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The *MRS1* gene of S. douglasii: co-evolution of mitochondrial introns and specific splicing proteins encoded by nuclear genes

Christopher J. Herbert, Catherine Macadre, Anne-Marie Bécam, Jaga Lazowska, and Piotr P. Slonimski

Centre de Génétique Moléculaire du CNRS, Laboratoire propre associé à l'Université Pierre et Marie Curie, Gif-sur-Yvette, France

We have developed a rapid and simple methodology to locate yeast genes within cloned inserts, obtain partial sequence information, and construct chromosomal disruptions of these genes. This methodology has been used to study a nuclear gene from the yeast S. douglasii (a close relative of S. cerevisiae), which is essential for the excision of the mitochondrial intron aI1 of S. douglasii (the first intron in the gene encoding subunit I of cytochrome oxidase), an intron which is not present in the mitochondrial genome of S. cerevisiae. We have shown that this gene is the homologue of the S. cerevisiae *MRS1* gene, which is essential for the excision of the mitochondrial introns bI3 and aI5β of S. cerevisiae, but is unable to assure the excision of the intron aI1 from the *coxI* gene of S. douglasii. The two genes are very similar, with only 13% nucleotide substitutions in the coding region, transitions being 2.5 times more frequent than transvertions. At the protein level there are 86% identical residues and 7% conservative substitutions. The divergence of the *MRS1* genes of S. cerevisiae and S. douglasii, and the concomitant changes in the structure of their mitochondrial genomes is an interesting example of the co-evolution of nuclear and mitochondrial genomes.

Although the mitochondrial genomes of all fungi encode the same "basic set" of rRNAs, tRNAs, and a few proteins involved in energy metabolism, there is considerable variation in their organization. This is due in part to the large variety of introns found in mitochondrial genes. These introns vary in their position within genes and in their sequence, but they all fall into two groups based on secondary structure considerations (Davies et al., 1982; Michel et al., 1982). The excision of introns from mitochondrial pre-mRNAs is a complex process: some introns can self-splice in vitro, but most, if not all, require proteins for splicing in vivo (for reviews see Grivell, 1989, and Cech, 1990).

The proteins involved in mitochondrial pre-mRNA splicing are both mitochondrial and nuclear in origin. The mitochondrial proteins (called RNA maturases; see Lazowska et al., 1980, 1989, and references therein), are encoded within introns and in general are only needed for the excision of the introns which encode them, the nuclear proteins may be involved in the excision of one or more introns. These nuclear proteins can be further subdivided into those that are only involved in mitochondrial

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Correspondence: Christopher J. Herbert, Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette Cedex, France Tel 33-1-69-82-30-30 Fax 33-1-69-07-53-22

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pre-mRNA splicing, such as CBP2 and MRS1 (Hill et al., 1985; Bousquet et al., 1990), and those required for splicing and other functions, such as the mitochondrial leucyl tRNA synthetase (Herbert et al., 1988a).

In order to identify the genes encoding these nuclear proteins two methods have been used. The first is the isolation of nuclear mutants deficient in mitochondrial splicing (*pet*) (McGraw and Tzagoloff, 1983); the second is the isolation of nuclear suppressors (*NAM*) of defined mitochondrial splicing mutations (Labouesse et al., 1985). These two approaches are not mutually exclusive, and in the case of the mitochondrial leucyl tRNA synthetase, they have both led to the identification of the same gene (Dujardin et al., 1980; Herbert et al., 1988a; and Tzagoloff et al., 1988).

One of the difficulties with these two approaches, when applied to splicing factors, has been to determine whether the genes isolated are directly involved in splicing, or whether the effects on splicing are a secondary consequence of the original defect. To overcome this problem Séraphin et al. (1987) constructed an intronless mitochondrial genome. A gene involved only in mitochondrial splicing will be dispensable in this strain; thus its inactivation should not affect growth on respiratory substrates. This system has been refined by Bousquet et al. (1990) in their study of *MRS1/PET157*. These authors constructed a large series of mitochondrial genomes with different intron compositions, and were thus able to identify which introns required the *MRS1/PET157* gene product in order to be spliced. The limitation of this approach is that it is valid only when the gene in question is involved solely in mitochondrial pre-mRNA splicing during respiratory growth.

In this paper we present a novel approach to clone a gene that is involved in mitochondrial pre-mRNA splicing using a heterologous system of interspecific hybrids. Saccharomyces cerevisiae and Saccharomyces douglasii are closely related yeasts; calculations based on limited nuclear sequence studies suggest that they diverged between 50 and 80 million years ago (Herbert et al., 1988b). The mitochondrial genomes of these two strains differ considerably (Kotylak et al., 1985; Claisse et al., 1987; Tian et al., 1991a,b); in particular there is a novel intron found in the *coxI* gene (subunit I of cytochrome oxidase) of S. douglasii which is not found in S. cerevisiae. In a previous publication Kotylak et al. (1985) called this intron aI4α; to avoid confusion we shall call the intron S. douglasii aI1. Interspecific hybrids with the mitochondrial genome of S. douglasii and the nuclear genome of S. cerevisiae are respiratory-deficient: because the S. cerevisiae nuclear genome cannot assume the excision of the S. douglasii intron aI1, no *coxI* messenger is made (Kotylak et al., 1985). When this intron is deleted from the mitochondrial DNA by recombination, the hybrid strains become respiratory competent. Using this nucleo-mitochondrial incompatibility, we have cloned an S. douglasii nuclear gene capable of promoting the splicing of the S. douglasii intron aI1 from the *coxI* pre-RNA, in an S. cerevisiae nuclear background. We have also developed a general and rapid methodology to locate and disrupt newly cloned genes, and obtain partial sequence information.

Materials and methods

Media, strains, and genetic methods

For standard cloning experiments and the propagation of M13 phages we used E. coli JM101 (*Δ[lac proAB], thi, supE,* F′ *traD36, pro AB, lac* I^Q, *lacZ ΔM15*). For the selection of *URA3* containing plasmids we used E. coli B15 *pyrF::Mu*, trp_{am}, $lacZ_{am}$, *hsdR*. The S. douglasii strains were 4707-22D (*MATaaa, HO, GAL, SUC, mal, αMG, his4, ura3, ade1, leu2, rho*$^+$, *mit*$^+$) and 4795-3B/150 (*MATααα, HO, GAL, SUC, mal, αMG, ura1, met1, ade2, leu1, tyr7, rho*°), CX1 is a cycloheximide resistant mutant derived from 4707-22D, and isolated after u.v. mutagenesis. The S. cerevisiae strains used were CW04 (*MATα, his3-11,15, leu2-3,112, ura3-1, trp1-1, rho*$^+$, *mit*$^+$ 777-3A) (Banroques et al., 1986), JC25/Δ (*MATα, kar1, ade2-1, his4-Δ15, rho*$^+$, *mit*$^+$ [intronless mitochondrial genome of Séraphin et al., 1987]) isolated during this study and W303/SD. The latter has the same nuclear genome as CW04 but the mitochondrial genome of S. douglasii and was constructed during this study. The media (glucose complete medium [YPGA], glucose minimal medium [WO], and glycerol complete medium [N3]) and the genetic methods used for yeast were as described in Dujardin et al. (1980). The media used for E. coli (LB and 2TY complete medium and M9 minimal medium) were as described in Maniatis et al. (1982). The bank used

to clone the S. douglasii gene was made by cloning a partial BamH I digest of genomic DNA from S. douglasii CX1 in the centromeric vector YCBL1 which carries the marker *TRP1* for the selection of clones in yeast and *amp*^{r} and *tet*^{r} for selection in E. coli. This bank was constructed and kindly donated by E. Petrochilo.

Growth curves

Growth curves on minimal ethanol/glycerol medium were determined as described by Glab et al. (1990).

Transformation

Yeast (both S. cerevisiae and S. douglasii) were transformed by the LiCl procedure of Ito et al. (1983) using cells grown in liquid medium, or using a modified protocol for cells grown on solid medium. E. coli were transformed by either the $CaCl_2$, or the $CaCl_2/RbCl_2$ method, as described in Maniatis et al. (1982).

DNA manipulations

Routine DNA manipulations, plasmid preparations, and restriction enzyme digestions were performed according to Maniatis et al. (1982). DNA sequencing was performed using the chain termination method of Sanger et al. (1977) in conjunction with the M13 phage system of Messing et al. (1981). To generate overlapping clones for sequencing, the 3.8 kb Hind III fragment of YEpCSI007 was subcloned into pUC19 to give the plasmid pCSI075. This was used as a source of the 3.8 kb Hind III fragment which was sonicated and end-repaired; the subfragments were cloned into Sma I cut dephosphorylated M13mp19. In this way the sequence of the central region around the EcoR I site encoding the complementing gene was determined. Synthetic oligonucleotides were used to fill in the sequence on the second strand when necessary.

RNA manipulations

The purification of mitochondrial RNA, Northern blotting, and hybridization were performed as in Bousquet et al. (1990).

Results

Cloning of an S. douglasii gene

In order to clone an S. douglasii gene able to promote the excision of the S. douglasii intron aI1, we made use of the nucleo-mitochondrial incompatibility that exists between S. cerevisiae and S. douglasii (Kotylak et al., 1985). Cloned fragments of S. douglasii genomic DNA which promote the excision of the S. douglasii intron aI1 should allow the growth on glycerol of an S. cerevisiae strain that contains S. douglasii mitochondria. W303/SD, which has the nucleus of S. cerevisiae and S. douglasii mitochondria, was transformed by the bank of S. douglasii genomic DNA described in Materials and Methods. After an initial selection for the plasmid, ~20,000 colonies were replica-plated onto glycerol medium. After incubation 7 glycerol-positive clones were isolated. Growth on a nonselective medium, followed by streaking for single colonies, showed that in 6 out of the 7 clones the capacity to grow on glycerol co-segregated with the plasmid marker. Plasmid DNA was isolated from these clones after passage through E. coli; restriction endonuclease analysis showed that the plasmids from all 6 clones contained a common 8 kb BamH I fragment. When the plasmid with the smallest insert (YCpCSI007) was used to transform W303/SD, all transformants were able to grow on glycerol. These data indicate that we have cloned a functional gene from S. douglasii able to restore respiratory growth and to circumvent the nucleo-mitochondrial incompatibility.

Localization of the complementing gene within the cloned fragment

In order to locate the complementing gene within the cloned fragment we used a functional test similar to transposon mutagenesis, which is outlined in Figure 1. The plasmid YCpCSI007 was digested with Sau3A I to give approximately one cut or less per molecule. This digestion was then ligated to the S. cerevisiae *URA3* gene isolated from the plasmid pFL34 as a Bgl II fragment, and thus compatible with Sau3A I. This ligation mixture was used to transform the E. coli strain B15, which is a *PyrF*$^-$ mutant (uracil auxotroph) that can be complemented by the S. cerevisiae *URA3* gene. This allowed a direct selection of recombinant plasmids that had incorporated the *URA3* gene; 60 such plasmids were obtained. Digestion of these plasmids by BamH I showed whether the *URA3* gene was located in the insert or the vector; of the 60 plasmids, 24 had the *URA3* gene located in the insert. These 24 plasmids were used to trans-

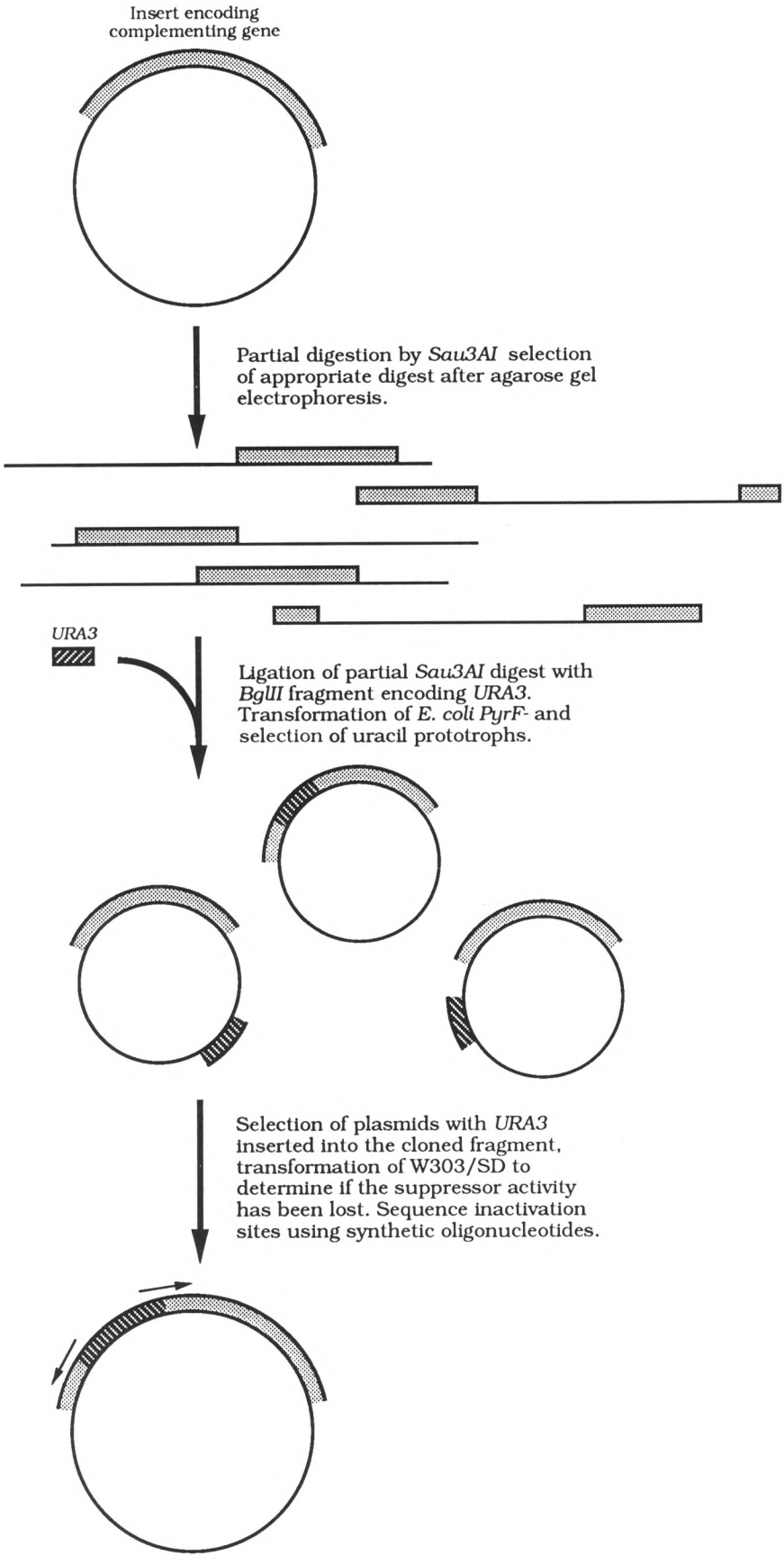

Figure 1. Schema showing the strategy used to locate the complementing gene within the cloned insert and to sequence the site of insertion.

form W303/SD, and the ability of the transformants to grow on glycerol was tested. In parallel with this, other restriction digestions were performed to locate the position of the *URA3* gene within the insert. Insertions of the fragment encoding the *URA3* gene which inactivate the original plasmid's ability to complement must be contained within the gene. Thus the combination of the growth on glycerol and the localization of the *URA3* gene define the region of the insert encoding the complementing gene. These results (summarized in Figure 2) indicate that the complementing gene is encoded in a 3.8 kb Hind III fragment, and that there is an EcoR I site within the gene.

Sequence of the cloned S. douglasii gene

Another advantage of the method used to locate the complementing gene within the cloned fragment (Fig. 1) is that it becomes simple to determine whether the cloned gene is already known. Using oligonucleotide primers complementary to the ends of the Bgl II fragment encoding the *URA3* gene (TGTTCGGAGATTACCGAATC and GGGAATCTCGGTCGTAATGA), it is possible to sequence from this fragment into the cloned genomic insert. When this is done on a plasmid in which the gene is inactivated by the insertion of the *URA3* encoding fragment, the sequence obtained should be within the gene; thus comparisons with yeast databases such as LISTA1 (Mosse et al., 1988) should show whether the gene has already been cloned and sequenced. This was done for the plasmids YEpCSI020 and YEpCSI054, in which the gene is inactivated by insertions on either side of the EcoR I site (see Fig. 2). Approximately 120 bp of sequence was obtained from each and, when compared with LISTA1, showed ~90% identity with the *MRS1* gene of S. cerevisiae (Kreike et al., 1987). The sequence of the central region of the 3.8 kb Hind III fragment was determined as described in Materials and Methods. An examination of the sequence showed the presence of an open reading frame of 1089 bp capable of encoding a protein of 363aa (MW 41.6 kDa; Fig. 3). This is the same size as the S. cerevisiae *MRS1* ORF; at the nucleic acid level the sequences show 952 identical bases out of 1089 (87%). At the protein level there are 86% identical residues (311aa), and 7% similar residues (25aa; Fig. 4). These results suggest that we have cloned the S. douglasii *MRS1* gene.

Inactivation of the *MRS1* gene of S. douglasii

To study further the function of the *MRS1* gene, we decided to inactivate the gene in S. douglasii using a one-step disruption procedure (Rothstein, 1983). For this we made use of the *URA3* insertion in the plasmid YCpCSI020, which inactivates the *MRS1* gene. YCpCSI020 was digested by Hind III, and the 4.9 kb fragment corre-

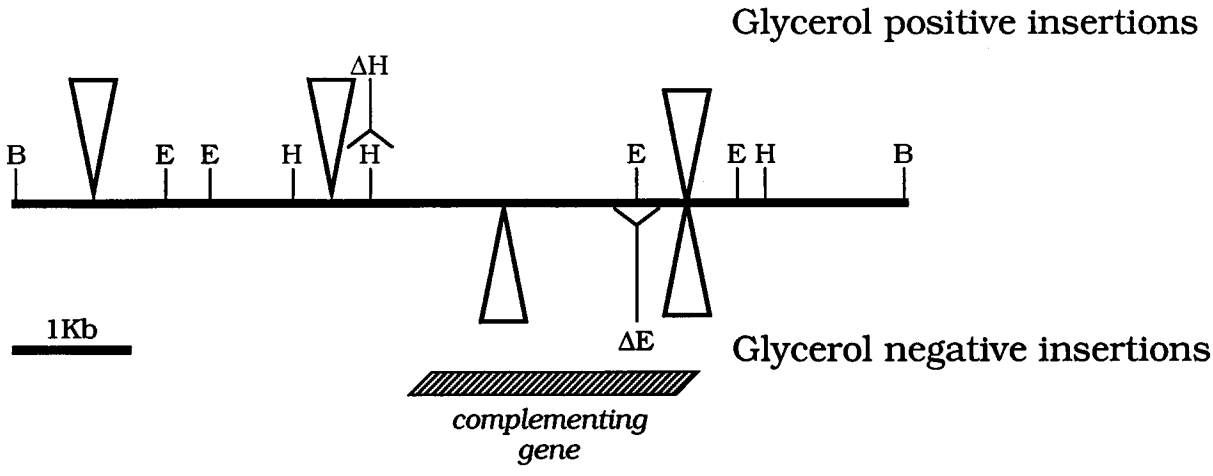

Figure 2. Partial restriction map of the cloned insert showing the positions of the *URA3* insertions (▽). Insertions have been located to the specific restriction fragments shown, but their positions within the fragments are not known. Insertions that inactivate complementation are marked below the line. The deduced position of the gene is shown by a hatched bar. H: Hind III; E: EcoR I; ΔH: Hind III site deleted; ΔE: EcoR I site deleted.

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   1 AGATCACGTGCATATTATTCTTGTCTCAACTGTAAGTCTTGATTAAGTCGAAGAAAAATG  60

  61 AAATATGCGATCCTAAATACCTAAAAGCCCAGCTATTTATGATAATCACGTTAGGGTCCC 120

 121 TCAAATGGATGTCCTTCAATACTTTTCGCTATTGGAAGATCGCATTCTTTTGGCGTTTTG 180

 181 TATCCTTATTATTCAAAACTTTGATCCGTTTCAACAATGCGGAAAAGGGAATTAAAAAAA 240

 241 GTGGTGGTGCAAAGTGAAAAATTATGAAAACTCGTTAAACGGCGAAGTTTAAGATGGTGC 300

                                                             M
 301 GGGTCAAGCAGCAATGGAAGTATAGGCTGGTATTGAAGAATCTGTGGTCATTTTGAAGAT 360

     S  P  K  N  L  T  R  S  V  V  P  A  I  D  L  Y  C  R  K  A
 361 GTCTCCGAAGAATTTAACAAGGTCTGTGGTCCCGGCTATCGACTTGTATTGCCGCAAGGC 420

     N  F  K  T  L  K  F  L  S  M  I  L  C  S  K  K  E  W  Y  D
 421 AAACTTTAAAACACTTAAATTTCTGTCGATGATCCTGTGCAGTAAAAAGGAGTGGTATGA 480

     N  T  K  A  P  V  R  N  F  L  V  S  R  C  A  V  F  E  Q  L
 481 TAATACAAAGGCGCCAGTGAGAAACTTTTTGGTCTCCCGGTGTGCTGTTTTCGAGCAGTT 540

     R  N  R  L  V  D  E  G  K  V  N  L  F  G  V  F  L  T  N  D
 541 ACGGAATCGTCTGGTTGACGAGGGCAAAGTCAACCTATTTGGCGTTTTCCTGACAAACGA 600

     S  F  S  F  C  K  M  T  V  D  D  K  F  D  T  S  L  V  D  W
 601 CTCATTTTCATTTTGCAAGATGACTGTTGATGATAAGTTCGACACTAGCTTGGTCGACTG 660

     Q  K  I  P  F  D  Y  S  F  A  T  E  R  R  Q  H  I  S  L  L
 661 GCAAAAGATACCCTTTGATTATTCATTCGCAACTGAGAGAAGACAGCATATTAGTTTGTT 720

     P  P  D  T  L  F  A  T  E  K  I  I  S  L  L  G  V  S  P  N
 721 ACCCCCTGATACACTTTTTGCAACTGAAAAGATCATATCACTACTTGGTGTATCTCCTAA 780

     M  A  N  L  V  S  I  E  R  Q  R  S  D  L  M  D  F  S  C  K
 781 TATGGCGAATCTTGTTTCCATAGAAAGACAGCGATCTGACCTGATGGATTTTAGTTGCAA 840

     L  Q  S  N  I  L  E  H  L  L  Y  A  K  C  Q  G  V  Q  V  T
 841 ACTGCAATCAAACATCCTAGAGCACCTGTTATACGCGAAGTGCCAAGGAGTGCAAGTAAC 900

     S  T  N  E  E  A  R  L  L  A  A  I  C  N  P  E  F  I  D  A
 901 TTCTACTAATGAAGAAGCTCGCTTGCTTGCAGCCATCTGCAATCCTGAATTCATTGACGC 960

     F  W  C  E  L  T  P  I  R  A  S  L  K  E  N  P  S  I  S  V
 961 CTTCTGGTGCGAGTTGACTCCCATAAGGGCCTCATTAAAGGAAAATCCTTCCATCTCCGT 1020

     P  Q  E  Y  Q  I  Y  D  P  V  I  R  A  T  I  K  E  V  V  A
1021 ACCTCAAGAATACCAGATATACGATCCGGTGATACGTGCCACCATAAAAGAGGTTGTCGC 1080

     K  R  L  L  R  S  A  F  D  N  D  I  D  P  L  M  R  L  R  L
1081 TAAGCGATTACTGCGATCCGCCTTCGATAATGACATTGACCCGCTAATGCGTCTGCGTTT 1140

     D  K  G  W  K  F  K  F  P  T  L  S  S  T  T  D  L  D  F  S
1141 GGATAAAGGCTGGAAGTTCAAATTTCCCACGCTCTCCTCGACAACCGACCTAGATTTCTC 1200

     L  K  D  C  L  S  L  D  T  R  R  D  A  Y  D  M  T  E  V  F
1201 CCTGAAGGATTGTCTTTCCCTGGATACACGAAGAGATGCATATGACATGACTGAGGTGTT 1260

     L  A  T  M  A  S  S  K  T  L  R  T  Y  S  N  L  V  D  I  V
1261 TCTTGCTACTATGGCATCGAGTAAAACTCTTCGTACGTATAGCAACCTCGTTGACATTGT 1320

     M  K  D  N  G  R  F  D  S  G  I  L  K  Q  F  N  D  Y  V  K
1321 GATGAAAGACAACGGTAGATTTGATTCAGGCATTCTAAAGCAATTCAACGACTACGTCAA 1380

     Q  E  K  L  N  L  Q  N  F  Q  A  G  S  S  E  F  L  K  G  V
1381 GCAAGAAAAGCTCAATCTACAAAATTTCCAGGCCGGTTCCTCAGAGTTTCTGAAAGGTGT 1440

     K  I
1441 AAAGATATAATCCTCTTATATAACATGTAAATAGAGAGTCCGTG 1484
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Figure 3. Nucleotide sequence of the S. douglasii *MRS1* gene and the deduced protein sequence. The Sau3A I sites where the fragment encoding the *URA3* gene is inserted in the plasmids YCpCSI020 (751) and YCpCSI054 (1095) are underlined.

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  1 MSPKNLTRSVVPAIDLYCRKANFKTLKFLSMILCSKKEWYDNTKAPVRNF  50
    ||||| |||| ||||||||||||||| ||||| |||||||  ||| | |
  1 MSPKNITRSVIPAIDLYCRKANFKTLKSLSMILGSKKEWYDTKKAPLRTF  50

 51 LVSRCAVFEQLRNRLVDEGKVNLFGVFLTNDSFSFCKMTVDDKFDTSLVD 100
    |||||  ||||| |||  |||||| |||||||||||||||||| |||||
 51 LVSRCGIFEQLRGRLVEDGKVNLFSVFLTNDSFSFCKMTVDDKFNTSLVD 100

101 WQKIPFDYSFATERRQHISLLPPDTLFATEKIISLLGVSPNMANLVSIER 150
    |||||||  ||| ||| ||||| |||||||||| ||||||| |||||||
101 WQKIPFDSTFATDRRQNISLLPVDTLFATEKIISILGVSPNMTNLVSIER 150

151 QRSDLMDFSCKLQSNILEHLLYAKCQGVQVTSTNEEARLLAAICNPEFID 200
     |||| || |||||||||||||||||| || ||| |||||| |||||||
151 ERSDLVDFNCKLQSNILEHLLYAKCQGVYVTPTNEKARLLAAVCNPEFID 200

201 AFWCELTPIRASLKENPSISVPQEYQIYDPVIRATIKEVVAKRLLRSAFD 250
     ||||||||| |||||||||| ||| |||| |||||||| |||||||||
201 TFWCELTPIRVSLKENPSISVPREYQMYDPVVRATIKEVVTKRLLRSAFD 250

251 NDIDPLMRLRLDKGWKFKFPTLSSTTDLDFSLKDCLSLDTRRDAYDMTEV 300
    ||||||| | |||||| ||| ||||| | |||||||||| || |||||
251 NDIDPLMCLHLDKGWKLKFPILSSTTGLNFSLKDCLSLDTGKDASDMTEV 300

301 FLATMASSKTLRTYSNLVDIVMKDNGRFDSGILKQFNDYVKQEKLNLQNF 350
    ||||| ||| ||||||| ||||||||| ||| |||||||||||||| |
301 FLATMESSKVLRTYSNLADIVMKDNGRLDSGVLKQFNDYVKQEKLNLQHF 350

351 QAGSSEFLKGVKI 363
    ||||| |||| ||
351 QAGSSKFLKGAKI 363
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Figure 4. Comparison of the S. douglasii (above) and S. cerevisiae (below) MRS1 protein sequences. The two sequences show 86% identical residues and 7% similar residues. Identical residues are shown with a vertical bar.

sponding to the S. cerevisiae *URA3* gene inserted in the S. douglasii *MRS1* gene was purified (see Fig. 2). This was used to transform the S. douglasii strain 4707-22D. After an initial selection for the *URA3* marker, 20 transformants were tested for their capacity to grow on glycerol; none of these transformants were able to do so. However, when they were crossed to a *rho*° tester strain (S. douglasii 4795-3B/50), the resulting diploids were all able to grow on glycerol, indicating that the inability of the original transformants to do so was not due to a defect in their mitochondrial DNA. Several transformants were further analyzed by Southern blot; in all cases the integration had occurred as expected. One of these transformants, C0701, was used in the subsequent experiments.

Does the S. douglasii *MRS1* gene have a function other than mitochondrial pre-mRNA splicing?

Recently it has become apparent that some genes involved in mitochondrial pre-mRNA splicing are also involved in other mitochondrial functions, such as translation (for a review see Grivell, 1989). Séraphin et al. (1987) have developed a method for determining whether a gene is involved only in splicing based on an intronless mitochondrial genome. If a gene is involved only in mitochondrial pre-mRNA splicing, it becomes dispensable when there are no mitochondrial introns. Thus if we compare the growth on glycerol of isogenic S. douglasii strains *MRS1* and *mrs1::URA3* with an intronless mitochondrial genome, we should be able to determine whether the S. douglasii *MRS1* gene is involved in functions other than mitochondrial pre-mRNA splicing. Using the S. cerevisiae strain JC25/Δ as a donor, the intronless mitochondrial genome of Séraphin et al. (1987) was introduced by cytoduction (Conde and Fink, 1976) into the S. douglasii strains 4707-22D/50 and C0701/50, both of which are *rho*°. Figure 5 shows the growth on glycerol of the resulting cytoductants 4707-22D/Δ and C0701/Δ; no difference can be de-

Figure 5. Growth on glycerol after 3 days incubation at 28°C of S. douglasii strains with a wild-type (*MRS1*) or inactivated (*mrs1::URA3*) *MRS1* gene, in the presence of the S. douglasii wild-type intron plus mitochondria (4707-22D and C0701) or an intronless mitochondrial genome (4707-22D/Δ and C0701/Δ). In the presence of the intronless mitochondrial genome the *mrs1::URA3* strain shows a wild-type growth on glycerol.

tected. Thus we may conclude that the *MRS1* gene of S. douglasii is involved only in mitochondrial pre-mRNA splicing.

The *MRS1* genes of S. douglasii and S. cerevisiae are functionally homologous

The sequence of the S. douglasii *MRS1* gene revealed that it is 87% identical at the nucleotide level to the S. cerevisiae *MRS1* gene. The *MRS1* gene was identified by Kreike et al. (1986) and shown to be involved in the excision of bI3. Recently Bousquet et al. (1990) have isolated the same gene and showed that in S. cerevisiae it is also needed for the splicing of the sixth intron of the *coxI* gene (aI5β), and that it is dispensable for glycerol growth when bI3 and aI5β are deleted from the mitochondrial genome. Tian et al. (1991a,b) have shown that although the mitochondrial genome of S. douglasii differs significantly from that of S. cerevisiae, the intron bI3 is almost identical, and aI5β is quite similar to their S. cerevisiae counterparts. Thus it is possible that the S. douglasii MRS1 protein may also function in the excision of these introns. To investigate further the possible functional similarity of the two MRS1 proteins, we decided to determine whether the S. douglasii *MRS1* gene can replace the S. cerevisiae *MRS1* gene. Again we made use of the plasmid YCpCSI020, in which the S. douglasii *MRS1* gene is inactivated by the insertion of a fragment carrying *URA3*. As the nucleotide sequences of the two *MRS1* genes are 87% identical, we decided to use a fragment of the S. douglasii *MRS1* gene to inactivate the S. cerevisiae gene in a one-step gene replacement experiment (Rothstein, 1983). In order to avoid artifacts due to changes in the adjacent genes, we used a Dra I-SnaB I fragment which contains the disruption and is internal to the *MRS1* ORF. This 2.06 kb Dra I-SnaB I was purified and used to transform the S. cerevisiae strain CW04; as expected, uracil-positive transformants were glycerol-negative. After a cross with a *rho*° tester strain to ensure that the transformants had retained their mitochondria, one transformant (CW75) was selected and transformed by the plasmids YCpCSI007 (*MRS1* S. douglasii), YEpIB001 (*MRS1* S. cerevisiae), or the vector controls YCBL1 and YEp13. The growth on glycerol of these strains is shown in Figure 6, and it is clear that both the S. douglasii and the S. cerevisiae *MRS1* genes are able to complement a disruption of the S. cerevisiae *MRS1* gene. Taken in conjunction with the other results this demonstrates that the S. douglasii and the S. cerevisiae *MRS1* genes are functional homologues.

The *MRS1* gene of S. cerevisiae when present on a multicopy plasmid, but not a single chromosomal copy, can stimulate some excision of the S. douglasii intron aI1

To complete our study of the *MRS1* genes of S. douglasii and S. cerevisiae, we decided to determine if the S. cerevisiae *MRS1* gene could

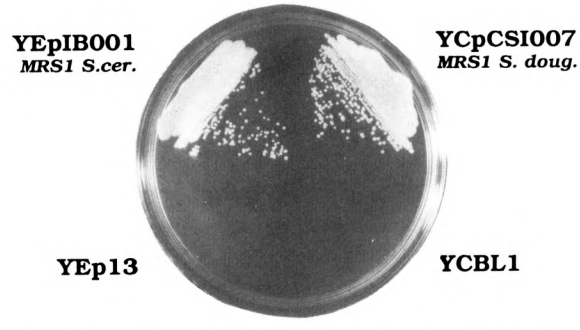

Figure 6. *MRS1* genes from both strains are iso-functional with S. cerevisiae mitochondria. Growth on glycerol after 3 days incubation at 28°C of a strain containing the S. cerevisiae nuclear and mitochondrial genomes, with an inactivated *MRS1* gene (*mrs1::URA3*) transformed by the S. cerevisiae *MRS1* gene on a multicopy plasmid (YEpIB001), the S. douglasii *MRS1* gene on a centromeric plasmid (YCpCSI007), and the vector controls. Both genes are able to efficiently complement the chromosomal disruption of the S. cerevisiae *MRS1* gene.

stimulate the excision of the S. douglasii intron aI1 when present on a multicopy plasmid. W303/SD was transformed with the multicopy plasmids YEp13 and YEpIB001 (the S. cerevisiae *MRS1* gene cloned in YEp13; Bousquet et al., 1990) and the centromeric plasmids YCBL1 and YCpCSI007 (the S. douglasii *MRS1* gene cloned in YCBL1). Transformants were plated on solid glycerol medium, and growth curves in minimal ethanol/glycerol medium were determined (Fig. 7). The vector controls showed no growth, cells transformed with YCpCSI007 grew well, and cells transformed with YEpIB001 grew significantly but not as well as those transformed by YCpCSI007 (it is important to note that under these conditions, the plasmid YEpIB001 is stably maintained in the cells). To confirm this, mitochondrial RNA was purified from these transformants and hybridized with probes for the *coxI* exons and the S. douglasii intron aI1. The results of these experiments are shown in Figure 7, and demonstrate clearly that when present on a multicopy plasmid, the *MRS1* gene of S. cerevisiae can stimulate the excision of the S. douglasii intron aI1 and restore growth on glycerol, while a single chromosomal copy present in its physiological environment results in no detectable excision of the intron. However, the growth on glycerol due to the S. cerevisiae *MRS1* gene cloned on a multicopy plasmid is less vigorous, and the pre-mRNA splicing less efficient than that observed with the *MRS1*

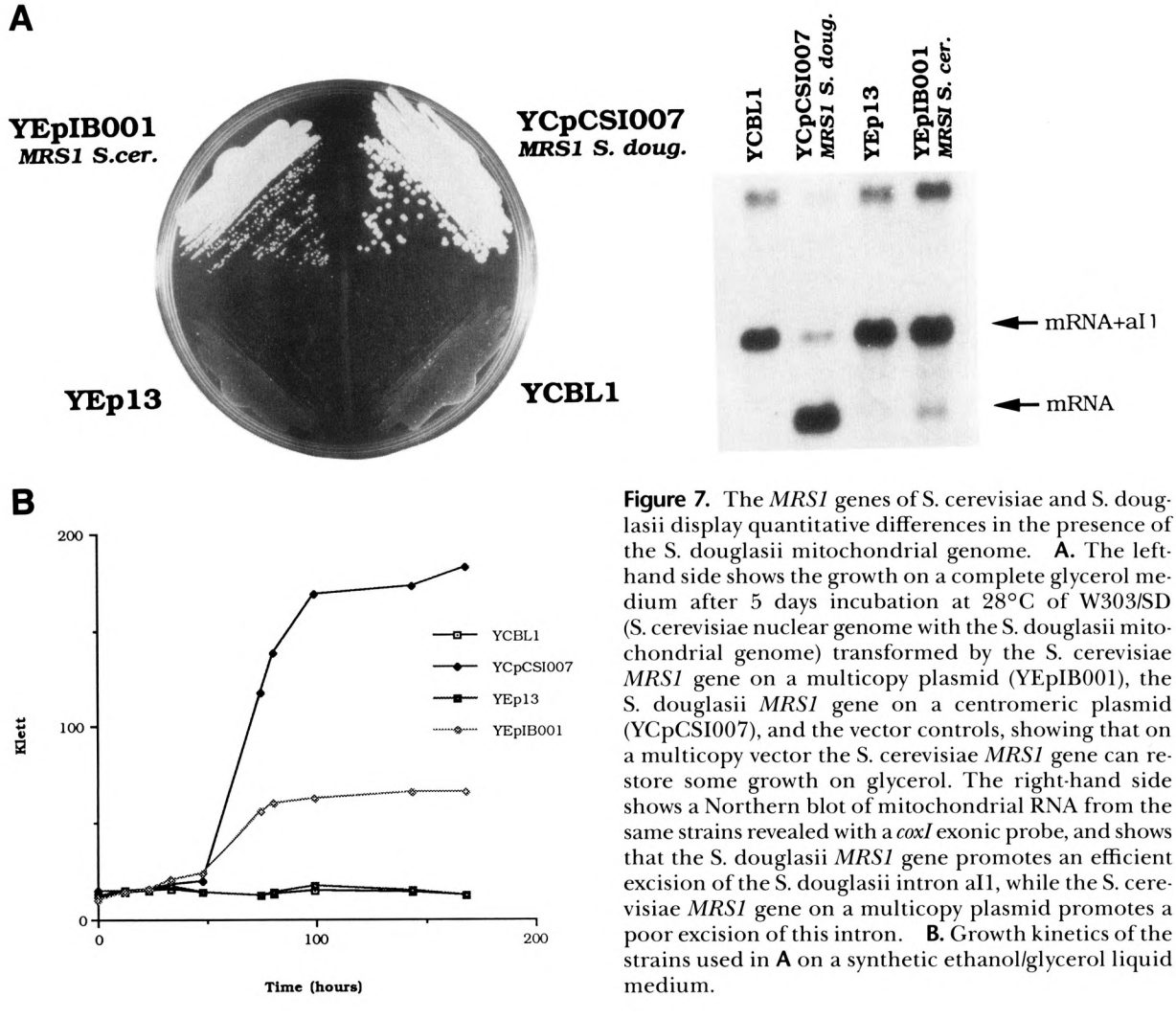

Figure 7. The *MRS1* genes of S. cerevisiae and S. douglasii display quantitative differences in the presence of the S. douglasii mitochondrial genome. **A.** The left-hand side shows the growth on a complete glycerol medium after 5 days incubation at 28°C of W303/SD (S. cerevisiae nuclear genome with the S. douglasii mitochondrial genome) transformed by the S. cerevisiae *MRS1* gene on a multicopy plasmid (YEpIB001), the S. douglasii *MRS1* gene on a centromeric plasmid (YCpCSI007), and the vector controls, showing that on a multicopy vector the S. cerevisiae *MRS1* gene can restore some growth on glycerol. The right-hand side shows a Northern blot of mitochondrial RNA from the same strains revealed with a *coxI* exonic probe, and shows that the S. douglasii *MRS1* gene promotes an efficient excision of the S. douglasii intron aI1, while the S. cerevisiae *MRS1* gene on a multicopy plasmid promotes a poor excision of this intron. **B.** Growth kinetics of the strains used in **A** on a synthetic ethanol/glycerol liquid medium.

gene from S. douglasii cloned on a centromeric plasmid.

Discussion

In this study we have used a novel inter-species complementation system to clone a gene from S. douglasii that is involved in mitochondrial RNA processing. In doing this we have developed a simple and rapid method for mapping functional genes within cloned inserts, obtaining partial sequence information, and producing chromosomal disruptions of the genes. In some ways this is similar to the linker insertion strategy developed by Legrain et al. (1991). However, the use of the *URA3* cassette for the mutagenesis instead of linkers has two significant advantages: (1) complementation of the *PyrF*$^-$ mutation of E. coli allows a positive selection of plasmids with an insertion, and (2) the constructions used to map the gene within the insert can be used to disrupt the chromosomal copy of the gene without further subcloning steps. The same strategy can be employed using the yeast *LEU2* gene to complement a *LeuB*$^-$ mutation of E. coli when selecting insertions (A. Harington, unpublished results).

The S. cerevisiae nuclear gene *MRS1* was originally isolated by Kreike et al. (1986) and shown to be involved in the excision of the intron bI3 (*cyt b* gene). More recently Bousquet et al. (1990) have shown that *MRS1* is also involved in the excision of aI5β (*coxI* gene), and that it is dispensable when these introns are deleted from the mitochondrial genome. In the present study we show that the *MRS1* gene of S. douglasii is essential for the splicing of the S. douglasii intron aI1 (*coxI* gene) and that this function cannot be replaced by a single chromosomal copy of the homologous gene from S. cerevisiae. When the S. cerevisiae *MRS1* gene is cloned on a multicopy plasmid, some splicing of the S. douglasii intron aI1 does take place, allowing limited growth on respiratory substrates (Fig. 7). Thus the *MRS1* gene of S. douglasii is more efficient than the S. cerevisiae gene at splicing the S. douglasii intron aI1.

This raises the question of whether the difference between the two *MRS1* genes is quantitative or qualitative. At present no definitive answer can be given to this question. A single copy of the S. cerevisiae *MRS1* gene is sufficient for the splicing of the introns bI3 and aI5β; it is only the splicing of the S. douglasii intron aI1 which is deficient (Kotylak et al., 1985). Cloned on a multicopy plasmid, the S. cerevisiae *MRS1* gene allows an inefficient splicing of the S. douglasii intron aI1 (~15% splicing; see Fig. 7); we feel it is improbable that under normal circumstances much larger amounts of the S. douglasii MRS1 protein are needed for the splicing of aI1 than for bI3 and aI5β. Thus we suggest that the difference between the two MRS1 proteins is qualitative. A precedent exists for this in the products of the *NAM2* genes of S. douglasii and S. cerevisiae. The wild-type S. douglasii *NAM2* gene has the suppressor activity of the mutant S. cerevisiae *NAM2* suppressor alleles. A Northern analysis showed that there was no significant difference in the level of expression of the two genes, and a fragment exchange experiment was able to identify part of the S. douglasii gene responsible for the difference (Herbert et al., 1988a,b).

We have shown that in a purely S. cerevisiae nuclear and mitochondrial background the *MRS1* genes of S. cerevisiae and S. douglasii are functionally equivalent (see Fig. 6). As the S. cerevisiae gene is essential for the excision of bI3 and aI5β, we can deduce that the S. douglasii gene is also competent in the excision of these introns. Interestingly, these introns are conserved both in position and sequence in the mitochondrial genome of S. douglasii (Tian et al., 1991a,b); thus it is reasonable to conclude that in S. douglasii the *MRS1* gene is needed for the splicing of three introns, bI3, aI1, and aI5β. This enlarged spectrum of the S. douglasii *MRS1* gene is consistent with the observation that in hybrid strains with an S. cerevisiae nuclear genome and an S. douglasii mitochondrial genome the cytochrome b transcript is fully processed, while a *coxI* transcript containing aI1 accumulates (Kotylak et al., 1985, and Fig. 7).

The precise function of the protein components involved in mitochondrial pre-mRNA splicing is unknown, but as the same basic mechanism of excision probably holds for all introns, and as some mitochondrial introns self-splice in vitro, it is reasonable to assume that the proteins involved in vivo have no direct catalytic role. Thus the protein components would be involved in obtaining the correct structure of the intron, to allow the RNA-catalyzed reactions to occur. We have shown that there is a functional difference in the *MRS1* genes of S. cere-

visiae and S. douglasii in respect to the splicing of the S. douglasii intron aI1. The simplest explanation of this is that the S. douglasii MRS1 protein has a higher affinity for the aI1 RNA than the S. cerevisiae protein. This is also consistent with the low-level excision of the S. douglasii intron aI1 seen when the S. cerevisiae protein is overexpressed. It might be expected that these differences would be reflected in the proteins. An examination of the deduced S. cerevisiae and S. douglasii MRS1 protein sequences shows 14% differences; however, there is no significant clustering of amino acid changes, which could delimit a particular domain of the protein which varies between S. cerevisiae and S. douglasii and thus explain the difference in specificity.

In S. cerevisiae a second ORF of 244 codons, present on the opposite strand, overlaps the *MRS1* ORF. Kreike et al. (1987) were able to show that this ORF was not responsible for the *MRS1* complementing activity, but were not able to determine if the ORF corresponded to a real gene and was expressed. In S. douglasii this second ORF is not maintained: in the region that overlaps the *MRS1* ORF there is a stop codon, and in the region outside the *MRS1* ORF there are two frameshift mutations. This shows that the second ORF is not functional in S. douglasii and by analogy probably does not correspond to a gene in S. cerevisiae either.

A comparison of the *NAM2* gene sequences of S. cerevisiae and S. douglasii leads to the conclusion that the two species diverged 50–80 million years ago (Herbert et al., 1988b). Table 1 shows a compilation of data from the three ORFs for which the comparison between S. cerevisiae and S. douglasii can be made. An examination of these data shows that there is remarkable uniformity for all parameters studied. Transitions are 3 times more frequent than transversions (a X^2 homogeneity test gives 2.7 for 2 degrees of freedom). Silent mutations are more frequent than replacement ones: they attain for all three genes a 50% value of all possible silent mutations, while the replacement mutations remain very low (4–7% of the possible sites). All these data strongly argue for a recent divergence of the two species. This uniformity contrasts markedly with the results of Tian et al. (1991b), who have shown that in the mitochondrial cytochrome b gene there is wide variation in the rate of fixation of mutations between the exons and the introns, and even between different introns. The exon sequences show less than 2% nucleotide substitutions, with transitions and transvertions being almost equal. Thus in the divergence of S. cerevisiae and S. douglasii the nuclear genes appear to accumulate mutations considerably faster than mitochondrial genes. Interestingly, this is the reverse of the situation in the vertebrate system, in which mitochondrial genes accumulate 5- to 10-fold more nucleotide substitutions than nuclear genes, with transitions being predominant (Brown et al., 1982).

Table 1. Comparison of evolutionary divergence for three homologous genes in S. cerevisiae and S. douglasii.

The *MRS1* data are from Figure 3 of this study for S. douglasii, and Kreike et al. (1987) for S. cerevisiae. The *NAM2* and *DY* data are from Herbert et al. (1988b) for S. douglasii, and Labouesse et al. (1987) for S. cerevisiae. The correction for the saturation of possible mutations have been calculated by the method of Perler et al. (1980) using the UWGCG diverge program (Devereux et al., 1984).

	Gene		
	MRS1	NAM2	DY
Codons	363	894	177
Base transitions	98	219	47
Base transversions	39	60	17
Actual mutations			
Silent	79	195	40
Replacement	58	84	24
Corrected saturation of possible mutations			
Silent	49%	50%	55%
Replacement	7%	4%	6%
Amino acid identity	86%	91%	89%

In conclusion, we have cloned the S. douglasii *MRS1* gene, shown that it is needed for the excision of the S. douglasii introns bI3, aI1, and aI5β, and that it is dispensable for respiratory growth in the presence of an intronless mitochondrial genome. Even though the two yeasts S. cerevisiae and S. douglasii are closely related, they display significant differences. In particular, since they diverged there has been either a concomitant gain of the intron aI1 and of function for the *MRS1* gene in S. douglasii, or a loss of both intron and function in S. cerevisiae. Thus the *MRS1* genes of S. cerevisiae and S. douglasii and their role in mitochondrial pre-mRNA splicing present an interesting example of the coevolution of nuclear and mitochondrial genomes.

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