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Homing of a group II intron in yeast mitochondrial DNA is accompanied by unidirectional co-conversion of upstream-located markers

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Group II introns ai1 and ai2 of the *Saccharomyces cerevisiae* mitochondrial *COXI* gene encode proteins having a dual function (maturase and reverse transcriptase) and are mobile genetic elements. By construction of adequate donor genomes, we demonstrate that each of them is self-sufficient and practises homing in the absence of homing-type endonucleases encoded by either group I introns or the *ENS2* gene. Each of the *S.cerevisiae* group II self-mobile introns was tested for its ability to invade mitochondrial DNA (mtDNA) from two related *Saccharomyces* species. Surprisingly, only ai2 was observed to integrate into both genomes. The non-mobility of ai1 was clearly correlated with some polymorphic changes occurring in sequences flanking its insertion sites in the recipient mtDNAs. Importantly, studies of the behaviour of these introns in interspecific crosses demonstrate that flanking marker co-conversion accompanying group II intron homing is unidirectional and efficient only in the 3′ to 5′ direction towards the upstream exon. Thus, the polar co-conversion and dependence of the splicing proficiency of the intron reported previously by us are hallmarks of group II intron homing, which significantly distinguish it from the strictly DNA-based group I intron homing and strictly RNA-based group II intron transposition.

Key words: allelic/group II/self-mobile introns/site-specific integration

Introduction

Although group I and group II introns have disparate RNA structures and splice via different pathways, they share two remarkable features. First, they are ribozymes that catalyse their own splicing, and second, they have the ability to act as mobile genetic elements (reviewed by Dujon, 1989; Michel *et al.*, 1989).

Group II introns are of particular biological interest because their splicing pathway resembles that of the nuclear pre-mRNA introns. Both types of intron are excised from pre-messenger RNAs as branched molecules, containing 2′-5′ phosphodiester bonds. It has been speculated that primitive self-splicing introns, like group II introns, gave rise to the RNA components of the spliceosome of the eukaryotic nucleus (Sharp, 1985; Cech, 1986; Jacquier, 1990). This hypothesis is made more attractive by the recent discovery of self-splicing group II introns in bacteria (Ferat and Michel, 1993; Ferat *et al.*, 1994). Until recently (Ferat and Michel, 1993), the distribution of group II introns was restricted only to fungal and plant organelle genomes (Michel *et al.*, 1989).

Group II introns are also of biological interest because they may act as mobile genetic elements. However, the mechanisms involved in their mobility are presently not well understood. Some fungal group II intron-encoded reverse transcriptase (RT)-like proteins have been shown recently to practice either *in vivo* homing (i.e. invasion at allelic locations; Meunier *et al.*, 1990; Skelly *et al.*, 1991) or transposition (i.e. invasion at non-allelic locations; Mueller *et al.*, 1993; Sellem *et al.*, 1993).

Two group II introns, ai1 and ai2 of the mitochondrial *COXI* gene of *Saccharomyces cerevisiae*, are the focus of this work. These introns are inserted at closely spaced sites (36 bp apart) and their open reading frames (ORFs) are closely related (50% amino acid identity; Bonitz *et al.*, 1980). Like many group II intron-encoded proteins, the ai1 and ai2 intron ORFs are clearly related to RTs, particularly those of non-LTRs, or LINE1-like, of retroid elements (Michel and Lang, 1985; Xiong and Eickbush, 1990). There is also genetic evidence that the activity of the ai1 and ai2 intron-encoded proteins in yeast mitochondria is implicated in both group I and group II intron loss (Gargouri *et al.*, 1983; Gargouri, 1989a; Levra-Juillet *et al.*, 1989). Recently, the RT-like proteins encoded by group II introns from some fungal mitochondria have been shown to be active intron-specific enzymes (Kennell *et al.*, 1993; Fassbender *et al.*, 1994). Remarkably, the group II intron-encoded proteins also have a maturase activity which functions in the splicing of the intron in which it is encoded (Carignani *et al.*, 1983, 1986).

We have reported previously that both group II introns, ai1 and ai2, of the *S.cerevisiae COXI* gene are transmitted to the intronless version of this gene with the same efficiency as mobile group I introns. Importantly, we have shown that the ai1 mobility is abolished in two splicing-deficient mutants; one carrying a *cis*-acting mutation that affects ribozyme structure, and another carrying a *trans*-acting mutation that affects maturase activity (Meunier *et al.*, 1990). This finding has provided the first significant difference from group I intron homing, which is not affected by *cis*-acting mutations (Wenzlau *et al.*, 1989). It also suggests that the mobility of group II introns occurs via different molecular mechanisms to that of group I introns; in particular, it would take place through an RNA intermediate.

It should be remembered that group I intron homing is a strictly DNA-based process, initiated by double-strand cleavage of an intronless allele by an intron-encoded endonuclease. The gene conversion initiated by group I

introns is accompanied by co-conversion of flanking exon sequences. The co-conversion events occur on both sides of the intron insertion site and are consistent with a bidirectional gene conversion gradient (reviewed by Dujon, 1989; Lambowitz and Belfort, 1993).

In this work we report the second important difference between group II and group I intron homing. The group II intron conversion events are accompanied by a very efficient unidirectional co-conversion extending in the upstream exon, strongly arguing that the genomic DNA is derived from the cDNA transcribed in reverse direction from the unspliced pre-mRNA by the intron-encoded RT. This is in contrast to the bidirectional co-conversion events accompanying group I homing.

Therefore, the unidirectional co-conversion accompanying group II intron homing also clearly distinguishes this process from strictly RNA-mediated group II intron transposition, which is conservative with respect to sequences flanking the integration site (Mueller *et al.*, 1993).

Results

Two group II introns of S.cerevisiae COXI gene are transmitted in a polar fashion to the intronless gene

The acquisition of an intron by an intronless gene can be easily detected by the quantitative analysis of the progeny of the crosses. In mitochondrial crosses, the transmission frequencies of the alleles which are represented in the progeny exhibit similar values for all loci except in polar regions, such as those surrounding some mobile group I introns (reviewed by Dujon, 1989). The transmission frequency is a characteristic feature of a given cross, and the relative appearance of non-polar markers constitutes the input ratio, which reflects the contribution of each parental mitochondrial DNA (mtDNA) to the mating pool (Dujon *et al.*, 1974).

To study the gene conversion of two group II introns which encode RT-like proteins and are present in the *COXI* gene of *S.cerevisiae*, we constructed donor strains having mitochondrial genomes with only one of these introns (ai1 or ai2) and various combinations of mitochondrial genetic markers (resistance or sensitivity to chloramphenicol, erythromycin and oligomycin; see Table I) known to be transmitted in a non-polar fashion (Dujon *et al.*, 1974; Meunier *et al.*, 1990).

The individual intron donor strains were crossed to recipients having an intronless mitochondrial genome, and the transmission of genetic markers to the progeny was analysed as described in Materials and methods. Tables II and III show the results of quantitative analyses of the progeny from two crosses: in the first cross, the ai1 intron was used as a donor (strain KF3/48-29; Table II), and in the second one, the ai2 intron was a donor (strain WF3/5-3; Table III). These results clearly demonstrate that both group II introns are transmitted individually to the progeny at the same and very high frequencies (nearly reaching 100%), while the transmission of markers located in non-polar genes is much lower (50–60%), indicating that the input of the parental genomes is equilibrated. In both cases the major class amongst recombinants (in bold in Tables II and III) results only from intron transfer to the recipient genome.

Table I. Yeast strains

Strain	Nuclear genotype	Mitochondrial genotype
YL1/1	*MATα, lys2*	*rho*$^+$, *C*^{R}, *D*^{S}, *E*^{S}, *O*^{S}, *int*$^-$, *ENS2*$^+$
CKYL1	*MATa, leu1, kar1-1*	*rho*$^+$, *C*^{R}, *D*^{S}, *E*^{S}, *O*^{S}, *int*$^-$, *ENS2*$^+$
KF3/48-29	*MATα, lys2*	*rho*$^+$, *C*^{S}, *D*^{S}, *E*^{R}, *O*^{R}, *ai1*$^+$, *ENS2*$^+$
KF3/48-5	*MATa, leu1*	*rho*$^+$, *C*^{S}, *D*^{S}, *E*^{R}, *O*^{R}, *ai1*$^+$, *ENS2*$^+$
KF4/3-4	*MATα, lys2*	*rho*$^+$, *C*^{R}, *D*^{S}, *E*^{S}, *O*^{S}, *ai1*$^+$, *ENS2*$^+$
YL20/1-2	*MATa, ura3*	*rho*$^+$, *C*^{S}, *D*^{S}, *E*^{R}, *O*^{R}, *ai1*$^+$, *ENS2*$^-$
WF3/5-1	*MATa, lys2*	*rho*$^+$, *C*^{S}, *D*^{S}, *E*^{R}, *O*^{R}, *ai2*$^+$, *ENS2*^{nf}
WF3/5-3	*MATa, leu1*	*rho*$^+$, *C*^{S}, *D*^{S}, *E*^{R}, *O*^{R}, *ai2*$^+$, *ENS2*^{nf}
KS158/3-1[a]	*MATα, leu2*	*rho*$^+$, *C*^{S}, *D*^{R}, *E*^{S}, *O*^{R}, *int*$^+$, *ENS2*$^-$
KS158/3-2[a]	*MATa, his, leu2*	*rho*$^+$, *C*^{S}, *D*^{R}, *E*^{S}, *O*^{R}, *int*$^+$, *ENS2*$^-$
YL6/1-23[a]	*MATa, leu1*	*rho*$^+$, *C*^{S}, *D*^{R}, *E*^{S}, *O*^{R}, *int*$^+$, *ai2*$^+$, *ENS2*$^-$

Mitochondrial markers conferring resistance (R) or sensitivity (S) to chloramphenicol (C), diuron (D), erythromycin (E) and oligomycin (O) are denoted. int$^-$ indicates that all *S.cerevisiae* mitochondrial introns are deleted; ai1$^+$ or ai2$^+$ indicate the only *S.cerevisiae* intron present in the mitochondrial genome; int$^+$ indicates that *S.douglasii* mitochondrial introns are present. *ENS2*: +, gene present and functional; nf, gene present but non-functional; −, gene absent.
[a]The interspecific hybrid strains between *S.douglasii* (mitochondrial genomes) and *S.cerevisiae* (nuclear genomes).

Table II. Polar transmission of the ai1 intron to the *S.cerevisiae* intronless *COXI* gene

Type of colony	Markers				Number of colonies
	cap	ery	oli1	ail	
P1	S	R	R	+	181
P2	R	S	S	−	1
Recombinant	R	R	R	+	3
Recombinant	R	R	S	+	2
Recombinant	R	S	R	+	14
Recombinant	S	R	S	+	34
Recombinant	S	S	R	+	12
Recombinant	**R**	**S**	**S**	**+**	**98**
Recombinant	S	S	S	+	13

Transmission of donor markers: C^S = 67.0% (240/358); E^R = 61.4% (220/358); O^R = 58.7% (210/358); **ail$^+$ = 99.7% (357/358)**. The results of quantitative analysis of the progeny (see Materials and methods) from the cross between the ai1 donor, strain KF3/48-29 (P1), and the intronless recipient, strain CKYL1 (P2), are shown. The 358 random diploid clones were checked on glycerol media supplemented with either chloramphenicol (cap), erythromycin (ery) or oligomycin (oli) for their phenotypes: R, resistance, S, sensitivity. Total DNA extracted from them was checked for the presence of the ai1 intron.

The unidirectional transfer of group II introns was confirmed by Southern blot analysis of mtDNAs from total progeny and parental strains. Figure 1 shows the results of qualitative analysis of the same cross (between KF3/48-29 and CKYL1 strains) for which a quantitative analysis was presented in Table II. Using a pure ai1 intron probe (Figure 1B, lanes 2 and 3), the results show that the mtDNA of the progeny is indistinguishable from the donor genome. However, using a pure exon probe, the results demonstrate that the fragment encompassing the ai1 insertion site and present only in recipient mtDNA (the 621 bp *Bst*NI fragment; see Figure 1A, C, lane 1 and D) has completely disappeared in the progeny mtDNA (Figure 1A and C, compare lanes 1 with lanes 2).

Table III. Polar transmission of the ai2 intron to the *S.cerevisiae* intronless *COXI* gene

Type of colony	Markers				Number of colonies
	cap	ery	oli1	ai2	
P1	S	R	R	+	125
P2	R	S	S	−	7
Recombinant	R	R	R	+	6
Recombinant	R	S	R	+	43
Recombinant	R	R	S	+	4
Recombinant	S	S	R	+	7
Recombinant	S	R	S	+	38
Recombinant	**R**	**S**	**S**	**+**	**126**
Recombinant	S	S	S	+	4

Transmission of donor markers: C^S = 48.3% (174/360); E^R = 48.1% (173/360); O^R = 50.3% (181/360); **$ai2^+$ = 98.1% (353/360)**. In all, 360 random diploid colonies amongst the progeny of the cross between the ai2 donor, strain WF3/5-3 (P1), and the intronless recipient, strain YL1/1 (P2), were analysed as described in Materials and methods. For other explanations see the legend to Table II.

Thus, the recipient intronless genome has been replaced entirely by a recombined intron-plus genome.

The results shown in Tables II and III and Figure 1 are interesting in two respects: (i) they demonstrate that each of the introns is highly mobile by itself and does not require the presence of the closely adjacent intron (they are separated only by a 36 bp-long exon in the original *COXI* gene; Bonitz *et al.*, 1980), and (ii) the mobility of the group II intron does not require the presence of any of the group I intron-encoded LAGLI-DADG-type endonucleases known to be active in gene conversion (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Colleaux *et al.*, 1986; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989; Sargueil *et al.*, 1991; Moran *et al.*, 1992; Séraphin *et al.*, 1992; Schapira *et al.*, 1993; Szczepanek *et al.*, 1994).

Both conclusions can be safely drawn due to the organization of the donor genomes which have been constructed in such a manner as to contain only one intron, either ai1 or ai2 (see Table I), in conjunction with non-polar markers used as controls (to assess the respective contributions of the parental mitochondrial genomes).

Furthermore, it should be emphasized that the ai1 or ai2 homing is not promoted by Endo-*Sce*I endonuclease (encoded by the *ENS2* gene), which cleaves at >30 sites in mtDNA and stimulates gene conversion at at least one of these sites (Nagakawa *et al.*, 1992). Evidence for this is 2-fold. Firstly, we constructed an ai1 intron donor strain (YL20/1-2; Table I and Materials and methods) in which the *ENS2* gene is completely deleted from its mtDNA. The results shown in Table IV (cross A) clearly indicate that intron transmission from this donor strain to the progeny is still extremely polar, and the same as that observed when mtDNA of the ai1 donor carries an active form of the *ENS2* gene (Table II). Secondly, all the strains used in this study having only the ai2 mobile intron (Tables I, III and IV) have the non-functional (interrupted by GC clusters; Nagakawa *et al.*, 1991) *ENS2* gene (data not shown). Thus, the group II intron homing we observe is not affected by the Endo-*Sce*I endonuclease activity.

With two introns, ai1 and ai2, there are four different configurations of crosses that can be studied: $ai1^+ai2°\times ai1°ai2°$; $ai1°ai2^+\times ai1°ai2°$; $ai1^+ai2^+\times ai1°ai2°$ and $ai1^+ai2°\times ai1°ai2^+$. The first two show very high mobility of individual introns (Tables II and III). As expected, the third cross, where the introns are in a *cis* configuration, gave a very high (close to 100%) and practically identical transmission (of both introns) to the progeny (Meunier *et al.*, 1990). In contrast, the results of the cross where ai1 and ai2 are the sole introns, but in *trans* configuration, gave quite unexpected results as shown in Table IV (cross

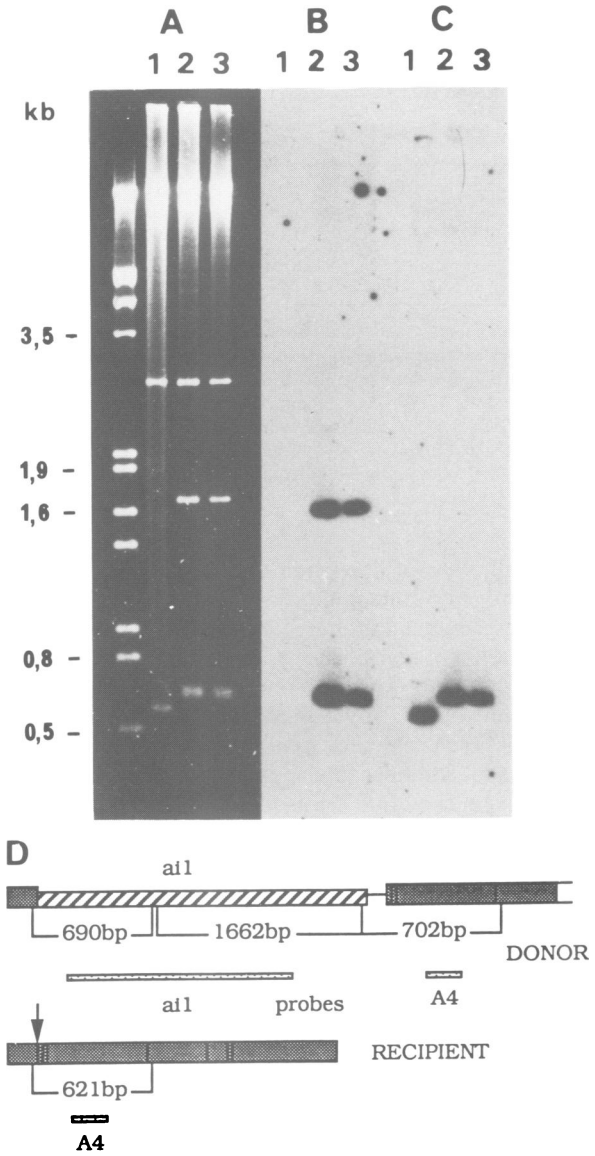

Fig. 1. Qualitative analysis of the ai1 intron transfer to an intronless *COXI* gene. mtDNA from total progeny of the cross shown in Table II and from parental strains was purified and digested with *Bst*NI. (**A**) An ethidium bromide-stained agarose gel with *Bst*NI fragments from mtDNA from recipient (lane 1), total progeny (lane 2) and donor (lane 3). Numbers to the left indicate sizes of *Eco*RI−*Hin*dIII fragments of bacteriophage λ DNA (first lane on the left) used as molecular weight markers. (**B** and **C**) Autoradiograms of blots hybridized with ai1-intronic and A4-exonic probes, respectively. (**D**) Structure of the 5′ part of the *COXI* genes of donor and recipient DNAs. Exons are depicted by solid boxes, intronic ORF by a hatched box and intronic non-coding sequence by a line. Vertical bars indicate the location of the *Bst*NI sites, and the fragment lengths deduced from sequencing are given. The arrow indicates the ai1 insertion site in the recipient gene.

Table IV. Influence of mitochondrial genome organization on the mobility of ai1 and ai2 introns

Cross	Transmission of markers (%)						Total number of diploids analysed
	C^R	D^S	E^R	O^R	ail^+	$ai2^+$	
A	–	–	50.0	54.4	97.3	–	182
B	52.3	–	41.8	54.5	70.8	99.2	351
C	–	19.6	19.6	–	–	63.0	92
D	–	18.9	19.6	–	19.6	–	92
E	–	66.9	66.9	–	69.2	–	354
F	–	58.8	62.0	–	94.5	–	345

Synchronous mating and quantitative analysis of progeny were as described in Materials and methods. The transmission of genetic markers present in genomes carrying the group II intron is given. A, absence of *ENS2* gene does not prevent ai1 mobility; the cross was $ai1^+$*ENS2*$^-$ (strain YL20/1-2) × GF167-7B (Séraphin *et al.*, 1987). B, in the *trans*-configuration ai1 is less transmitted than ai2; the cross was $ai1^+ai2°$ (strain KF4/3-4) × $ai1°ai2^+$ (strain WF3/5-3). C, the ai2 is capable of homing into the *S.douglasii COXI* gene; the cross was $ai1°ai2^+$ (strain WF3/5-1) × $ai1°ai2°$ (strain KS158/3-1). D and E, ai1 is incapable of homing into the *S.douglasii COXI* gene; the crosses were $ai1^+ai2°$ (strains KF3/48-5 and KF3/48-29) × $ai1°ai2°$ (strains KS158/3-1 and KS158/3-2). F, ai1 is capable of homing into the recombined *S.douglasii COXI* gene resulting from the homing of ai2 into *S.douglasii*; the cross was $ai1^+ai2°$ (strain KF3/48-29) × $ai1°ai2^+$ (strain YL6/1-23). For strain descriptions see Table I and Materials and methods.

B). In all, 99% of the genomes of the $ai1^+ai2°$×$ai1°ai2^+$ cross progeny contain intron ai2, but only 70% of the genomes contain intron ai1 (it should be noted that the transmission of non-polar markers indicates that the inputs are equilibrated). This difference is statistically significant and is emphasized by the observation that amongst the four possible classes of genomes, the only class that is present, in addition to the most abundant recombinant class $ai1^+ai2^+$ (244/351), is the $ai1°ai2^+$ class (104/351); the reciprocal classes, $ai1°ai2°$ and $ai1^+ai2°$ are totally (0/351) or nearly absent (3/351).

Since a homing event of $ai1^+$ to the $ai1°ai2^+$ recipient should give $ai1^+ai2^+$, and a homing event of $ai2^+$ to the $ai1^+ai2°$ recipient should do the same, why does the $ai1°ai2^+$ genome still persist? One possible explanation would be that some mutual hindrance of homing occurs in the *trans* configuration. Alternatively, a co-conversion initiating at the $ai2^+$ insertion point and occurring unidirectionally towards the $ai1^+$ intron could efface the latter's presence and create a recombinant $ai1°ai2^+$ molecule. This will be discussed later.

S.cerevisiae introns ai1 or ai2 can be either mobile or non-mobile when confronted with mitochondrial genomes from closely related yeast species

It is known that different yeast species display a variety of situations with respect to the presence/absence of introns. In particular, the *Saccharomyces douglasii* and *Saccharomyces capensis* mitochondrial genomes are devoid of any intron located at the positions where ai1 and ai2 are inserted in *S.cerevisiae* (Wenzlau *et al.*, 1989; Tian *et al.*, 1993; Szczepanek *et al.*, 1994).

We have addressed the question whether these two introns can invade the mitochondrial genomes of their close relatives. To avoid the influence of nuclear genes, we first transferred the mitochondrial genomes of *S.douglasii* and *S.capensis* into the nuclear genome of *S.cerevisiae* by cytoduction, thus creating interspecific nucleo-mitochondrial hybrid strains (Kotylak *et al.*, 1985; Claisse *et al.*, 1987; Lazowska *et al.*, 1992; Szczepanek *et al.*, 1994). In this manner the *S.douglasii* and *S.capensis* mitochondrial genomes could be used as recipients for the study of *S.cerevisiae* ai1 and ai2 mobility.

The results of quantitative analyses of the progeny issued from crosses between *S.douglasii* recipients (see Materials and methods and Table I for strain descriptions) and *S.cerevisiae* donors of individual group II introns strains are presented in Table IV (crosses C–E). Surprisingly, the transmission of the ai1 intron to the progeny (Table IV, crosses D and E) is the same as that of the non-polar markers, indicating that ai1 is not mobile in these crosses. In contrast, the ai2 intron is transmitted in a polar fashion (Table IV, cross C). Particularly striking is a comparison of donor marker transmissions to the progeny, from two crosses in which the same recipient strain (KS158/3-1) was mated to either the ai1 donor strain (cross D) or to the ai2 donor (cross C). Although the input of parental mtDNAs is biased in both crosses (~20% for non-polar and ai1 markers), the frequency of ai2 intron transmission (63%) is significantly higher.

Homing of intron ai2 to the *S.douglasii COXI* gene is confirmed by Southern blot analysis of mtDNA purified from total progeny of cross C (Table IV). The results shown in Figure 2 clearly indicate the presence, in this mtDNA (lane 2), of a new abundant fragment of 2.7 kb, which strongly hybridized with both intron- and exon-specific probes (Figure 2A and B, compare lanes 2 with lanes 1 and 4). The same fragment is present in mtDNA purified from one subcloned recombinant (strain YL6/1, lane 3), which was isolated as being representative of a major class of recombinants detected by quantitative analysis. This new (*Eco*RI–*Hin*dIII) fragment results from the *S.cerevisiae* ai2 intron homing to the *S.douglasii* recipient *COXI* gene, as depicted in the diagrams shown in Figure 2C.

The immobility of ai1 and the mobility of ai2 were also observed when the *S.capensis COXI* gene was used as the recipient in the crosses (data not shown).

The main conclusion from these results is that the two *S.cerevisiae* group II introns act as mobile elements in the *S.cerevisiae* mitochondria, but only intron ai2 has the ability to move into mtDNA of *S.douglasii* and *S.capensis*.

A plausible explanation for the non-mobility of the ai1 intron in interspecific crosses can be proposed on the basis of the divergence of sequences flanking the intron insertion sites amongst the three yeasts. As shown in Figure 3, in

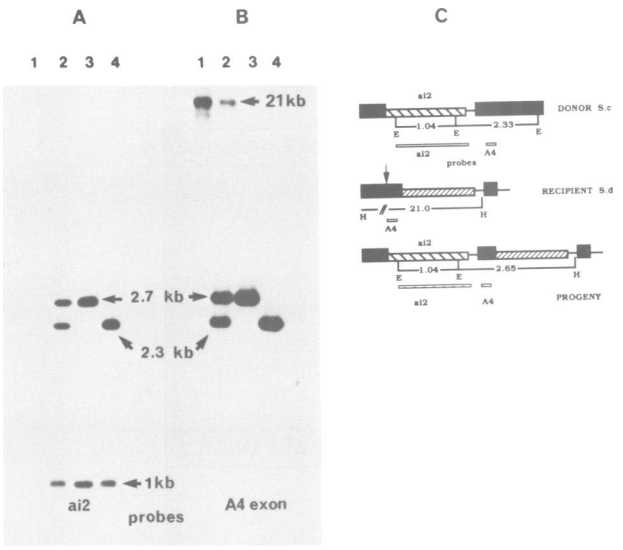

Fig. 2. Qualitative analysis of the *S.cerevisiae* ai2 intron transfer to the *S.douglasii COXI* gene lacking it. mtDNA from recipient (strain KS158/3-1, lane 1), donor (strain WF3/5-3, lane 4), total progeny (lane 2) and one subcloned individual recombinant (strain YL6/1, lane 3) was purified, digested with *Eco*RI + *Hin*dIII, transferred to nitrocellulose blots and hybridized with ai2 intron-specific (**A**) and A4 exon-specific (**B**) probes. (**C**) Structure of the 5′ parts of the *COXI* gene of donor, recipient and recombinant (progeny), with the location of the *Eco*RI (E) and *Hin*dIII (H) sites, the lengths of fragments and the location of molecular probes indicated. The arrow indicates the ai2 insertion site in the recipient gene. For other explanations see the legend to Figure 1.

which we schematically summarize the behaviour of the two group II introns in interspecific crosses, there are several nucleotide substitutions, in particular in the exon sequences surrounding the ai1 insertion point in the *S.douglasii* (Figure 3, bases in bold and underlined) and *S.capensis COXI* genes (bases within brackets), when compared with the cognate sequence of *S.cerevisiae*. Importantly, only two nucleotide substitutions, more distal to the intron insertion site (at positions −20 and +29; Figure 3), are the same in the *S.douglasii* and *S.capensis* sequences. This finding suggests that both, or one of the two, nucleotide substitutions could prevent ai1 mobility.

It should be pointed out that not all nucleotide substitutions prevent intron homing, since the ai2 intron is mobile in interspecific crosses (Table IV, crosses B and C; Figure 2) in spite of a few changes occurring in sequences flanking its insertion point (Figure 3). The allelic integration of this intron has been confirmed by restriction mapping and Southern blot analysis of mtDNA from recombinant strains YL6/1 (Figure 2) and YL18/2 (data not shown), the latter resulting from the transfer of ai2 to the *S.capensis COXI* gene. Sequence determination of the COXI mRNA from recombinant strains showed that in both cases the sequences flanking the intron insertion site were co-converted, i.e. they became identical to the donor exon sequences (Figure 3).

We took advantage of the creation of these genomes, which consist almost entirely of the *S.douglasii* (YL6/1-23) or *S.capensis* sequence (YL18/2-4) except for a few nucleotide changes surrounding the ai2 insertion site, to determine whether the lack of mobility of ai1 to the *S.douglasii* and *S.capensis* genomes is due to the general organization and properties of these genomes, in which case they should still resist the homing of ai1. Or alternatively, the determining factor is the local variation of sequence which extends for at least 56 bp (Figure 3), in which case they should become susceptible to ai1 homing. The results (Table IV, cross F) clearly show that the second possibility is true, since very efficient transfer of ai1 was observed.

Two important conclusions can be drawn from the study of group II intron homing in mitochondria from related *Saccharomyces* species. First, sequence analysis of hybrid *COXI* genes (strains YL6/1-23 and YL18/2-4) resulting from the mobility of ai2 clearly shows that this intron homing event is accompanied by co-conversion of exonic sequences flanking its insertion point. It can be seen that the polymorphic changes occurring in the *S.douglasii* and *S.capensis* sequences that flank the ai2 insertion site (Figure 3) are tolerated and do not prevent intron homing. Second, the transfer of intron ai1 is sequence-specific and, apparently, the *S.cerevisiae* type of sequence is needed for its homing. Amongst 10 nucleotide substitutions which occur in the *S.douglasii* and *S.capensis* exonic sequences flanking the ai1 insertion site, those two (Figure 3, positions −20 and +29) at the more distal locations from the intron border are identical, suggesting their possible involvement in preventing the mobility of this intron.

A polar co-conversion of exon sequences extending preferentially in the 3′ to 5′ direction accompanies group II intron homing

Having demonstrated the occurrence of co-conversion of flanking exonic sequences, which follows the intron ai2 mobility, we have addressed the question of symmetry of the co-conversion events. In other words, we have asked whether co-conversion affects the sequences located on both sides of the intron insertion site to the same extent, or preferentially affects one side more than the other. It should be remembered that DNA-based group I intron homing involves the co-conversion of sequences flanking the intron up to 700–1000 bp on either side, and that the co-conversion rate decreases with increasing distance from the intron insertion site (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985).

To answer this question we have analysed a statistically significant number of independent ai2 homing events in the *S.douglasii* recipient mitochondrial genome. The interspecific hybrid strain which has the *S.douglasii* wild-type mitochondrial genome (SD12; see Materials and methods) was crossed to the ai2 donor (strain WF3/5-3; Table I), and only recombinant clones resulting from the transfer of ai2 (the recombinant genomes contain simultaneously the ai2 and cyt. b gene introns, and can be easily distinguished by hybridization from the parental genomes which contain them separately) were chosen for further analysis.

The 12 polymorphic positions indicated in Figure 4A have been analysed to determine the extent of co-conversion. They comprise single base substitutions in exons (eight out 12 positions analysed) and the presence/absence of introns. The nature of the sequence present in individual recombinants was determined using a rapid screening

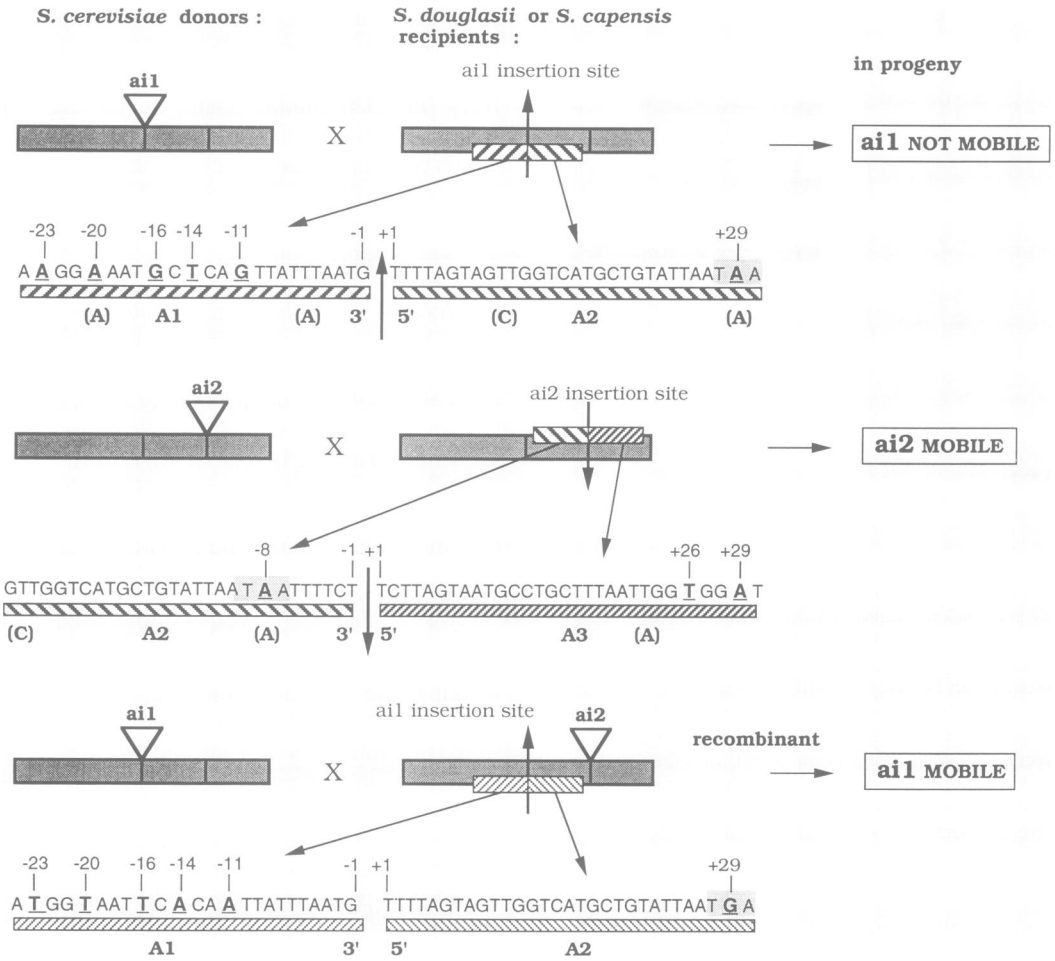

Fig. 3. The sequence-specific integration of the *S.cerevisiae* ai1 intron to the *S.douglasii* and *S.capensis COXI* genes lacking it. Exons are depicted by solid boxes. The smaller boxes variously hatched indicate the section of exonic sequences surrounding the homing sites of both introns in which the polymorphic changes amongst *S.cerevisiae*, *S.douglasii* and *S.capensis* are found. The nucleotides are numbered from −1 and +1, extending upstream and downstream of the intron insertion site (heavy arrow). Variable bases at indicated positions are in bold, those occurring in the *S.douglasii* sequence are underlined and those occurring in *S.capensis* are in brackets. The triplets TAA in exon 2 sequences are shadowed to indicate overlapping in this short 36 bp exon.

technique (di Rago *et al.*, 1990), based on hybridization with synthetic oligonucleotides overlapping the polymorphic change, and the relative stability of homo- and heteroduplexes during differential dehybridization (see Materials and methods). As shown in the example in Figure 4C, the mismatched hybrids (in this case, co-converted sequences) present in 12 out of 34 recombined mtDNA are clearly distinguished from the correct pairings which remain stable at the same washing temperature (53°C, near the calculated T_m value; see Materials and methods and legend to Figure 4).

The results obtained by this method were confirmed by direct sequencing of total mtRNA extracted from several recombinants (data not shown).

The nature of the flanking markers located nearest to either side of the intron insertion site was established in 161 selected individual recombinants, and for distal downstream markers in a lower number (see Figure 4A, lower portion). The absence of distal upstream markers limited our estimation of the extent of co-conversion to 59 bp (corresponding to position −23 in Figure 3) from the intron border.

The results shown in Figure 4A (lower portion) and B clearly indicate that the co-conversion detected here is almost unidirectional. The flanking markers of the recipient gene located upstream of the intron insertion site have been co-converted to the *S.cerevisiae* donor sequences in almost all recombinants (98%) at position −8, and in 86% of them at positions −50 to −56. In contrast, the flanking markers located downstream at positions +26 and +29 are co-converted in only 34% of the same set of screened recombinants. It is likely that this value is somewhat overestimated, taking into account the background transmissions of the more distal markers (18% on average) probably resulting from recombination events other than that involved in the ai2 intron homing. Because the number of polymorphic sites analysed upstream is small, it is unclear whether there is some kind of a 'gradient' with diminishing probability as some function of the distance from the exon−intron border. What comes through clearly are the upstream−downstream exon differences and a possibility of a real 'gradient' from 34 to 11% (seven polymorphic sites analysed) as one proceeds 5′ to 3′ on the downstream exon. It should be pointed out that the *S.douglasii* introns are not mobile, are transmitted into the progeny with comparable background values

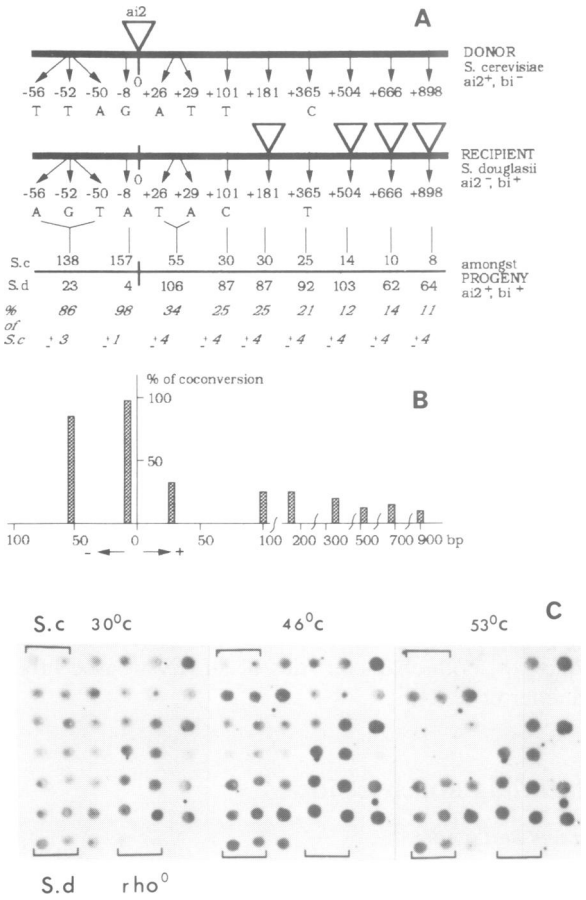

Fig. 4. Determination of co-conversion accompanying the mobility of the ai2 intron. (**A**) Structure of donor and recipient *COXI* genes [*S.douglasii COXI* gene data from Tian *et al.* (1993)] and the nature and position of molecular markers analysed. The markers located upstream from the ai2 intron insertion site (0 point) at positions −56, −52 and −50 correspond to those shown in Figure 3 (in exon 1 sequence) at positions −20, −16 and −14, respectively. Vertical bars indicate the numbers of colonies examined for each position and their type (S.c for *S.cerevisiae* and S.d for *S.douglasii*). Numbers in italics indicate the percentage (and SD values) of molecular markers from the donor mtDNA amongst the selected progeny. (**B**) Graphical representation of the extent of co-conversion. (**C**) One example of differential dehybridization. DNA was probed with 5′ end-labelled oligonucleotide complementary to the *S.douglasii* sequence (flanking position +101). Filters were progressively washed for 3 min at selected temperatures and autoradiographed after each washing. Total DNA of a rho° mutant strain (devoid of mtDNA) was used as a control for non-specific hybridization, and parental DNAs were used as a control for correct (S.d., not eluted at a temperature near the T_m calculated value) and mismatched (S.c., eluted at the same temperature) pairings. The presence or absence of intronic markers was checked by dot-blot hybridization using the intron-specific probes (see Materials and methods).

(Tian *et al.*, 1993; data not shown) and therefore do not influence the extent of co-conversion brought about by intron ai2.

These data provide evidence that the co-conversion of the flanking marker accompanying group II intron homing is polar, and is very efficient only in the upstream exon. This finding is important because it provides a further distinction between group II and group I intron homing, and also between group II intron homing and transposition (Mueller *et al.*, 1993).

Discussion

As shown previously by us (Meunier *et al.*, 1990), the two group II introns (ai1 and ai2) of the *S.cerevisiae COXI* gene are able to invade an intronless allele with the same high efficiency as neighbouring ai3 (Szczepanek *et al.*, 1994) and ai4 (Wenzlau *et al.*, 1989) group I mobile introns. We have also shown that this ability depended on the intron being splicing-competent. This finding provided the first significant difference between endonuclease-mediated group I intron homing and RT-mediated group II intron homing.

In this work we asked three questions concerning the group II intron mobility. (i) Is their homing process influenced by other genetic elements residing in mtDNA? (ii) What are the features of their mobility in mitochondria from closely related yeast species? (iii) Does their homing involve solely the intronic sequences (which would suggest that reverse splicing is an underlying mechanism) or does it extend to neighbouring exonic sequences?

By constructing several strains with adequate mitochondrial donor genomes in which only one group II intron, ai1 or ai2, is present, we have shown that each group II intron is self-sufficient in carrying out a very efficient homing (approaching 100%). Clearly the ai1- and ai2-mediated gene conversions are independent of each other and are also independent of the activities of group I mobile intron-encoded endonucleases (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985; Colleaux *et al.*, 1986; Delahodde *et al.*, 1989; Wenzlau *et al.*, 1989; Sargueil *et al.*, 1991; Moran *et al.*, 1992; Séraphin *et al.*, 1992; Schapira *et al.*, 1993; Szczepanek *et al.*, 1994).

Importantly, we also demonstrated that group II intron homing is not affected by the activity of the Endo-*Sce*I endonuclease, encoded by the *ENS2* gene, which is known to mediate gene conversion in yeast mitochondria (Nagakawa *et al.*, 1992). This is indicated by the finding that the polar transmission of group II introns to the progeny is exactly the same, whether the *ENS2* gene is present and functional, present but non-functional (ORF interrupted by GC clusters; Nagakawa *et al.*, 1991), or completely deleted from donor genomes.

Some unexpected features of group II intron homing events have arisen from the study of the mobility of *S.cerevisiae* introns into mitochondria from closely related species. Two related yeasts, *S.douglasii* and *S.capensis*, are of particular interest since their *COXI* genes lack group II introns; further, their exon sequences show several polymorphic nucleotide substitutions (Wenzlau *et al.*, 1989; Tian *et al.*, 1993; Szczepanek *et al.*, 1994) with regard to their *S.cerevisiae* counterpart (Bonitz *et al.*, 1980).

The ai1 intron is not mobile in interspecific crosses, while ai2, as in intraspecific crosses, is still mobile. This lack of mobility of the ai1 intron could be correlated with the divergence of sequences flanking its insertion sites observed amongst the three relevant *COXI* gene sequences. Homing of ai1 into *S.douglasii* and *S.capensis* mitochondrial DNA can proceed provided all the polymorphic changes have been corrected *in vivo* to the *S.cerevisiae*-type sequence, taking advantage of the recombination events accompanying the ai2 homing process (see below). The extreme effectiveness of both introns in mediating

gene conversion events in *S.cerevisiae* mitochondria, and the sequence-specific transfer of the ai1 intron observed during interspecific crosses, implicitly suggest that recognition of target sequences by a site-specific endonuclease is involved in group II intron homing. It should be noted that the RT-like protein encoded by retrotransposon R2 from the silkworm *Bombyx mori* has a site-specific DNA endonuclease activity which cleaves the target gene at the R2 insertion site (Xiong and Eickbush, 1988). The group II intron ORF products, having a striking similarity to those encoded by non-LTR elements (Michel and Lang, 1985; Xiong and Eickbush, 1990), might also contain a site-specific endonuclease activity.

In this work, we report a novel distinctive feature of group II intron homing. It consists of the occurrence of unilateral flanking marker co-conversion as a result of group II intron inheritance. This phenomenon was strongly suggested by the observation that unequal transfer, with that of ai2 being most prominent, is observed in progeny from crosses in which ai1 and ai2 are the sole introns, but in a *trans* configuration. Direct evidence is given by sequencing of recombinant strains resulting from the ai2 intron homing to the *S.douglasii* and *S.capensis COXI* genes, showing that polymorphic markers surrounding the intron insertion site in both recipient strains are co-converted to the donor-type sequences. Finally, we demonstrate, on the basis of molecular analyses of significant numbers of independent ai2 conversion events into *S.douglasii* mitochondria, that the co-conversion we detected here is unidirectional and occurs towards the upstream exon. According to the distribution of available markers, our estimation of the extent of co-conversion of upstream-located nucleotide substitutions is at least 59 nucleotides; the lack of abundant downstream sequence co-conversion is already detectable at 26 nucleotides from the intron and does not change significantly for several hundred nucleotides from the intron border. Although the number of nucleotide substitutions analysed is relatively small, it is clear that at 59 nucleotides upstream of the intron insertion position the frequency of co-conversion is at least twice greater than that at half of that distance downstream. The extent of the polar co-conversion at a given distance from the intron insertion site is quite comparable with that which accompanies group I intron-directed gene conversion (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985).

In contrast to the well-established group I intron homing pathway, which is DNA-mediated, the group II intron mobility pathway mediated by RT-like proteins is presently not well understood (Belfort, 1993).

Our finding that group II intron homing is abolished by *cis*-active splicing-deficient mutations (Meunier *et al.*, 1990; J.Lazowska *et al.*, manuscript in preparation) suggests that intron excision is required for mobility. The possibility that only the excised intron could be an intermediate in mobility via reverse splicing or direct integration into DNA is excluded by the polar co-conversion demonstrated here. This implies a process involving recombination of homologous exon sequences. Our results indicate that the cDNA intermediates involved in group II intron homing most probably derive from reverse transcripts of unspliced precursor RNAs via the activity of the RT encoded by the intron. In the case of ai2, such cDNA intermediates (referred to as 3.1 kb cDNAs in Kennell *et al.*, 1993), initiated immediately downstream in exon A3 and extending upstream into ai1, were found to be abundant in the whole population of cDNAs. Concerning the intron ai1, the RT activity was easily detectable in some strains but not in others. In our strains the ai1 homing process is very efficient and equally so is that of ai2, indicating that ai1 by itself is sufficient for mobility.

The role of splicing and the mechanism of site-specific integration of the cDNA involved in the group II intron homing process remain unclear. Therefore, the hallmarks of this process, which are the polar co-conversion and dependence on the splicing proficiency of the intron, suggest that the group II intron homing pathway is a novel RNA−DNA-based mechanism probably involving RNA−DNA pairing, reverse transcription and DNA endonucleolytic steps, where DNA would be cleaved by RNA, protein or a complex of the two. It is significantly different from the group II intron transposition to ectopic sites, which is also dependent on the intron being splicing-competent but does not involve co-conversion of flanking sequences, both characteristics of an RNA-based process (Mueller *et al.*, 1993).

Materials and methods

Strains and media

The yeast strains constructed in this study are listed in Table I. The *S.cerevisiae* strains used as a donor with only one mitochondrial intron (ai1 or ai2) interrupting the *COXI* gene were constructed by a series of crosses between a strain with no introns (GF167-7B; Séraphin *et al.*, 1987) and strains with decreased numbers (from 13 to three) of them. All introns present in constructed strains originated from strain 777-3A. Initially, each antibiotic-resistant marker (erythromycin, E^R; oligomycin, O^R; chloramphenicol, C^R) was obtained from GF167-7B by screening for spontaneous antibiotic resistance mutations. The mutation sites were assigned to either the locus of LSrRNA (E^R and C^R) or oli1 (O^R) by the allelic test using the tester strains from the laboratory collection.

YL20/1-2 haploid strain was obtained after sporulation of a YL20/1 diploid arising from a cross between strain KF3/48-29 (Table I) and a rho^- clone (GF266-9; kindly given by G.Faye); the mitochondrial genome has a basic repeat length of 6.0 kb starting at the end of the *COXI* gene and is devoid of the *ENS2* sequence (G.Faye, personal communication).

Strains having an intronless mitochondrial genome and carrying genetic markers were used mainly as recipient genome, along with two interspecific hybrid strains having the *S.cerevisiae* nuclear genome and the *S.douglasii* mitochondrial genome.

SD12 (*MAT***a**, *leu1*, *kar1-1*, rho^+, *S.douglasii*) interspecific hybrid strain is respiratory-deficient because of nucleo-mitochondrial incompatibility (Kotylak *et al.*, 1985; Tian *et al.*, 1991, 1993).

KS158/3 is derived from SD12 (Kotylak *et al.*, 1985). This *S.douglasii* recipient genome is respiration-competent because it contains the *COXI* gene lacking its first intron (deletion of this intron abolished the nucleo-mitochondrial incompatibility observed in the interspecific hybrid; Tian *et al.*, 1993) and carries two non-polar genetic markers conferring oligomycin resistance (the same locus as donor strains) and diuron (D^R) resistance in the cob-box locus (Table I).

The YL6/1-23 strain was obtained by sporulation of one subcloned diploid, which was representative of the major class of recombinants issued from cross between WF3/5-3 and KS158/3-1 (Table III and Figure 2).

The following media were used for growth of yeast: YPGA (1% yeast extract, 1% peptone, 2% glucose, 20 mg/l adenine); YP10 (1% yeast extract, 1% peptone, 10% glucose); N3 (as YP10 but 2% glycerol instead of glucose); WO (0.67% yeast nitrogen base without amino acids, 2% glucose); WO10 (as WO but 10% glucose). Where required, N3 medium was supplemented with appropriate antibiotics.

Measurement of transmission of mitochondrial genetic markers

Haploid cells with homoplasmic genomes containing only one intron (donor genomes) were crossed to haploid cells whose genome had either no introns or some of them in a different configuration (recipient genomes). After synchronous mating performed in a complete non-selective medium (YP10), and segregation of initially heteroplasmic to homoplasmic cells (~15 generations) in minimum anaerobic medium (WO10, non-selective for mitochondrial genome), the progeny of the cross were subjected to quantitative and qualitative analyses as described in Szczepanek *et al.* (1994).

For quantitative analysis the cells were plated on minimal medium (WO); individual prototrophic diploid colonies were then picked at random and grown on complete medium (YPGA, 300 µl/well) for 2 days in 96-well microtitration plates. Plates were replicated on selective media (WO, N3 and glycerol with appropriate antibiotics) to determine the transmission of genetic non-polar markers. Total DNA extracted from the same colonies was checked for transmission of individual introns by dot-blot hybridizations.

For the qualitative analysis of the cross, mtDNA from the parental haploid strains and total progeny was purified (Gargouri, 1989b), digested with appropriate restriction endonucleases and the digestion products subjected to Southern blot analysis (Maniatis *et al.*, 1982).

Molecular probes

The COXI exon probe is a pYJL17 recombinant plasmid containing 151 bp of exon A4 (Tian *et al.*, 1991). The *S.cerevisiae* ai1 intron probe is a recombinant plasmid containing 766 bp of ai1 sequence (gift from M.Labouesse). The *S.cerevisiae* ai2 intron probe is a recombinant plasmid containing 2200 bp of ai2 sequence (gift from E.Bergantino). The *S.douglasii* intron-specific probes pYJL21, pYJL26-2, pYGT4 and pYGT9 are described in Tian *et al.* (1991).

Measurement of the co-conversion accompanying the ai2 intron conversion

After synchronous cross and quantitative analysis of progeny, performed as described in Szczepanek *et al.* (1994), DNA from selected recombinants (resulting from the mobility of the ai2 intron into the recipient genome) was analysed by a rapid screening technique based on oligonucleotide hybridization with mtDNA described by di Rago *et al.* (1990). All oligonucleotides used were complementary to the recipient wild-type sequences (sequence data from Tian *et al.*, 1993); thus, the mutated sequences could be differentiated by a decrease in the duplex fusion temperature (T_m) of ~4–8°C, depending on the nature and position of the mismatch. The following oligonucleotides, which overlap the polymorphic changes (bases in bold) between donor and recipient mtDNA, occurring at the positions referred to in Figure 4 were synthesized: 5′-CATTAAATAACTG**AG**C**A**TT**T**CC-3′ (positions −50, −52, −56; T_m = 58°C); 5′-GCACAGAAAATT**A**TTAATAC-3′ (position −8; T_m = 50°C); 5′-CCAAA**T**CC**A**CCAATTAAAG-3′ (positions +26, +29; T_m = 52°C); 5′-GTAATACTCAAAA**G**GCAATG-3′ (position +101; T_m = 54°C); 5′-CTGTAAT**A**AAAATTGATCATAC-3′ (position +365; T_m = 54°C). Hybridization with 5′ end-labelled oligonucleotides was performed as described by di Rago *et al.* (1990). Filters were progressively washed for 3 min at selected temperatures and autoradiographed after each washing. The nature of the polymorphic changes was confirmed by direct sequencing of total mtRNA which was extracted from at least four individual recombinants for each class of molecule showing the same extent of flanking marker co-conversion (screened by differential dehybridization). For each class, the mtRNAs were mixed and the cDNA was dideoxy sequenced.

Sequence determination

Total mtRNA was extracted from cells grown in YPGA. Purification of mtRNA and sequencing by primer extension with avian myeloblastosis virus reverse transcriptase were performed as described by Tian *et al.* (1993).

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