or two loci in the mitochondrial genome (Kostriken *et al.*, 1983; Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989). It has been demonstrated that, at an early stage of meiosis, chromosomal

DNA is cleaved sequence-specifically at two hot spots or initiation sites of homologous recombination (Sun *et al.*, 1989; Alani *et al.*, 1990; Cao *et al.*, 1990). It has therefore been postulated that sequence-specific endonuclease(s) define the initiation sites for homologous recombination. Since it has been estimated that there are as many initiation sites for homologous recombination as there are genes (reviewed by Whitehouse, 1982), it is plausible that endonuclease(s) with broad recognition specificities attack many specified sites. However, only a few candidates for such endonucleases have been discovered in eukaryotic cells, and the involvement of endonuclease(s) with multiple cutting sites in homologous genetic recombination has not yet been proven.

We have purified a sequence-specific endonuclease, Endo.SceI, from *S.cerevisiae* (Watabe *et al.*, 1981, 1983). This endonuclease is expected to be an initiator of homologous recombination, mainly because the cutting sequence of Endo.SceI is multiple. We have deduced an asymmetrical consensus sequence within a region of 26 bp around the cleavage sites from the results of experiments involving several bacteriophage and plasmid DNAs (Shibata *et al.*, 1984; Kawasaki *et al.*, 1991). The frequency of scission is comparable to that of restriction enzymes with 6 base recognition sequences. In addition, Endo.SceI causes double-strand scission in DNA, producing cohesive ends with four bases protruding at the 3' termini (Watabe *et al.*, 1983; Shibata *et al.*, 1984), as in the case of the sequence-specific endonucleases of yeast which have been shown to be involved in site-specific gene conversion (Szostak *et al.*, 1983; Colleaux *et al.*, 1986; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989).

The active form of the endonuclease is a heterodimer with subunits of 75 and 50 kDa (Watabe *et al.*, 1983; Nakagawa *et al.*, 1988). The 75 kDa subunit, encoded by a nuclear gene (*ENS1*), is transported from the cytosol into the mitochondria (Morishima *et al.*, 1990), and the 50 kDa subunit, encoded by a mitochondrial gene (*ENS2*), is synthesized in mitochondria (Nakagawa *et al.*, 1991). Since the active endonuclease is localized in mitochondria, its most probable target is mitochondrial DNA (mtDNA). Through *in vitro* mapping of Endo.SceI cutting sites in mtDNA of yeast, we have found that Endo.SceI cleaves mtDNA every 2–3 kb (to be reported elsewhere). In addition, several fragments of the mtDNA amplified in *Escherichia coli* were also cleaved by Endo.SceI at identical sites to those observed in yeast mtDNA. There is no indication that there is any covalent modification in yeast mtDNA that would confer resistance to Endo.SceI digestion. Our results suggest that, upon mating, Endo.SceI could cleave mtDNAs from both parents in zygotes, where fusion of parental mitochondria and thus mitochondrial recombination take place, although the cutting efficiency would not be high enough to break mtDNA up into pieces. If double-strand scission caused by Endo.SceI does trigger the recombination event, one would observe reciprocal recombination between the mitochondrial

genomes of both parents because of the equivalence of the mtDNAs for the break. However, the detection of Endo.SceI-dependent recombination would be hampered because mitochondrial crossing over occurs at a significantly high frequency even in the absence of Endo.SceI (see Results). To examine the involvement of Endo.SceI in the homologous recombination of mtDNA, we constructed mating pairs so that recombination induced by Endo.SceI could be distinguished from background recombination, expecting that the asymmetry of parental mtDNAs might abolish the equivalence of the DNAs in recombination.

Some yeast strains lack the gene for the 50 kDa subunit of Endo.SceI within the mitochondrial genome (designated as Δ ens2), indicating that the 50 kDa subunit of Endo.SceI is not essential for the function of mitochondria. Due to the deletion of the *ENS2* sequence, the mtDNA of the Δ ens2 strain was quite different from that of the *ENS2* strain. By using a well designed set of mitochondrial markers in combination with either *ENS2* or Δ ens2 alleles, we detected the genetic recombination of mtDNA which is dependent on the Endo.SceI activity. In this paper, we discuss the probable function of endonucleases with multiple cutting sites as initiators of homologous genetic recombination.

Results

Experimental design for the detection of Endo.SceI-dependent recombination

Through the analysis of Endo.SceI cutting sites in mtDNA, one cutting site was detected around the 3' region of *oli2*, which encodes oligomycin sensitive ATPase subunit 6 (the mapping study of Endo.SceI cutting sites will be reported elsewhere). The *oli2* region is followed by the *ENS2* sequence (the gene for the 50 kDa subunit of Endo.SceI), which shows polymorphism as to its gene structure (Nakagawa *et al.*, 1991). We decided to use the oligomycin resistant mutation as a marker for the detection of Endo.SceI-dependent recombination.

For mapping the Endo.SceI sites *in vitro*, mtDNA samples isolated from haploid strains were cleaved with *HinfI* and Endo.SceI. The double digests of mtDNAs were hybridized with the 3' sequence of *oli2* (*HinfI*–*EcoRI* region of the Δ ens2 strain, Figure 1A) so that the Endo.SceI site could be mapped from the *HinfI* site. Figure 1B shows that cleavage by Endo.SceI generated a specific fragment of either 390 bp (OP19, *ens2*; YKN1423, Δ ens2) or 1.5 kb (OP20, *ENS2*), the size difference depending on the conservation of the *HinfI* site within the *oli2* sequence (Figure 1A). Fine mapping of the Endo.SceI site revealed that double-strand scission was caused within the sequence shown in Figure 1C (see Materials and methods). The Endo.SceI site within the *oli2*–*ENS2* locus was located at the 3'-terminal region of the *oli2* coding sequence. No additional Endo.SceI site could be detected within the *oli2*–*ENS2* locus.

Of the mtDNAs tested, the cleavage site of mtDNA of YKN1423 (Δ ens2) is several fold more sensitive to Endo.SceI (Figure 1B) than those of the others. The Endo.SceI site within the *oli2* locus roughly matches the consensus sequence for Endo.SceI recognition determined previously with phage and plasmid DNAs (Shibata *et al.*, 1984; Kawasaki *et al.*, 1991): the recognition sequence in Δ ens2 mtDNA deviates from the consensus sequence by two nucleotides, while those of the other strains exhibit three

mismatches. The difference in cutting efficiency seems to be due to this variation in the cutting sequences. Since the cutting efficiency could affect the mode of recombination, we will discuss this point later.

Sequence analysis of the oligomycin resistant mutant that we used revealed that the mutation is due to a single nucleotide change ~220 bp upstream of the Endo.SceI site within the *oli2* region (see Materials and methods). Assuming that the mode of mitochondrial recombination could be affected by a difference in the configuration around the cleavage site, we analyzed mitochondrial recombination between oligomycin resistant (*Oli*^r) and sensitive (*Oli*^s) strains, which had either the *ENS2* or Δ ens2 marker, as parental haploid strains (Figure 1A). Unlinked markers (erythromycin, Ery; antimycin, Ant) and the non-functional allele of *ENS2* (designated *ens2*, whose coding sequence is interrupted by the insertion of GC clusters; Nakagawa *et al.*, 1991) were used as controls. Erythromycin resistance (Ery^r) and antimycin resistance (Ant^r) are conferred by a mutation in the 21S rRNA locus (Dujon, 1980) and *cob-box* locus (Colson and Slonimski, 1979), respectively. We did not exactly locate the Ery^r and Ant^r mutations used in this study. Our preliminary data showed that Endo.SceI cleavage sites are far away (more than ~1 kb) from any Ery^r and Ant^r mutation site previously reported (Dujon, 1980; di Rago and Colson, 1988), and these cleavage sites were less sensitive to Endo.SceI than that in the *oli2* locus. We could not detect recombination at the Ery and Ant markers induced by Endo.SceI as described below.

Transmission and recombination patterns of mitochondrial markers upon mating

A single *Saccharomyces* cell contains 40–50 mtDNA molecules, which are homogeneous (homoplasmy). A typical mitochondrial cross involves the formation of zygotes through the mating of homoplasmic haploid cells with different mitochondrial markers. Although zygotes initially contain the parental mtDNAs and recombinant molecules, mtDNA molecules rapidly segregate after ~20 generations. In the mitochondrial crosses, the transmission frequencies of the alleles which are represented in the progeny exhibit similar values for all loci except the polar regions such as ω (reviewed by Dujon, 1981). The appearance of markers is usually unequal, however, between the parental mtDNAs, because the contribution of mtDNA molecules from each parent to the mating pool in the zygote is not necessarily equal (variation in the input ratio). The transmission frequency is a characteristic feature of a given cross. One can deduce the input ratio by measuring the transmission frequencies of nonpolar markers.

After synchronous mating between tester strains with different *ENS2* alleles, diploid progenies were analyzed in order to determine the transmission frequency and to assess recombination (Table I). In all the possible combinations of the crosses of the *ENS2* markers, the frequencies of the appearance of control markers (Ery and Ant) were identical (Figure 2). The frequency of either the Ery or Ant marker in these crosses is considered to be the input ratio, which reflects the contribution of each parental mtDNA to the mating pool.

Unlike the antibiotic resistant markers described above, the *Oli* marker exhibited significantly polarized transmission in a specific situation (Figure 2, cross 2). In the cross

Fig. 1. Structure of the *oli2-ENS2* locus and its *Endo.SceI* site. (A) Schematic representation of the *oli2-ENS2* locus in the mitochondrial genome carrying different *ENS2* alleles. The gene structures of the following strains are shown: YKN1424 (*ENS2*); YKN1423 (*Δens2*); YKN1425 (*ens2*). Boxes represent the coding regions, except for the black box in *Δens2* strains, which indicates a heterologous non-coding sequence (Sraphin *et al.*, 1985). The non-functional *ens2* coding region is a little larger than that of *ENS2* because of the insertion of GC clusters, which break the frame (Nakagawa *et al.*, 1991). The restriction sites for *HinfI* and *EcoRI*, and a cutting site for *Endo.SceI* are shown. Asterisks indicate the position of the base substitution in the *oli2* region which confers oligomycin resistance. A DNA probe is represented by a thick line. (B) Southern blot analysis of the cleavage by *Endo.SceI* at the *oli2* locus. MtDNAs were completely digested with *HinfI* and then treated with *Endo.SceI* (400 U/g) for 30 min (Watabe *et al.*, 1984). The digestion products were electrophoresed and then transferred onto a nylon membrane for Southern blot analysis. The specific signals of 1.5 kb (*ENS2*) and 390 bp (*Δens2*, *ens2*) are indicated by arrows. (C) The *Endo.SceI* cutting sequence of the *oli2* gene. The cutting site in the *oli2* region of the *Δens2* mtDNA was determined as described in Materials and methods. The bottom strand of the cutting sequence and the corresponding sequence of the *ENS2* strain are shown in reference with the consensus sequence previously determined for plasmid and phage DNAs (Shibata *et al.*, 1984; Kawasaki *et al.*, 1991). Bases not matching the consensus sequence are denoted by lower case letters. The arrow indicates the position of the *Endo.SceI* cleavage.

between the *ENS2* (a) and *Δens2* (α) strains, the *Oli*^s marker inherited from the a mating type parent appeared in over half the diploid progeny, while the other two markers were transmitted to ~20% of the progeny. This disparity suggests that the genetic information of the *Oli*^s marker of the *ENS2* strain was efficiently copied onto the corresponding locus of the *Δens2* strain (i.e. gene conversion). No polarized

transmission was observed, however, for crosses 1 and 3–6, where the transmission frequency of the *Oli*^s marker was almost equal to those of the *Ery* and *Ant* markers in each cross (i.e. input ratio).

Strong gene conversion of the *oli2* locus was observed under the conditions that the mating pool included both the *Endo.SceI* activity and the asymmetry in the regions around

Table I. Transmission and recombination of mitochondrial markers

Cross	Mating type a	mating type α	Ery ^r Ant ^r /Oli markers								Ery ^r Ant ^s + Ery ^s Ant ^r (%)
			<i>rrs</i>	<i>rsl</i>	<i>srl</i>	<i>rrt</i>	<i>sss</i>	<i>rss</i>	<i>srs</i>	<i>ssr</i>	
1	OP19 (Ery ^r Ant ^r <i>ens2</i>)	× YKN1423 (Oli ^r Δ <i>ens2</i>)	30	12	8	5	7	6	8	224	11.3
2	OP20 (Ery ^r Ant ^r <i>ENS2</i>)	× YKN1423 (Oli ^r Δ <i>ens2</i>)	35	5	7	0	84	23	14	132	16.3
3	OP19 (Ery ^r Ant ^r <i>ens2</i>)	× YKN1424 (Oli ^r <i>ENS2</i>)	26	21	3	7	7	4	8	224	12.0
4	OP20 (Ery ^r Ant ^r <i>ENS2</i>)	× YKN1424 (Oli ^r <i>ENS2</i>)	36	26	7	7	16	8	9	191	16.7
5	OP19 (Ery ^r Ant ^r <i>ens2</i>)	× YKN1425 (Oli ^r <i>ens2</i>)	19	15	10	10	5	5	10	226	13.3
6	OP20 (Ery ^r Ant ^r <i>ENS2</i>)	× YKN1425 (Oli ^r <i>ens2</i>)	19	13	8	6	12	2	12	228	11.3

For each cross, 300 diploid clones were randomly selected and their resistance to erythromycin, antimycin and oligomycin was determined. The phenotypes (r, resistance; s, sensitivity) are listed in the same order as the antibiotics. The markers inherited from the parent of mating type a (Ery^r, Ant^r, Oli^r) are italicized. The numbers represent clones appearing among the 300 random diploids in each case.

the markers of parental DNAs (*ENS2* × Δ *ens2*). The presence of the *ENS2* marker alone was not sufficient for the polarized transmission. On the mating of *ENS2* × *ENS2* strains (Figure 2, cross 4), the appearance of the Oli marker was almost equal to those of the Ery and Ant markers (input ratio). Similarly, the asymmetry alone did not generate such a gene conversion phenomenon. Although the *ens2* strain had a similar configuration around the *oli2* locus to that of the *ENS2* mtDNA (Figure 1A), it did not show polarized transmission of the Oli marker when crossed with the Δ *ens2* strain (Figure 2, cross 1).

Gene conversion at the *oli2* locus might occur without the synthesis of the 50 kDa protein (the gene product of *ENS2*), if the *ENS2* sequence of wild type is recombinogenic. To exclude the possibility that the *ENS2* sequence is a *cis*-acting factor, we examined the transmission frequency of the Oli marker from a respiration deficient strain (ρ^-), whose mitochondrial genome comprised the Oli^s and *ENS2* sequences as a repeating unit. After crossing the ρ^- strain with the respiratory competent strains with the Oli^r marker, the appearance of Oli^s marker in respiratory competent progeny was measured. In contrast to the case of crossing between ρ^+ haploid strains, no polarized transmission of the Oli^s marker was observed with either *ENS2* allele, whereas the Oli^s marker appeared in <1% of the respiratory competent progeny (the appearance of ρ^- progenies was ~20%, independent of the *ENS2* alleles). This suggests that the translation product of the *ENS2* gene, and therefore the Endo.SceI activity, is required for gene conversion.

The requirement of both the functional *ENS2* gene and the asymmetrical configuration for the observed gene conversion was not specific to the strains used in cross 2. Using another Δ *ens2* strain (crosses 7–9), we also observed that polarized transmission only occurred with the combination of the *ENS2* and Δ *ens2* strains (Figure 3, where the transmission frequencies of the Ery and Oli markers are shown), while neither the combination of Δ *ens2* × Δ *ens2* (cross 8) nor *ens2* × Δ *ens2* (cross 9) exhibited such polarized transmission. Similarly, after crossing another *ENS2* strain (mating type α) with strains carrying different *ENS2* alleles (crosses 10–12), the combination of *ENS2* × Δ *ens2* was the only case in which the Oli marker was transmitted to the progeny in a biased manner. These results also indicate that the gene conversion occurs irrespective of the allelic form of the *oli2* gene, because both Oli^s (cross 7) and Oli^r (cross

Fig. 2. Transmission of mitochondrial markers. The three-dimensional bars represent the percentages of progeny which inherited the antibiotic markers (either sensitive or resistant to erythromycin (Ery), antimycin (Ant) and oligomycin (Oli)) from the parent of mating type a. These values were calculated from the results in Table I. The numbers of crosses correspond to those shown in Table I: cross 1 (*ens2* × Δ *ens2*), cross 2 (*ENS2* × Δ *ens2*), cross 3 (*ens2* × *ENS2*), cross 4 (*ENS2* × *ENS2*), cross 5 (*ens2* × *ens2*), cross 6 (*ENS2* × *ens2*). The combinations of the *ENS2* alleles are indicated with the following symbols: +, *ENS2*; -, *ens2*; 0, Δ *ens2*. The black bars represent the results for cross 2, showing biased transmission of the Oli marker (see the text).

11) of the *ENS2* strain were efficiently transmitted to the progeny.

The observed gene conversion was independent of the mating type combinations. Crosses 7 and 11, both of which were between *ENS2* and Δ *ens2* strains, were reversed combinations with respect to the mating types. As shown in Figure 3, the Oli marker of YKN394 (a, *ENS2*) was preferentially transmitted to the progeny (cross 7). In the opposite mating type, YKN1424E (α , *ENS2*) exhibited similar biased transmission (cross 11). In this case, the appearance of the Oli marker inherited from the parental a haploid (Δ *ens2*) was significantly less than that of the Ery marker (cross 11). It should be noted that, in the cross between the *ENS2* and Δ *ens2* strains, the *oli2* locus of Δ *ens2* mtDNA behaved as a dominant recipient of the genetic information.

One of the indices of recombination is the percentage of the progeny having non-parental markers. Recombination between the Ery and Ant loci, for example, can be expressed as the ratio of non-parental types (Ery^rAnt^s and Ery^sAnt^r) in crosses 1–6) to the total progeny analyzed. As stated above, recombinants appeared in each cross (11.3–16.7%), being independent of the presence or absence of active Endo.SceI

Fig. 3. Biased transmission of the *oli2* marker depends on the combination of the *ENS2* alleles, not on the mating type. The results of crossing of YKN394 (*a*, *ENS2*), YKN395 (*a*, *Δens2*) and YKN398 (*a*, *ens2*) with an identical *α*, *Δens2* strain (YKN1423E, crosses 7–9) and an identical *α*, *ENS2* strain (YKN1424E, crosses 10–12) are shown as described in Figure 2. The appearance of the Ery marker is expressed as the input ratio (see the text). Note that in the combination of the *a*, *Δens2* and *α*, *ENS2* strains (cross 11), the level of the Oli marker from the *a* parent was significantly lower in progeny than the input ratio (transmission of the Ery marker), because the Oli marker of the *α* parent was efficiently transmitted to progeny.

(Table I). The frequencies of recombination between the Ery and Ant loci were not far from the theoretical values expected from random pairing and recombination of mtDNAs (Dujon *et al.*, 1974). This result suggests that another endonuclease(s) is involved in the *Endo.SceI*-independent recombination (discussed later).

In vivo cleavage of the *Endo.SceI* site within the *oli2* gene

To determine if the *Endo.SceI* site within the *oli2* gene is cleaved upon mating by this endonuclease, mtDNAs from mating mixtures were analyzed. Total DNA samples were digested with *HinfI* and then hybridized with a probe containing the 3' region of the *oli2* gene. For the detection of weak *Endo.SceI* activity, a large amount of DNA (~20-fold more than that used for *in vitro* cleavage analysis) was subjected to Southern blot analysis.

Because the restriction length polymorphism depends on the conservation of a *HinfI* site (Figure 1A), *in vivo* cleavage of the *oli2* region within *Δens2* mtDNA could be distinguished from that which had occurred in the *ENS2* mtDNA. As shown in Figure 4A, a specific fragment of 390 bp was detected in a *HinfI* digest of mtDNA prepared from the *ENS2* × *Δens2* mating mixture at 4 h and thereafter. Such a specific break could not be observed in the mtDNA at time 0, suggesting that cleavage of *Δens2* mtDNA by *Endo.SceI* did not occur during preparation of the DNA. The size of the hybridized band indicates that *Δens2* mtDNA was cleaved at the *Endo.SceI* site within the *oli2* region *in vivo* to generate the 390 bp fragment upon *HinfI* digestion. The time course of the specific cleavage is consistent with the fact that zygote formation between *a* and *α* cells is accomplished within 4–6 h after mixing of the mating haploids, at which time the first diploid bud emerges (reviewed by Thorner, 1981). The frequency of cleavage at the *Endo.SceI* site was roughly estimated to be <10% of the total mtDNAs through measurement of the band intensity. In the cross between *ens2* and *Δens2*, however, no *in vivo* cleavage at the corresponding site could be detected (Figure 4A), suggesting that the appearance of the specific signal was dependent on the functional *ENS2*. These results suggested that *Endo.SceI* caused a double-strand break

Fig. 4. *In vivo* cleavage of mtDNA by *Endo.SceI*. (A) Total DNA was prepared from the *ENS2* × *Δens2* mating mixture at appropriate time intervals. The DNA samples were digested with *HinfI* and then subjected to 6% PAGE for Southern blot analysis. A *HinfI*–*EcoRI* fragment of *Δens2* mtDNA (Figure 1A) was used as a probe. *ens2*, OP19; *Δens2*, 1423; *ENS2*, OP20. (B) *In vivo* cleavage of mtDNA during the vegetative phase. The *ENS2* strain (OP20) was grown in YPD medium (Sherman *et al.*, 1986) to the exponential growth phase. MtDNA was prepared from the cells and then examined by Southern blot analysis as in (A). As negative controls, mtDNAs of *ens2* (OP19) and *Δens2* (YKN1423) strains were also analyzed.

at the cleavable site within the *oli2* gene upon mating, when gene conversion events were proceeding. As to the scission which would have been made within *ENS2* mtDNA, a specific fragment of 1.5 kb could not be clearly demonstrated, probably due to the strong signal of the original *HinfI* fragment (1.7 kb).

The *in vivo* activity of *Endo.SceI* does not seem to be specific to the mating period, however, because the specific fragment of 390 bp was detected even after 20 h mating reaction (Figure 4A). The mating process of *Saccharomyces* is completed within about 4 h and the diploid progeny of an early generation contain either or both of the parental mtDNAs as well as recombinant molecules if recombination of the parental mtDNAs had taken place. The persistence of the specific signal of 390 bp up to 20 h after the initiation of mating suggests that *Δens2* mtDNA is still susceptible to cleavage by *Endo.SceI* in the diploid progeny of an early

generation. It is important, therefore, to examine whether the *Endo.SceI* cleavage could occur in cells during vegetative growth. As shown in Figure 4B, mtDNA of the *ENS2* strain was to some extent cleaved *in vivo* during vegetative growth. Therefore it is probable that a fraction of the mtDNA molecules, which coexist with *Endo.SceI* in mitochondria, are cleaved during the vegetative phase, while the cutting efficiency was estimated to be ~3% of the total number of cleavable sites in the *oli2* region.

Coconversion of the *ENS2* marker with biased transmission of the *Oli* marker

The results described above suggest that *Endo.SceI* induces gene conversion through scission at the cleavage site, and therefore that the *Endo.SceI* site within the *oli2* gene functions as an initiation site for the conversion of the *Oli* marker gene. Genetic studies have shown that gene conversion in *S.cerevisiae* is often associated with the coconversion of flanking markers on both sides of the conversion site (Fogel *et al.*, 1979). It is reasonable to suspect that a flanking marker downstream of the cleavage site is coconverted when the upstream *Oli* marker is converted. In the crosses involving the *ENS2* strain, the

Fig. 5. Coconversion of the *ENS2* marker with the *Oli* marker. The appearance of the *ENS2* marker in diploid progeny was analyzed by colony hybridization. The 1.7 kb *EcoRI* fragment containing the *ENS2* sequence was used as a probe; it hybridized to both *ENS2* and *ens2* mtDNAs, but not to *Δens2* mtDNA (Nakagawa *et al.*, 1991). Cross 1, *ens2* × *Δens2*; cross 2, *ENS2* × *Δens2*; cross 7, *ENS2* × *Δens2*; cross 9, *ens2* × *Δens2*; cross 11, *Δens2* × *ENS2*.

transmission of the *ENS2* allele was analyzed by colony hybridization. Since *Δens2* mtDNA completely lacks the *ENS2* sequence, the *Δens2* allele can be clearly distinguished from those containing the *ENS2* sequence. As shown in Figure 5, the *ENS2* marker exhibited the disparity (biased transmission) only when the *ENS2* strain was mated with the *Δens2* strain, as in the case of the *Oli* marker (crosses 2, 7 and 11). The transmission frequency of the *ENS2* sequence was almost equal to that of the *Oli* marker in all the crosses, including those showing the biased transmission of the *Oli* marker. In addition, all progeny that had inherited the *Oli* marker from the *ENS2* parent gave a positive colony hybridization signal with the *ENS2* probe. These results suggest that the *ENS2* allele was coconverted with the *Oli* marker.

The conversion of *Δens2* to *ENS2*, accompanied by that of the *Oli* marker, was confirmed by Southern blot analysis. Twenty recombinant progeny which inherited the *Oli* marker from the *ENS2* parent, and both the Ery and Ant markers from the *Δens2* strain in crosses 2, 7 and 11, were randomly selected and subjected to Southern blot analysis of the mtDNAs after *EcoRI* digestion. The *ENS2* allele can be clearly distinguished from the *Δens2* allele because the former exhibits a 1.7 kb *EcoRI* fragment which hybridizes to the cloned *ENS2* gene while the latter does not (Nakagawa *et al.*, 1991). Without exception, these progeny possessed the 1.7 kb *EcoRI* fragment, suggesting that they had inherited the intact *ENS2* gene (data not shown). It is concluded that the *ENS2* locus is coconverted whenever *oli2* is converted. This result is consistent with the idea that double-strand scission in the 3' region of *oli2* triggers gene conversion, resulting in the coconversion of the flanking regions on both sides.

Discussion

This study shows that *Endo.SceI* plays a role in the initiation of mitochondrial gene conversion, a type of genetic recombination. Our previous studies had suggested that *Endo.SceI* had a role in genetic recombination: they revealed that *Endo.SceI* has characteristics similar to those of the HO, ω and α 4 endonucleases (Shibata *et al.*, 1984; Nakagawa

Table II. Yeast strains

Strain	Nuclear genotype	Mitochondrial genotype
OP19	a <i>ura3 leu2-3,112 trp1</i> (OP11C-1)	ω^+ Ery ^r Ant ^r <i>ens2</i> (S30)
OP20	a <i>ura3 leu2-3,112 trp1</i> (OP11C-1)	ω^+ Ery ^r Ant ^r <i>ENS2</i> (S30)
YKN1423	α <i>ura3-52 leu2 met3</i> (CG378 × S30)	ω^+ Oli ^r <i>Δens2</i> (CG379)
YKN1424	α <i>ura3-52 leu2 met3</i> (CG378 × S30)	ω^+ Oli ^r <i>ENS2</i> (CG379)
YKN1425	α <i>ura3-52 leu2 met3</i> (CG378 × S30)	ω^+ Oli ^r <i>ens2</i> (CG379)
YKN394	a <i>ade5 leu2-3,112 trp1-289 ura3-52 can1-1</i> (CG378)	ω^+ <i>ENS2</i> (CG379)
YKN395	a <i>ade5 leu2-3,112 trp1-289 ura3-52 can1-1</i> (CG378)	ω^+ <i>Δens2</i> (CG379)
YKN398	a <i>ade5 leu2-3,112 trp1-289 ura3-52 can1-1</i> (CG378)	ω^+ <i>ens2</i> (CG379)
YKN1423E	α <i>ura3-52 leu2 met3</i> (CG378 × S30)	ω^+ Ery ^r Oli ^r <i>Δens2</i> (CG379)
YKN1424E	α <i>ura3-52 leu2 met3</i> (CG378 × S30)	ω^+ Ery ^r Oli ^r <i>ENS2</i> (CG379)
CG378-1	a <i>ade5 leu2-3,112 trp1-289 ura3-52 can1-1</i>	ϱ^0
CG379	α <i>his7-2 leu2-3,112 trp1-289 ura3-52</i>	ω^+ <i>Δens2</i>
OP11C-1	a <i>ura3 leu2-3,112 trp1</i>	ϱ^0
S30	α <i>leu2-1 met3</i>	ω^+ <i>ens2</i>
N548-1	α <i>kar1 his4-5</i>	ϱ^0

In the upper part of the Table, the haploid tester strains are described. The origins of their genetic backgrounds are given in parentheses. In the lower part, the haploid strains used for the construction of the tester strains are shown.

et al., 1991; Kawasaki *et al.*, 1991), all of which are involved in the initiation of site-specific gene conversion. It was shown (i) that these endonucleases show complex sequence recognition, since in contrast to prokaryotic restriction endonucleases, the sequences recognized and required for digestion are very long (~20 bp or more) and asymmetrical; (ii) that the cleavage of double-stranded DNA by these enzymes creates cohesive ends with four base 3' overhangs; and (iii) that the smaller subunit (50 kDa peptide) of Endo.SceI contains two conserved motifs comprising dodecameric amino acid sequences, which are identified in the endonucleases for the initiation of site-specific recombination (Nakagawa *et al.*, 1991).

However, we found a significant difference between Endo.SceI and site-specific endonucleases involved in site-specific recombination, suggesting that Endo.SceI was involved in a type of genetic recombination that was not site-specific. Except for HO endonuclease, which is encoded by a chromosomal gene, site-specific endonucleases involved in site-specific recombination are encoded in optional introns of the mitochondrial genome (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989). These endonucleases cleave specific sites in an intronless sequence in mtDNA of the opposite mating type. In contrast to them, Endo.SceI is encoded by two independent genes (Morishima *et al.*, 1990; Nakagawa *et al.*, 1991). A much more significant point which distinguishes Endo.SceI from other site-specific endonucleases is the number of cutting sites in DNA. Endo.SceI exhibits broad sequence specificity (Shibata *et al.*, 1984): in fact, it cleaved yeast mtDNA at >30 sites randomly distributed in the DNA both *in vitro* and *in vivo* (Morishima *et al.*, unpublished observation), whereas all other site-specific endonucleases for site-specific recombination have only one active cutting site in the whole yeast genome. Any of these cutting sites of Endo.SceI in mtDNA appear to be able to serve as the initiation site for genetic recombination. The recombination, which we found occurred between a pair of homologous genetic loci, depends on Endo.SceI. Therefore, Endo.SceI is very likely to be involved in the initiation of homologous recombination in mitochondria.

We emphasize that the disparity in gene conversion at the *oli2* locus required the activity of Endo.SceI. As far as we know, homologous recombination depending on an endonuclease has not been found in any other organism. The related observations are as follows: RecBCD enzyme nicks at the Chi site to increase homologous recombination of the phage genome to ~10-fold higher than the background level in *E. coli* (reviewed by Kobayashi *et al.*, 1984). At an early stage of meiosis, chromosomal DNA was demonstrated to be cleaved sequence-specifically at two hot spots or initiation sites of homologous recombination (Sun *et al.*, 1989; Alani *et al.*, 1990; Cao *et al.*, 1990). These cuttings are supposed to be mediated by sequence-specific endonuclease(s), but the enzyme(s) involved have not been identified.

Another significant difference between Endo.SceI and site-specific endonucleases (ω , $\alpha 4$ and HO endonucleases) is in the mode of protection of the DNA encoding each endonuclease. This difference would reflect the difference in the regulation mechanism for the relevant recombination. As to the recombination initiated by the site-specific endonucleases, their recognition sequences are absent or protected in one of a pair of the parental loci to be recombined; i.e. in mitochondrial site-specific recom-

bination, recognition sequences for the endonucleases are interrupted by their own coding sequences (Colleaux *et al.*, 1986; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989) and, thus, these endonucleases cleave mtDNAs only when haploid cells containing the endonucleases are mated with cells whose mtDNAs lack the coding sequences. In the case of HO endonuclease, one of the parental loci to be recombined (*HML* or *HMR*) is constitutively protected from cleavage by a function of *SIR* genes (Klar *et al.*, 1981). Therefore, cleavage by these site-specific endonucleases can only occur in one of the pair of parental loci, which causes extensive disparity in gene conversion; the cleaved locus always serves as the recipient of genetic information. In contrast to these site-specific endonucleases, we showed that both parental DNAs can be cleaved by Endo.SceI at the *oli2* locus both *in vitro* and *in vivo*, but we still observed a high level of disparity at the *oli2* locus, as described above. There are two possible explanations for this disparity: (i) the cutting site on a parental DNA having $\Delta ens2$ ($\Delta ens2$ mtDNA) is much more sensitive to cleavage by Endo.SceI than that on the parent having the *ENS2* gene, and/or (ii) the asymmetrical structure around the Endo.SceI cutting site at the *oli2* locus causes the disparity. The Endo.SceI cutting site at the *oli2* locus of $\Delta ens2$ mtDNA is actually several-fold more sensitive to cleavage by Endo.SceI than that of *ENS2* mtDNA (Figure 1B). This difference in sensitivity is apparently consistent with the fact that the *oli2* locus of $\Delta ens2$ mtDNA behaved as a dominant recipient in the cross with the *ENS2* strain. It should also be considered that the DNA ends at the *oli2* locus created by Endo.SceI could differ in their recombinogenic nature, depending on the flanking sequences of the 3' side. The flanking sequence of ~1.7 kb downstream of the cutting site of *ENS2* mtDNA is heterologous to $\Delta ens2$ mtDNA, whereas the heterologous region of $\Delta ens2$ mtDNA to *ENS2* mtDNA is significantly shorter (~60 bp) than that of *ENS2* mtDNA (Figure 1A). This difference could also explain the observed disparity in gene conversion at the *oli2* locus.

Possible homologous crossing over, another type of homologous recombination, in an Endo.SceI-dependent manner could not be readily detected because of several reasons described below. Firstly, even in the absence of Endo.SceI, the mitochondrial genome showed crossing over at a significantly high frequency (11.3–16.7%, Table I). The frequency was not far from the theoretical value of mitochondrial recombination, which is the upper limit expected from the recombination frequency of two unlinked loci at the input ratio of ~20% (Dujon *et al.*, 1974). Therefore crossing over induced by Endo.SceI scission is likely to be obscured by the 'background' recombination. The involvement of Endo.SceI in crossing over could be examined using strains which have no mitochondrial endonucleases apart from Endo.SceI (described below). Secondly, in a mitochondrial cross, a pair of recombination products generated through a single recombination event cannot be directly analyzed. In contrast to nuclear recombination, each mitochondrion contains multiple copies of mtDNA and mitochondrial recombination is a consequence of multiple rounds of pairing and recombination among the heterologous mtDNAs (reviewed by Dujon, 1981). This multiround crossing over would result in the high frequency of the event. Thus, we can detect Endo.SceI-dependent recombination only from the disparity in gene conversion.

The high frequency of homologous recombination in the absence of *Endo.SceI* might be explained if one supposes that another sequence-specific endonuclease(s) like *Endo.SceI* is present in mitochondria. The recognition sites for the probable endonuclease could be dispersed over the whole mtDNA. Since several intronic sequences (other than ω and $\alpha I4$ endonucleases) and related open reading frames (RF1 and RF2) within the mitochondrial genome contain the dodecameric conserved sequences described above (Coruzzi *et al.*, 1981; Michel, 1984), some proteins encoded by such sequences could possess endonucleolytic activity. Boulet *et al.* (1990) observed that the absence of introns does not abolish mitochondrial recombination. It is interesting to examine the recombination frequency of strains without RF1 and RF2 as well as all the intronic sequences, because the contribution of *Endo.SceI* to crossing over could be evident in such strains. An alternative possibility is that a nuclear-encoded endonuclease is involved in mitochondrial recombination. NUC1 endonuclease, which is a nuclear-encoded mitochondrial non-specific endonuclease, was a candidate because of its prevalence in yeast mitochondria. However, recent studies have shown that this endonuclease does not have a great effect on the frequency of recombination, although it does affect the length of conversion tracts (Zassenhaus and Denniger, 1992).

It has been postulated that sequence-specific single-strand or double-strand breaks define the initiation sites for homologous recombination, especially during meiosis. This conjecture is based on the results of classical genetic studies (Whitehouse, 1963; Meselson and Radding, 1975; Orr-Weaver and Szostak, 1983). The number of recombination initiation sites where sequence-specific breaks would be made is estimated to be as large as the number of genes in the genome (Whitehouse, 1982). It is unlikely, however, that cells contain a large repertoire of sequence-specific endonucleases for each break. Rather, it is likely that a limited number of endonucleases attack many specified sites. The present study shows that a sequence-specific endonuclease with multiple cutting sites in a genome initiates genetic recombination, which is very likely to be homologous. Because *Endo.SceI* remains the only known example of a eukaryotic sequence-specific endonuclease which recognizes and cleaves DNA with multiple sequences, it can be regarded as a model enzyme for nuclear endonucleases with broad specificities which have not yet been identified.

Materials and methods

Media and strains

The yeast media and genetic techniques were essentially as described by Sherman *et al.* (1986). Solid media contained 2% Bacto agar (Difco). The selection media for antibiotic resistant strains contained 1% yeast extract (Difco), 2% Bacto peptone (Difco) and 3% glycerol buffered with 50 mM sodium/potassium phosphate (pH 6.25). The media for the antimycin selection included 2% ethanol instead of glycerol. The working concentrations of antibiotics were: erythromycin (5 mg/ml), antimycin (0.1 μ g/ml) and oligomycin (3 μ g/ml). All the antibiotics were obtained from Sigma. q^0 and q^- derivatives were isolated by the treatment of cells with ethidium bromide (Goldring *et al.*, 1970; Deutsch *et al.*, 1974). Desired q^- clones retaining particular regions of mtDNA were identified by colony hybridization (Zinn and Butow, 1985) using appropriate fragments of mtDNA as probes.

The yeast strains used in this study are listed in Table II. All the respiration competent strains we used had the ω intron in the 21S rRNA locus of the mitochondrial genome (ω^+), which thus showed no biased transmission of the ω marker between them (Dujon, 1981).

In each set of tester haploid strains, isonuclear haploids were constructed

from either CG378-1, OP11C-1 or haploid progenies obtained through crosses between derivatives of CG378 and S30 to avoid a possible effect of the nuclear background on mitochondrial recombination. Initially, each antibiotic resistant marker (*Ery^r*, *Ant^r* and *Oli^r*) was obtained from S30 by screening for spontaneous antibiotic resistance mutations. The mutation sites were assigned to either the locus of 21S rRNA (*Ery^r*), *cob-box* (*Ant^r*) or *oli2* (*Oli^r*) by the q^- -mediated marker rescue test (Slonimski and Tzagoloff, 1976). The mutation site in *oli2*, determined by DNA sequencing, was located at codon 171 (T to G substitution), which had been previously reported (Macino and Tzagoloff, 1980). These resistance markers were subcloned into q^- derivatives whose mtDNA consisted of a relatively short repeat unit to cover a part of a single gene. The q^- strains were mated, for the transfer of mitochondrial markers, with q^+ recipient strains carrying the mitochondrial genome of either CG379 or S30 (Table II). In these crosses, regions other than the marker locus on the recipient mitochondrial genome remained the same as those of the original recipient. The organization of the mtDNAs constructed was examined by restriction digestion analysis. Similarly, different alleles of the *ENS2* locus (Nakagawa *et al.*, 1991) were generated in the desired mitochondrial genome. The tester strains, including OP19, OP20, YKN394, YKN395 and YKN398, were constructed from q^0 strains (CG378-1 or OP11C-1) via cytoduction involving a *kar1* strain. The other tester strains (YKN1423, YKN1424, YKN1425, YKN1423E and YKN1424E) were obtained through sporulation of diploid strains constructed by mating of derivatives of CG378 and S30 (Table II).

The tester strains all exhibited similar growth rates, irrespective of the allele on the *ENS2* locus. Respiration deficient mutants appeared spontaneously from these clones at a similar frequency (<0.1% per generation), which was also independent of the *ENS2* allele.

Measurement of transmission and recombination of mitochondrial markers

The tester haploid strains were grown for 2 days at 30°C in the presence of antibiotics. Synchronous mating was performed by the method of Sena *et al.* (1973). Prototrophic diploid cells were grown anaerobically in synthetic minimal medium containing 10% dextrose for ~20 generations. The diploid progenies were spread on YPG or YPDG plates (Sherman *et al.*, 1986) and then incubated several days to allow respiration competent cells to form visible colonies. From each cross, 300 colonies were randomly selected and their antibiotic markers examined. For the detection of the functional *ENS2* alleles of diploid progenies, the colonies were transferred to BAS85 nitrocellulose membranes (Schleicher & Schuell) and then analyzed by colony hybridization (Zinn and Butow, 1985).

DNA and enzymes

MtDNA was purified from *S.cerevisiae* mitochondria by ultracentrifugation in the presence of cesium chloride and Hoechst 33258 (Hudspeth *et al.*, 1980). Total DNA was prepared from yeast cells according to the method of Sherman *et al.* (1986).

Restriction endonucleases, Klenow enzyme, T4 DNA ligase and bacterial alkaline phosphatase were purchased from Takara Biomedicals (Kyoto, Japan). Manipulation of DNA was performed essentially as described by Maniatis *et al.* (1982).

Endo.SceI was purified from ~2 kg of *S.cerevisiae* IAM 4274 cells as described previously (Nakagawa *et al.*, 1988; Kawasaki *et al.*, 1991).

Hybridization probe

The DNA probe was constructed with a Multiprime Labelling Kit (Amersham) with [α -³²P]dATP (~110 TBq/mmol).

Southern blot analysis

The digestion products were subjected to 6% PAGE for Southern blot analysis (Southern, 1975) using a Tris-borate-EDTA buffer system (Maniatis *et al.*, 1982). DNAs separated on a polyacrylamide gel were electrophoretically blotted onto a Hybond N⁺ nylon membrane (Amersham) with a TransBlot apparatus (Bio-Rad). The blotted membrane was hybridized to a ³²P-labelled DNA probe in 5 $\times$ SSPE (SSPE: 10 mM NaH₂PO₄ (pH 7.7), 1 mM EDTA and 0.18 M NaCl), 5 $\times$ Denhardt's solution (0.2% Ficoll 400 (Pharmacia LKB), 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin), 50% formamide, 0.5% sodium dodecyl sulfate and 0.1 mg/ml salmon sperm DNA. The hybridization was performed overnight at 42°C. After hybridization, the membrane was washed twice with 0.5 $\times$ SSC (SSC: 0.15 M sodium chloride and 15 mM sodium citrate) and 0.1% sodium lauroylsarcosine at 65°C.

DNA sequencing

Fragments of mtDNA were subcloned into plasmid pUC118/119 (Vieira and Messing, 1987) for sequencing by the chain termination method (Sanger *et al.*, 1977; Messing *et al.*, 1981) using fluorescent derivatives of

dideoxyribose nucleotides (Du Pont—New England Nuclear). The products of the fluorescent labelling reactions were analyzed with a GENESIS 2000 DNA analysis system (Du Pont—New England Nuclear).

Determination of the *Endo.SceI* cleavage site by DNA sequencing

Endo.SceI fragments of the *oli2-ENS2* region were prepared from *Δens2* mtDNA as follows. *Δens2* mtDNA was initially cut with purified *Endo.SceI* under the standard reaction conditions described previously (Watabe *et al.*, 1984), repaired with the Klenow enzyme and then digested with *HapII*. *HapII* sites were located ~0.9 kb upstream and 0.6 kb downstream of the *Endo.SceI* site within the *oli2* sequence (Figure 1A). The DNA fragments were cloned into the *AccI* and *SmaI* sites of plasmid pUC119. The junction of the *Endo.SceI* site was sequenced in both directions. The sequences determined upstream and downstream of the *Endo.SceI* cutting site were 4 nt apart, because *Endo.SceI* cleavage produced four base overhangs at the 3' termini, which were removed through the action of the Klenow enzyme.

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