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MSS18, a yeast nuclear gene involved in the splicing of intron aI5β of the mitochondrial *cox1* transcript

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We have isolated and characterized a nuclear gene, *MSS18*, which is implicated in the splicing of intron aI5β of the mitochondrial *cox1* transcript (subunit 1 of the cytochrome *c* oxidase). Northern blotting and S1 nuclease protection experiments as well as the analysis of mitochondrial point revertants suggest that *mss18* mutations block (perhaps indirectly) the cleavage of the 5′ exon–intron junction of aI5β. Mitochondrial point revertants also indicate that up to 13 bases of the aI5β exon sequence, upstream of the 5′ splice site of aI5β, are involved *in vivo* in the splicing of this intron. The implications of this result on the splicing of group I introns are discussed.

Key words: Saccharomyces cerevisiae/group I intron/gene disruption/revertants/intron deletion

Introduction

In *Saccharomyces cerevisiae* the mitochondrial genes encoding 21S rRNA, cytochrome *b* (*cob*) and subunit 1 of cytochrome *c* oxidase (*cox1*) are split by intronic sequences (Grivell, 1984) which belong either to group I or group II introns (Michel and Dujon, 1983; Waring and Davies, 1984). Although several of them are endowed with self-splicing capacity (Cech and Bass, 1986; Van der Horst and Tabak, 1985; Schmelzer and Schweyen, 1986; van der Veen *et al.*, 1986; Peebles *et al.*, 1986; Tabak *et al.*, 1987), numerous proteins coded either by the mitochondrial or nuclear genome participate to help or conduct mitochondrial splicing reactions (Lazowska *et al.*, 1980; Faye and Simon, 1983b; Hill *et al.*, 1985; Kreike *et al.*, 1986; Schmelzer and Schweyen, 1986; Labouesse *et al.*, 1985). These proteins are supposed to stabilize RNA structures which are implicated in cleavage or ligation reactions, though such protein–RNA interactions remain to be proven.

We previously isolated a pet$^-$ mutation, *mss18*, which affects the maturation of the *cox1* transcript (Faye and Simon, 1983b). In the present work we demonstrate that protein MSS18 is necessary for the efficient excision of intron aI5β of *cox1*. Mitochondrial point revertant analyses further suggest that exon sequences are implicated in mitochondrial splicing.

Results

We have previously isolated from strain FF1210-6C a pet$^-$ mutant, N295 (Bolotin-Fukuhara *et al.*, 1977), whose cytochrome absorption spectrum revealed the absence of cytochrome aa_3 but a normal level of cytochrome *b* (unpublished result). Mutant N295 grows slowly on glycerol medium. Northern blots of the mitochondrial RNA (mt RNA) from strain N295, probed with a fragment of *cox1* exon4, showed the accumulation of an ~4.0 kb long RNA intermediate, suggesting that the splicing of some *cox1* intron(s) was impaired (Faye and Simon, 1983b). We named the corresponding gene *MSS18*.

Cloning and sequencing of the MSS18 gene

Gene *MSS18* was cloned by complementation in yeast as described in Materials and methods (cf. Figure 1). The sequence complementing the mutation *mss18-4* is contained in the 1.5 kb *Sac*I–*Hin*dIII fragment shown in Figure 2B. A long open reading frame is observed which should correspond to the *MSS18* gene; it may encode a protein of 268 amino acids with a calculated mol. wt of 30 961 daltons. This putative protein contains a slight excess of acidic over basic amino acids (38 Asp and Glu versus 31 Lys and Arg). A codon bias index of −0.03 indicates that *MSS18* is poorly expressed (Bennetzen and Hall, 1982). On the other hand, no protein sequence significantly homologous with *MSS18* was found in Genbank (Rel42, May 86). To intervene in mitochondrial splicing, the *MSS18* protein must be imported into mitochondria. Cytoplasmically synthesized mitochondrial proteins usually have an N-terminal presequence rich in basic amino acids and in serine and threonine residues. Since such a presequence is absent from protein *MSS18* we cannot yet decide whether this gene plays a direct role in the process of *cox1* pre-mRNA, or whether it is necessary for the expression of another gene which more directly performs this function.

Characterization of the MSS18-transcript

Poly(A)$^+$ mtRNA of strain AB320 was purified (Faye and Simon, 1983a), subjected to electrophoresis on a 1.1% agarose gel, then transferred to a nitrocellulose filter. When probed with the *Pst*I–*Hin*dIII fragment (probe C) ^{32}P-labelled by nick-translation, a 1.0 kb-long RNA was detected (Figure 3, lane C). With the single-stranded probe J-*Hin*dIII (probe B) labelled according to Hu and Messing (1982) (cf. Figure 3, lane B), two transcripts, 0.4 and 1.0 kb long respectively, were observed, whereas no RNA was revealed with the single-stranded probe *Eco*RI-J″ (probe E, Figure 3, lane E). These results suggest that, owing to its location and its size and direction of transcription, the 1.0 kb transcript corresponds to gene *MSS18*.

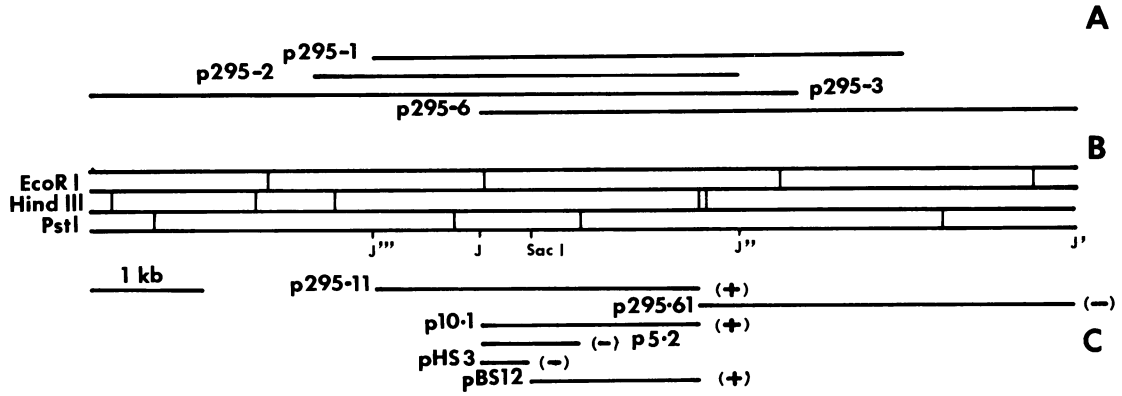


Fig. 1. Localization of the gene *MSS18* on the cloned DNA segments. (**A**) Extent of DNA fragments borne by recombinant plasmids complementing the mutation *mss18-4* (strain BS7-133-5). (**B**) *Eco*RI, *Hin*dIII and *Pst*I restriction maps. Only one of several *Sac*I sites is represented. J''', J, J'' and J' indicate ends of cloned DNA fragments. (**C**) Cloned DNA fragments borne by plasmids p295-1 and p295-6 were subcloned. Subclones able to restore respiration of strain BS7-133-5 are marked with (+), the others are marked with (−).

Disruption of the MSS18 gene

We have disrupted the *MSS18* gene of strain GRF18 by inserting the yeast *LEU2* gene in place of its *Hpa*I−*Hpa*I segment (which caused an almost complete deletion of the *MSS18* gene; cf. Figure 2B). This gene disruption was confirmed by genomic blotting (data not shown). The mutant strain obtained, named BS106-3 (and the corresponding mutation *mss18-3*), was still able to grow slowly, like N295, in glycerol medium. This indicates that the process in which the *MSS18* gene product is involved is not completely blocked when this gene is destroyed. Low stringency Southern blottings suggest that this leakiness cannot be accounted for by the presence of a closely related gene (data not shown).

Strains GRF18 and BS106-3 were made rho^0 by ethidium bromide treatments (Simon and Faye, 1984), after which mitochondria, either of strain D273-10B/A, 777-3A, or GF167-7B (the latter is devoid of mitochondrial introns; Séraphin *et al.*, 1987), were introduced by cytoduction (Conde and Fink, 1976; Lancashire and Mattoon, 1979). Cytoductants carrying the nucleus of GRF18 were named respectively BS104-1, BS104-8 and BS104-10, and those harboring the nucleus of BS106-3 were called respectively BS108-1, BS108-8 and BS108-10. These strains are therefore all isonuclear (except for the *MSS18* and *LEU2* genes). Compared with the former strains (those in which the *MSS18* gene is intact) the latter strains grew slowly on glycerol or lactate plates, except BS108-8 which grew even more slowly on glycerol and did not grow at all on lactate plates. The generation times of some of these strains grown in glucose or lactate medium are presented in Table I. The data indicate that a strain with the *mss18-3* null allele is unable to use lactate as a carbon source when it contains the 777-3A mitochondrial genome. It is known that, when compared with that of strain D273-10B/A, the mitochondrial genome of strain 777-3A contains five supplementary introns: aI5α, aI5β, bI1, bI2 and bI3 (Grivell, 1984). Furthermore, observation of the cytochrome absorption spectrum of strain BS108-8 indicates that cytochrome *b* synthesis is almost normal whereas the amount of cytochrome aa_3 is clearly reduced (data not shown). Thus, this suggests that *MSS18* should play a role in the splicing of intron aI5α or aI5β. However, since the growth rate of strain BS108-10 (which is devoid of mitochondrial introns) is reduced on lactate medium, we have to suppose that *MSS18* is also implicated in a process other than splicing (unless an as yet undiscovered intron is present in GF167-7B mitochondria).

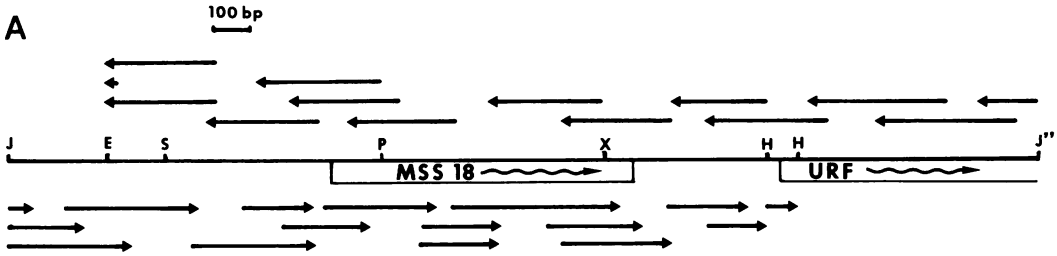
The availability of the *mss18-3* null allele allowed us to show that the gene we have cloned is actually the wild-type allele of the *N295* pet^- mutation. First, a strain carrying the *mss18-3* null allele has a leaky phenotype on glycerol medium like that of the original *N295* pet^- mutant. Second, when a strain carrying the *mss18-3* null allele is crossed with strain N295, the respiratory competency is not restored. Third, the analysis of 25 tetrads obtained from the cross FF314 × BS106-3/2 shows that Leu^+ phenotype cosegregates with the lactate minus phenotype.

Extragenic revertants of the mss18-3 null allele present in strain BS108-8

Numerous spontaneous or $MnCl_2$-induced revertants of strain BS108-8 were recovered from lactate plates (cf. Materials and methods). Genetic tests on 60 revertants showed that 52 were mitochondrial whereas the remaining eight were nuclear. Two of these nuclear revertants were dominant, two semi-dominant and four recessive. Total DNA of mitochondrial revertants was extracted using a rapid procedure (Davis *et al.*, 1980), digested with the restriction enzymes *Eco*RI and *Bam*HI, then submitted to electrophoresis on an agarose gel. The positions of stained mitochondrial bands were compared to those of the parental strain. The band pattern of ~90% of the mitochondrial revertants was identical to that of the parental strain. The other mitochondrial revertants suffered either a 4.0, 2.3 or 1.6 kb deletion in one of the mitochondrial bands. These mtDNA alterations were further analyzed by restriction mapping and Southern blotting (data not shown). These experiments revealed that all three introns aI5α, aI5β, aI5γ or both aI5β and aI5γ or aI5β alone had been deleted from gene *cox1*. Sequencing across the borders of the deletion of one of the revertants which had lost both aI5β and aI5γ (BS108-8/R1) established that a precise deletion of both introns occurred (Figure 4A). This fact excludes the existence of the mini-exon

1882	Thr	Tyr	Tyr
	ACT	TAT	TAT

inside intron aI5β as suggested by Hensgens *et al.* (1983a) (unless such an exon is not required for *cox1* function). These



B

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                                                                                                                    120
GAGCTCATCAAGAACAAACTGAGAAAACCTTGAAACAGGCCGCTTATGTGGCTGCGTTTCTTTGGGTTTCCCCAATGATCTGGCATTTGGTGAAAAAGCAATGGAAATAATTCCCCACTG
SacI                                                                                                                240
AAGAAAAAAGATTGCATCTATGAATGACACTAAAATATGTTTCTCTTTTTGTATTTGCTTTTGATTAAATAATGGTCAATTTGCGCCCCCCACCTTATATAGTTTAGAGAAAGACGTGTA
                                                                                                                    360
AATATTTTTTAAAAAATTGATACTGCTCTAATGAGCCTAAATCTCCTTTCTTCGCTTGTATATATGCGTATTCTGTATGTTTGTCCAGGCAGATTTTTCGGCTTTTTTTACCAGCTTTTA
                                                                                                                    480
                                                                                                  M  G  L  P  E  V  N  F  L  R
TTATTAAAATGTGATAACCTTAATTGAAGGTTGCGATACAAAATAATCCTTTATTACGCATAACCAAGGAAAAGAGAAAATCAGTGCACATATGGGTTTACCTGAAGTTAACTTTCTTCG
                                                                                                        HpaI        600
 K  N  C  I  L  V  E  L  K  L  F  Y  Q  T  V  Y  P  P  K  E  L  Y  W  N  N  R  I  T  A  E  L  S  T  F  S  N  I  K  Y  A
AAAAAACTGCATACTTGTGGAGTTAAAATTATTCTATCAAACAGTATACCCACCGAAGGAACTATATTGGAATAACCGAATAACTGCAGAATTGAGTACATTCTCCAATATTAAGTATGC
                                                                                      PstI                          720
 R  P  T  F  A  V  N  N  G  T  F  Q  R  T  R  P  K  L  D  L  V  L  A  S  S  D  I  R  K  L  A  T  V  L  F  N  L  K  A  L
AAGGCCGACTTTCGCAGTAAATAATGGCACATTTCAGAGAACAAGACCGAAATTAGACTTGGTACTGGCTTCATCTGATATTCGTAAGCTGGCTACGGTTTTATTTAATTTAAAAGCTCT
                                                                                                                    840
 I  M  N  T  K  G  E  E  P  T  L  T  T  M  T  S  V  Q  T  N  E  E  K  N  D  N  L  E  Q  K  Y  S  S  L  L  D  R  W  N  G
CATAATGAATACGAAGGGTGAGGAACCCACTCTAACCACGATGACTAGTGTCCAAACGAACGAAGAGAAAAATGATAACCTGGAACAGAAATATTCAAGTCTGCTAGATAGATGGAATGG
                                                  SpeI                                                              960
 K  V  E  V  H  D  S  P  F  L  Q  L  Q  R  D  S  N  L  L  F  A  E  R  P  V  R  Y  V  S  T  T  E  G  E  G  V  D  I  S  S
GAAAGTAGAAGTGCATGATTCTCCTTTCCTTCAACTCCAACGGGACAGCAACCTTTTGTTTGCTGAGAGGCCGGTACGTTACGTTAGCACTACAGAAGGTGAAGGCGTGGACATATCTAG
                                                                                                                   1080
 E  E  F  F  R  L  E  E  E  Q  C  R  R  N  Y  D  V  L  V  D  E  H  S  T  P  S  V  G  M  K  D  G  Q  Y  G  P  N  I  I  H
TGAAGAGTTTTTCAGGTTAGAGGAAGAACAGTGCCGAAGGAATTATGATGTTTTGGTCGATGAACATTCTACACCATCAGTCGGGATGAAAGACGGCCAATACGGACCAAATATAATTCA
                                                                                                                   1200
 F  E  P  S  L  Y  H  T  Y  S  S  L  P  M  S  M  K  F  W  L  N  G  L  E  D  D  E  T  T  M  M  N  I  D  E  K  S  A  E  N
CTTTGAACCGTCGCTGTACCACACATATTCTTCTCTACCAATGAGTATGAAATTTTGGTTAAATGGTCTAGAAGACGACGAAACGACGATGATGAACATTGATGAGAAGTCAGCCGAAAA
                                                                                                                   1320
 L  D  I  L  L  H  G  F  K  G  F  S  N  K  R  V  K  G
TCTGGATATATTATTACATGGTTTCAAAGGATTTTCGAACAAGCGCGTTAAAGGATGAATTAATCATCCTCTTCATGTACTACTTATGTCCAATTTGAGTGTAAAATCACAGGTACAAGG
                                                                                                                   1440
CTTATCCATATTCGTATATATCTTCATAATGTTTCATAATTGCTTCAGAAAATTATTCAAAACGTCTTGTGATATTCATTAGGTGAAATCGTAGTTTGCCTGATACTTGAAAGAATCTCA
                                                                                                                   1560
CCTGGAGAATGGGTGTTTTCACTAAGATTCGTTAATTTCCACTAATGATTAATTGTTAACTGTTTAACGCGACGCGTAATAAAGTTTCCTGAATACGCCAACATATGGGAACATATAGAT
                                                                  HpaI
TAAATTAATAAGAAAGCTT
          HindIII
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Fig. 2. DNA sequence of the *MSS18* gene. (**A**) Sequencing strategy. Open boxes represent reading frames of *MSS18* and of an URF which stretches downstream of *MSS18*. The wavy arrows indicate the direction of transcription. E, *Eco*RI; S, *Sac*I; P, *Pst*I; X, *Xba*I; H, *Hin*dIII restriction sites. J is the left end and J'' the right end of the cloned DNA fragment borne by plasmids p295-6 and p295-2, respectively. (**B**) The *MSS18* coding sequence is shown with its upstream and downstream flanking sequence. A peptide sequence, homologous to the P1 and P2 dodecapeptides generally encountered in the maturases of group I, is underlined:

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P1:  Y  L  A  G  L  *  D  G  D  G  +  F
     W     S           E              I
P2:  W  L  *  G  F  /  D  A  D  G  S  F
        I              E     E     T
MSS18: Y  V  S  -  T  T  E  G  E  G  V  D
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*, majority of apolar amino acids; +, majority of non-ionic polar amino acids; /, any amino acid (Séraphin *et al.*, 1985).

data lead us to the conclusion that the *MSS18* gene is necessary for the efficient splicing of intron aI5β.

Mitochondrial mutants presenting a wild-type restriction pattern might well carry small rearrangements or point mutations which could be localized in or near the aI5β intron. To test this hypothesis, we determined the sequence of intron aI5β and that of the two adjacent exons aE5β and aE5γ both in revertant BS108-8/R19 and parental strain BS108-8 (Figure 4B). Compared with that of strain BS108-8, the sequence of revertant BS108-8/R19 revealed two nucleotide changes at position 125 and 309, respectively (corresponding to position 1488 and 1673 in Hensgens *et al.*, 1983a). Multiple base changes were not unexpected since a high concentration of $MnCl_2$ (6 mM) had been used to obtain this revertant (De La Salle *et al.*, 1982). We also sequenced the 5' exon (aE5β) and the 5' part of intron aI5β (encompassing the region mutated in strain BS108-8/R19) of two other independent point revertants, BS108-8/R20 and BS108-8/R26. Each revertant differed only by one nucleotide from the wild type strain: an A to T transition at position 125 in strain BS108-8/R20 and at position 128 in strain BS108-8/R26 (Figure 4B). We have not genetically mapped the reversion sites of these revertants but the fact that the same nucleotide change occurred independently in two strains

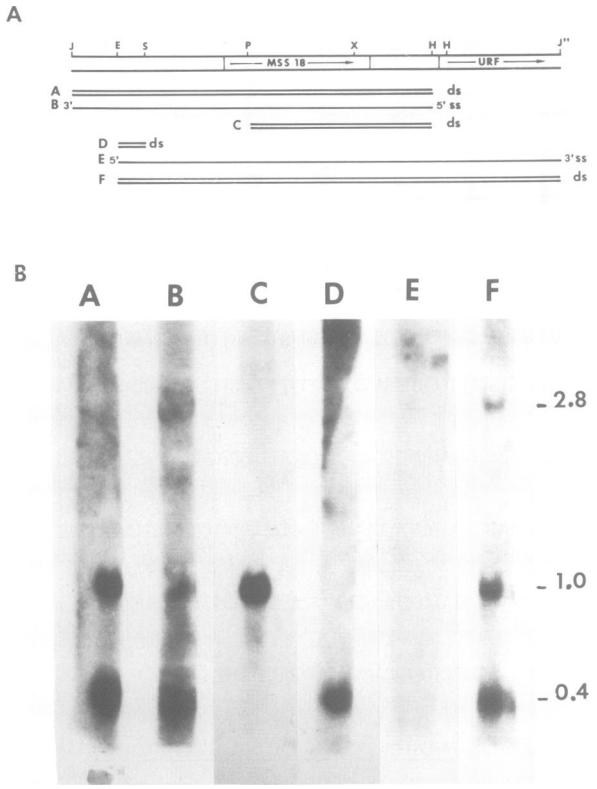

Fig. 3. Northern blot analysis of the *MSS18* transcript. **Panel A.** Description of the probes used. The restriction map of the DNA fragment containing gene *MSS18* is drawn in the upper part of the Figure (E, *Eco*RI; S, *Sac*I; P, *Pst*I; X, *Xba*I; H, *Hin*dIII). J is the left end and J'' the right end of the cloned DNA fragment borne by plasmids p295-6 and p295-2, respectively (cf. Figure 1). URF, unknown reading frame. Double-stranded probes A, C, D and F were ^{32}P-labelled by nick-translation. Single-stranded probes B and E were labelled according to Hu and Messing (1982); the labelled strands were those depicted in the Figure). **Panel B.** Poly(A)$^+$ mRNA of strain AB320 was hybridized with each of the probes shown in **Panel A**. Probe A (**lane A**), two RNA species were detected. Probe B (**lane B**), the same RNA species were observed. Probe C (**lane C**), only the 1.0 kb long RNA was visible. Probe D (**lane D**), only the 0.4 kb long RNA was observed. Probe E (**lane E**), no RNA was detected. Probe F (**lane F**), an additional RNA species (2.8 kb long), probably corresponding to the URF sequence, was detected.

as well as the clustering of the substitutions in the 5′ exon leave no doubt that they are the suppressor mutations. Since BS108-8/R19 and /R20 have the same growth rate on lactate medium (Table I), we suspect that the nucleotide change at position 309 does not participate in the reversion phenomenon.

Northern blotting

In order to follow the effect of the *mss18* gene disruption on the processing of the *cox1* primary transcript, we carried out Northern experiments using mtRNA from various strains and probes derived either from exons or introns of the *cox1* gene. The main conclusions drawn from the resulting autoradiograms shown in Figure 5B are the following.

(i) Strain BS108-8 (lane 5, Figure 5B) accumulates a 3.8 kb long RNA intermediate (◀ in Figure 5B) which should correspond to the mature *cox1* mRNA plus intron aI5β (2.2 + 1.7 kb) since in addition to the aI5β probe (p73-A25), both exon 1 (pEX1-1) and exon 6 (pAE6) probes hybridize to this RNA [the same RNA was also detected in

Table I. Growth rate

Strains	*MSS18* gene	Mitochondria	Generation time	
			Glucose	Lactate
BS104-1	*MSS18*	D273-10B/A	1 h 35	4 h 00
BS108-1	*mss18-3*	D273-10B/A	1 h 35	7 h 25
BS104-8	*MSS18*	777-3A	1 h 35	4 h 00
BS108-8	*mss18-3*	777-3A	1 h 45	no growth
BS108-8/R1	*mss18-3*	777-3A[a]	1 h 35	6 h 50
BS108-8/R19	*mss18-3*	777-3A[b]	1 h 40	9 h 05
BS108-8/R20	*mss18-3*	777-3A[b]	1 h 40	9 h 05
BS108-8/R26	*mss18-3*	777-3A[b]	1 h 40	10 h 00
BS104-10	*MSS18*	GF167-7B	1 h 45	4 h 00
BS108-10	*mss18-3*	GF167-7B	1 h 45	6 h 14

Cells were grown in complete liquid media (1% extract, 1% bactopeptone) supplemented with either 2% glucose or 1% lactate and 50 mM phosphate buffer, pH 5.5. Optical densities of cultures were measured at 600 nm. All strains studied carry the nucleus of strain GRF18 (they are therefore isonuclear) except for the *MSS18* and *LEU2* genes. Mitochondria of strains D273-10B/A, 777-3A or GF167-7B were introduced by cytoduction using strains Kar(273), Kar(777) and Kar(167), respectively (Séraphin *et al.*, 1987b). 777-3A[a]: mitochondrial revertant with the aI5β and aI5γ introns deleted. 777-3A[b]: mitochondrial point revertants.

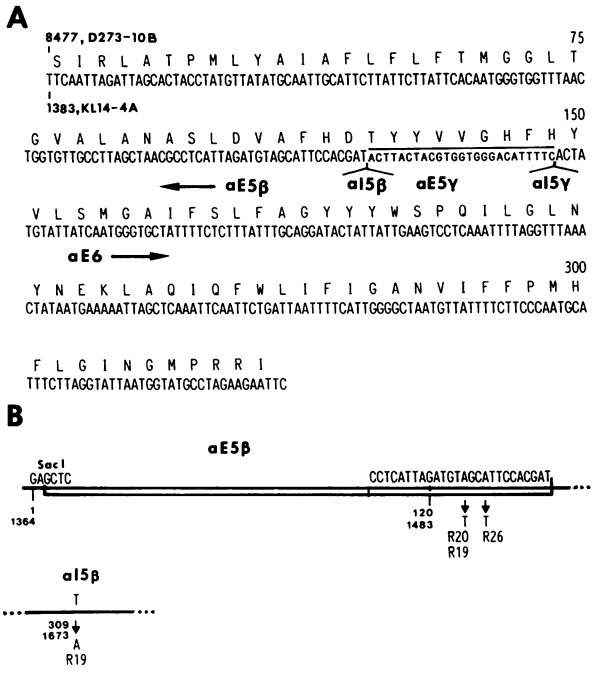

Fig. 4. Mitochondrial extragenic revertants of the *mss18-3* null allele present in strains BS108-8. (**A**) Revertant BS108-8/R1. The DNA sequence reveals that introns aI5β and aI5γ have been deleted. Two nucleotide changes (positions 109 and 196) are observed when compared with the sequence of strain D273-10B (Bonitz *et al.*, 1980). In addition, this sequence reveals that intron aI5γ is inserted in a histidine (CAC) codon, so the yeast *cox1* sequence in this region (HFHYVL) is homologous to that of other organisms (Lang, 1984). We have applied the codon recognition rule of yeast mitochondria, where TGA is used for Trp, CTN for Thr and ATA for Met. The two numbers 8477 and 1383 refer to the *cox1* sequences published by Bonitz *et al.* (1980) and Hensgens *et al.* (1983a), respectively. (**B**) Point mitochondrial revertants BS108-8/R19, R20 and R26. The position of the nucleotide change(s) in each revertant is indicated. The upper numbering starting from the *Sac*I site refers to our sequencing results (note that mitochondria of strain BS108-8 originate from strain 777-3A), the lower numbering is according to Hensgens *et al.* (1983a).

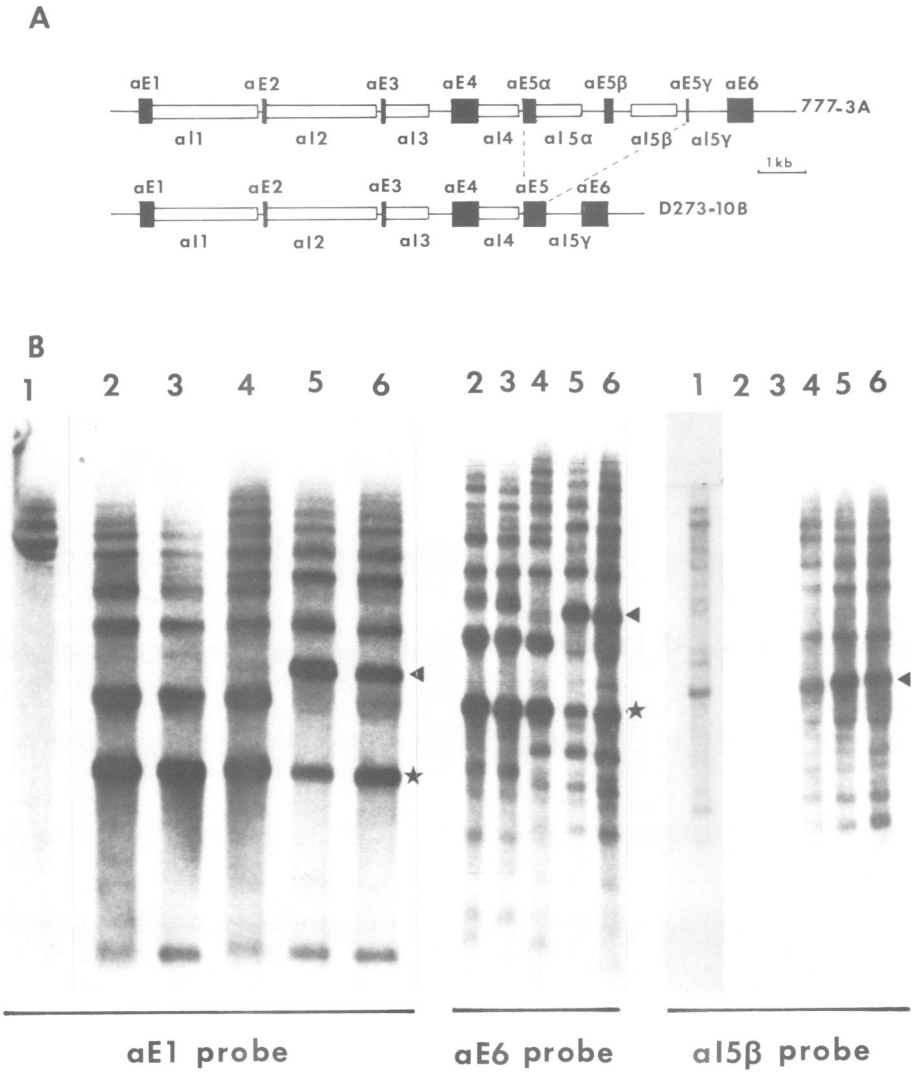

Fig. 5. Northern blots of mtRNA from strains MH41-7B/L721 (O_1P_2) (**1**), BS104-1 (**2**), BS108-1 (**3**), BS104-8 (**4**), BS108-8 (**5**) and BS108-8/R19 (**6**). (**A**) Structure of the *cox1* gene of strain 777-3A (and MH41-7B) (Hensgens *et al.*, 1983b) and strain D273-10B (Bonitz *et al.*, 1980). Exons are depicted by thick closed boxes, while thinner open boxes represent intron ORFs. (**B**) Autoradiograms. The numbers indicate the origins of mtRNAs. The arrowheads indicate the position of the 3.8 kb RNA intermediate and the stars the position of the 2.2 kb mature *cox1* mRNA.

strain N295 using an exon 4 probe (Faye and Simon, 1983b)]. This RNA intermediate is also present in strain BS108-8/R19 (lane 6) though in a lower amount than in strain BS108-8.

(ii) In strain BS108-8 (lane 5), detection of a small amount of *cox1* mRNA (* in Figure 5B) suggests that the splicing defect is not complete even with a deletion of the *MSS18* gene. This may account for the leakiness observed on glycerol medium. Compared with strain BS108-8, an ~2-fold increase in the level of the mature *cox1* mRNA is observed in strain BS108-8/R19 (lane 6).

(iii) Minor differences in the pattern of *cox1* RNA are observed in strains BS108-1 (lane 3), BS108-8 (lane 5) and BS108-8/R19 (lane 6) as compared with the corresponding wild-type strains. This may indicate that *MSS18* has some other relatively minor effect on the splicing of introns other than aI5β.

(iv) The *cox1* sequence is transcribed in the *rho*$^-$ strain O_1P_2 (lane 1) in which the *cox1* RNA undergoes some processing reactions. For instance with probe aI5β eleven bands are clearly visible on a gel (Figure 5B), the size of the major band is ~3.8 kb but this RNA species does not contain the exon aE1 sequence since no 3.8 kb long band is detected with the aE1 probe.

Mitochondrial translation products

We have mentioned above that even in the presence of a mitochondrial genome devoid of introns, a strain with the disrupted gene *MSS18* grows poorly on lactate medium (Table I). To test whether the expression of mitochondrial proteins other than *cox1* is disturbed by the *mss18-3* disruption, we analyzed the mitochondrial translation products of several strains. Autoradiograms are shown in Figure 6. We observed a faint amount of cytochrome c oxidase subunit 1 in strain BS108-8 (lane 5), whereas the level of this protein was appreciably higher in strain BS108-8/R19 (lane 6). Compared with strain BS104-1 (lane 2), strain BS108-1 (lane 3) presented a slight diminution of the amount of *cox1* protein. The synthesis of ATPase 9 and/or 8 was also reduced in strain BS108-1, BS108-8 and BS108-8/R19 as well as the synthesis of cox3 and/or ATPase 6 proteins in strain BS108-8 and BS108-8/R19. In addition, the mito-

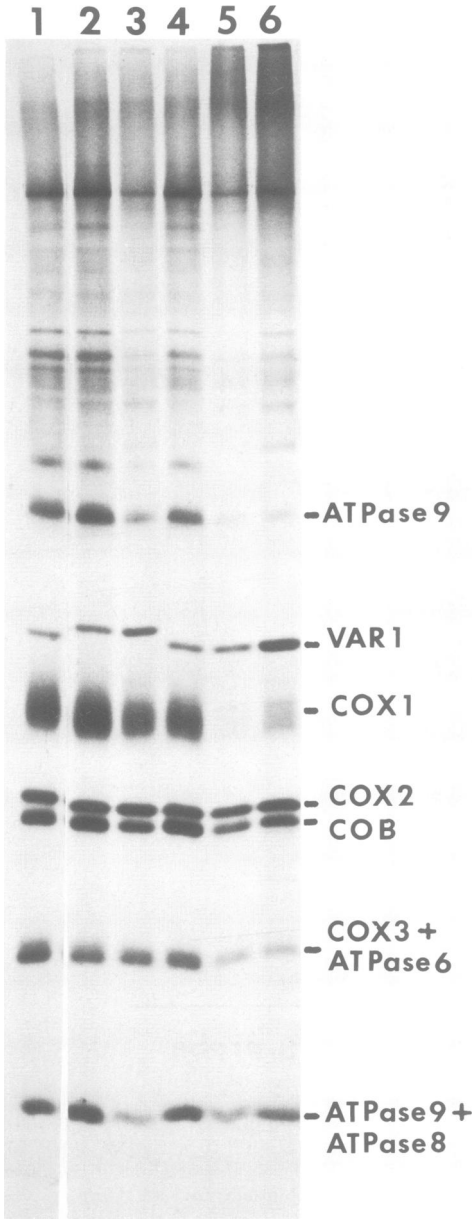

Fig. 6. ^{35}S-Labelled mitochondrial translation products of strain GRF18 (**1**), BS104-1 (**2**), BS108-1 (**3**), BS104-8 (**4**), BS108-8 (**5**) and BS108-8/R19 (**6**). The positions of the mitochondrially synthesized polypeptides are indicated: (VAR1) var1 ribosomal protein; (cox1, cox2, cox3) subunits I, II and III of cytochrome c oxidase; (COB) cytochrome b; (ATPase 6, ATPase 8, ATPase 9) subunits 6, 8 and 9 of mitochondrial oligomycin-sensitive ATPase.

chondrial protein pattern of strains BS104-1 and BS108-1 or of strains BS104-8 and BS108-8 differed by some new as yet unidentified bands. Thus the *mss18-3* mutation mainly affects the synthesis of *cox1* polypeptide, although the synthesis of some other mitochondrial translation products is also, directly or indirectly, altered to some extent.

S1 nuclease mapping

We suspected that point reversions in strain BS108-8 generated a new 5′ splice site allowing excision of aI5β at a rate sufficient to grow on lactate medium [cf. Figure 7(1B)]. To test this supposition we carried out S1 nuclease protection experiments. Single-stranded DNA probes covering exon aE5β from the *Sac*I site and part of intron aI5β [Figure 7(2)] were hybridized to purified mtRNA from strain BS104-8, BS108-8 or O_1P_2 (for probe S18) and from strain BS108-8/R19 (for probe S3). Results are shown in Figure 7(3). DNA fragments protected on ~135 bases were observed with each of the four different RNA species. This is the protection expected for probes that hybridized with RNA molecules cleaved at the correct aE5β−aI5β 5′ splice junction. Furthermore, these S1 mapping experiments indicate that the splicing rate of aI5β is low in strain BS108-8 when compared with strain BS104-8 [Figure 7(3), lanes 4 and 5] and that it is slightly enhanced in the revertant strain BS108-8/R19 [as a different probe is used, compare the ratio of spliced to unspliced products in lane 3, Figure 7(3) with the corresponding ratio in the other lanes]. No new 5′ splice site was revealed with RNA of this latter strain. A cleavage occurs at the 5′ junction of intron aI5β in the *cox1* transcript of the rho$^-$ strain O_1P_2 [Figure 7(3), lane 6]. This result accounts for the fact that the major O_1P_2 RNA which hybridized with probe aI5β (Figure 5B) has no counterpart among those hybridizing with probe aE1: a cleavage occurs at the junction aE5β−aI5β. However, we have to note that in this latter S1 experiment probe and RNA were not homologous as the DNA probe was issued from '777-3A' mitochondria and O_1P_2 RNA from 'MH41-7B' mitochondria (Grivell, 1984).

Discussion

From both biochemical and genetic approaches we may infer that the *MSS18* gene is implicated in the splicing of the mitochondrial intron aI5β. As we have not demonstrated that the *MSS18* gene product enters mitochondria it remains possible that in fact *MSS18* controls the synthesis or the activity of one (or several) gene(s) product(s) directly involved in the excision of aI5β. Nuclear reversions of the *mss18-3* mutation may concern such nuclear genes.

On the other hand, our studies throw new light on the following aspects of the mitochondrial splicing.

Action site of the nuclear gene products involved in mitochondrial splicing

The step(s) of the mitochondrial intron excision process in which the nuclear genes so far identified are participating [*CPB2 (Hill et al.*, 1985), *MRS1* (Kreike *et al.*, 1986), *NAM2* (Labouesse *et al.*, 1985)] are unknown. We have shown in the present study, both by Northern blotting and S1 nuclease protection experiments, that *mss18* mutations block the cleavage of the 5′ exon−intron junction of aI5β. This block will consequently impede the realization of the 3′ cleavage (Cech, 1986). The finding that three independent mitochondrial point revertants harbor sequence changes just upstream of the 5′ junction further sustains the idea that the *mss18* mutations do affect the 5′ cleavage of aI5β.

The putative aI5β maturase (as any other mitochondrial maturases) is not required for the cleavage of the junction aE5β−aI5β since this cleavage does occur in a rho$^-$ strain. Interestingly, the *MSS18* protein contains a short sequence (amino acids 157 to 167, Figure 2) presenting homology with the P1 and P2 dodecapeptides found in group I maturases (Michel *et al.*, 1982; Hensgens *et al.*, 1983a; Waring *et al.*,

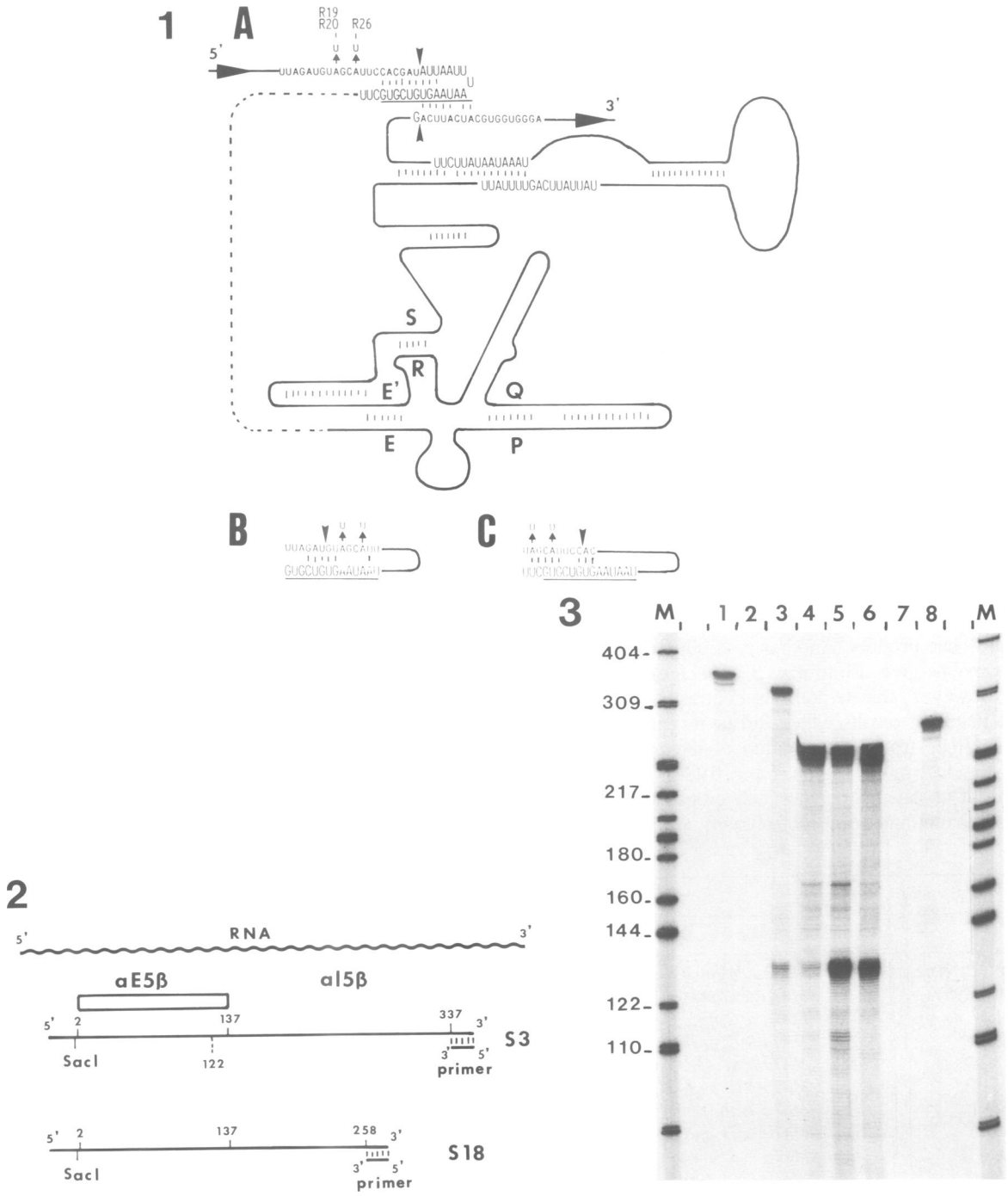

Fig. 7. S1 nuclease mapping. **Part 1** (**A**) A model for the secondary structure of intron aI5β drawn from Waring and Davies (1984). Exon sequences are indicated in small letters and intron sequence in capital letters. E, P, Q, R, E′ and S symbolize the six internal conserved nucleotide sequences typical of class I introns (Waring and Davies, 1984). The internal guide sequence near the 5′ end of intron aI5β is underlined. **Part 1** (**B**) Putative secondary structure between the internal guide sequence and exon aE5β which may be stabilized by the point reversions R19, R20 or R26. **Part 1** (**C**) Putative secondary structure which may be destabilized by the point reversions R19, R20 and R26. Arrowheads, cleavage points. **Part 2** of the figure illustrates the building of probes S3 and S18. The mitochondrial template DNAs from which probes S3 and S18 were synthesized come from strains BS108-8/R19 and BS108-8 respectively. We used the same nucleotide numbering as in Figure 4B. Position 122 is that of the putative alternative 5′ cleavage site suggested in Part 7(1B). **Part 3** of the figure shows autoradiograms. (**1**) Probe S3 was used as size marker; (**2**) probe S3 hybridized with *E.coli* tRNA; (**3**) probe S3 hybridized with mtRNA of strain BS108-8/R19; (**4**) probe S18 hybridized with mtRNA of strain BS108-8; (**5**) probe S18 hybridized with mtRNA of strain BS104-8; (**6**) probe S18 hybridized with mtRNA of strain MH41-7B/L721 (O_1P_2); (**7**) probe S18 hybridized with *E.coli* tRNA; (**8**) probe S18 used as size marker. M, end-labelled restriction fragments of pBR322 cut with *Hpa*II and used as size markers.

1982). It is tempting to speculate that the *MSS18* protein may share some properties with mitochondrial maturases and to favor the hypothesis that *MSS18* is a *trans*-acting factor which, probably by binding to the *cox1* RNA in the vicinity of the aE5β−aI5β junction, facilitates its cleavage. An alternative hypothesis in which the absence of the *MSS18* gene product would decrease the rate of the 5′ cleavage by destabilizing a *cox1* RNA structure or disorganizing a RNP complex remote from the aE5β−aI5β junction appears less likely.

Exon sequences engaged in the splicing of group I introns

The model of Waring and Davies (1984) postulates that up to six bases of the exon sequences upstream of group I introns interact with an intron sequence, the internal guide sequence, generating a folded structure propitious for the cleavage–ligations reactions [cf. Figure 7(1A)]. Direct interactions between group I or group II introns and the upstream exon were observed (Waring *et al.*, 1986; Been and Cech, 1986; Jacquier and Michel, 1987). The position of the mitochondrial point reversions of the *mss18-3* mutation indicates that the 3′ terminal part of exon aE5β engaged in the splicing process of intron aI5β is at least 13 bases long. This is twice as long as the sequence implicated in the pairing of the 5′ splice site with the internal guide sequence. Several hypotheses may be envisaged to explain why these point reversions partially restore the excision of intron aI5β. First, it is noteworthy that the aE5β nucleotide stretch ^{125}AGCAUUCCAC, which is partially complementary to the internal guide sequence [eight nucleotides out of ten; cf. Figure 7(1C)], may form an alternative hairpin structure competing with the sequence which is thought to direct the splicing reaction. One possibility would be that the point reversions, by destabilizing this alternative structure, prevent a 5′ cleavage occurring at a wrong place in the absence of the *MSS18* gene product. Another possibility could be that these reversions give rise to new 5′ cleavage sites [Figure 7(1B)]. However, this hypothesis contrasts with our S1 nuclease mapping results which fail to reveal any cryptic splice site [the strong conservation of the cytochrome *c* oxidase subunit 1 in this region (Lang, 1984), suggests that activation of cryptic splice sites will probably lead to the synthesis of a non-functional *cox1* protein]. A more attractive hypothesis is that the point revertants in the aE5β exon may directly, or through the formation of a secondary structure, affect the binding of the *trans*-acting factor(s) mentioned above.

Nuclear gene products involved in mitochondrial splicing may accomplish several actions

Besides its role on the splicing of intron aI5β, the *MSS18* gene seems to be involved in less well characterized processing phenomena (cf. Figure 5). The *MSS18* gene product may also be required for other functions, as suggested by the fact that a strain with a disrupted *MSS18* gene and devoid of mitochondrial introns does not recover its full respiratory competency. This characteristic is not peculiar to this gene—several nuclear gene products implicated in mitochondrial splicing seem to have multiple functions (Labouesse *et al.*, 1985; Pape *et al.*, 1985; Akins and Lambowitz, 1987). This may also be true for mitochondrial encoded maturases (Kotylak *et al.*, 1985) which besides their involvement in mitochondrial splicing seem to participate in recombination or reverse transcription, since some of them present homologies with reverse transcriptases (Michel and Lang, 1985).

Materials and methods

Yeast strains

The strains used are given in Table II. All genetic procedures and media were as described by Sherman *et al.* (1983). Yeast transformation, mtDNA preparation and DNA manipulation were as described (Faye and Simon, 1983a; Séraphin *et al.*, 1987; Maniatis *et al.*, 1982).

Table II. Yeast strains

Strains	Genotype	Source
MH41-7B/L721 (O_1P_2)	*MATa ade2 his1* [*rho*$^-$]	Bolotin-Fukuhara *et al.* (1977)
FF1210-6C/N295	*MATα* ura1 ura3 mss18-4 [*rho*$^+$]	
PS23-8D/1	*MATa leu2 trp1* [*rho*$^-$]	P.Shalit
BS7-133-5	*MATa leu2 trp1 ura mss18-4* [*rho*$^+$]	Meiosis of FF1210-6C/N295 × PS23-8D/1
AB320	*MATa/MATα HO ade2 lys2 trp5 leu2 can*^{R} *ura met* [rho$^+$]	M.Olson
GRF18	*MATα leu2 his3 can*^{R} [*rho*$^+$]	Donahue *et al.* (1983)
D273-10B/A	*MATα met6* [*rho*$^+$]	A.Tzagoloff
777-3A	*MATα ade1 op1* [*rho*$^+$]	L.Clavillier
GF167-7B	*MATα lys2* [*rho*$^+$]	Séraphin *et al.* (1987)
Kar(273)	*MATa Kar1-1 trp5 can*^{R} [*D273-10B/A*]	
Kar(777)	*MATa Kar1-1 trp5 can*^{R} [*777-3A*]	
Kar(167)	*MATa Kar1-1 trp5 can*^{R} [*GF167-7B*]	
FF314	*MATa leu2 trp1 ura3 ade5 can*^{R} [*rho*$^+$]	F.Fabre
BS106-3	*MATα leu2 his3 can*^{R} *mss18-3* [rho$^+$]	
BS106-3/2	*MATα leu2 his3 can*^{R} *mss18-3* [*rho*^{0}]	
BS104-1	*MATα leu2 his3 can*^{R} [*D273-10B/A*]	
BS104-8	*MATα leu2 his3 can*^{R} [*777-3A*]	
BS104-10	*MATα leu2 his3 can*^{R} [*GF167-7B*]	
BS108-1	*MATα leu2 his3 can*^{R} *mss18-3* [*D273-10B/A*]	
BS108-8	*MATα leu2 his3 can*^{R} *mss18-3* [*777-3A*]	
BS108-10	*MATα leu2 his3 can*^{R} *mss18-3* [*GF167-7B*]	
BS111-3B	*MATa leu2 trp1 ura3 ade5 mss18-3* [*rho*$^+$]	Meiosis of BS106-3/2 × FF314
BS111-3B/2	*MATa leu2 trp1 ura3 ade5 mss18-3* [*rho*^{0}]	

Mitochondrial genomes are indicated between brackets. The mitochondrial genome of strain 777-3A contains the thirteen mitochondrial introns: ω, aI1, aI2, aI3, aI4, aI5α, aI5β, aI5γ, bI1, bI2, bI3, bI4 and bI5. The mitochondrial genome of strain D273-10B/A lacks the following introns: aI5α, aI5β, bI1, bI2 and bI3. Introns bI1, aI1, aI2 and aI5γ belong to group II, the others to group I. ω: 21S rRNA intron, aI: cytochrome oxidase subunit 1 intron, bI: cytochrome b intron. *mss18-3* is a mutant allele of the gene *MSS18*; it was obtained by integrating the *LEU2* gene between its *Hpa*I sites (see Materials and methods).

Cloning and DNA sequencing

A yeast library constructed with the yeast−*Escherichia coli* vector YEp13 (Broach *et al.*, 1979), and the whole fragmented genome of strain AB320 (Nasmyth and Tatchell, 1980) was used to transform strain BS7-133-5. *LEU2*$^+$ transformant colonies were replated on glycerol complete medium to select for glycerol positive transformants (Faye and Simon, 1983a). Plasmids extracted from four such colonies were amplified in *E.coli*. Strain BS7-133-5 transformed with any of these plasmids (p295-1, -2, -3 and -6) recovered respiratory competency. These plasmids overlap each other over a 2.6 kb long segment in which gene *MSS18* is probably located (Figure 1). The cloned DNA fragments borne by p295-1 and p295-6 were cut with *Sac*I, *Pst*I and/or *Hin*dIII enzymes; DNA fragments therefrom were inserted into vectors YEp13 or pEMBLYe31 (Baldari and Cesarini, 1985), then introduced into BS7-133-5. The ability of transformants to use glycerol as a carbon source was tested. Results are presented in Figure 1 and suggest that the sequence complementing the mutation *mss18-4* is contained in the 1.5 kb *Sac*I-*Hin*dIII fragment.

The fragments J-*Hin*dIII and *Eco*RI-J″ (Figure 2) were cloned with opposite orientations in M13mp19 vector (Messing, 1983). Subclones obtained according to the strategy of Lin *et al.* (1983) were submitted to sequence analysis by the Sanger dideoxy sequencing technique (Sanger *et al.*, 1977). The sequencing strategy is depicted in Figure 2. The entire sequence of fragment JJ″ was determined but only the *Eco*RI−*Hin*dIII DNA segment was sequenced nearly completely on both strands.

To sequence mtDNA of strain BS108-8 and of revertants isolated from it we first cloned the *Sac*I−*Eco*RI DNA fragment containing the aI5β region in pTZ vectors (Pharmacia). We then followed the sequencing strategy of Lin *et al.* (1983).

Gene disruptions

The *Eco*RI−*Hin*dIII fragment containing the *MSS18* gene was cloned in vector pUC19 between the *Eco*RI and *Hin*dIII sites (Yanisch-Perron, 1985). A recombinant plasmid, pHE295, was isolated. The 2.4 kb *Hpa*I restriction fragment containing the yeast *LEU2* gene was prepared from vector YEp73 (Broach *et al.*, 1979). It was inserted in place of the *Hpa*I−*Hpa*I fragment of pHE295. The new plasmid obtained, p295-H1, possessed only the first seven amino acid residues of *MSS18*. DNA of plasmid p295-H1 was digested with restriction enzymes *Xba*I and *Hin*dIII and then the *Xba*I−*Hin*dIII fragment, harboring the *LEU2* inserted gene, was used to replace the chromosomal copy of the wild type *MSS18* gene in strain GRF18, according to the method developed by Rothstein (1983).

Selection of respiratory competent revertants

One ml of a fresh culture of strain BS108-8 (Table II) was diluted in 19 ml of YPglu. $MnCl_2$ was added to 2 ml aliquots of this cell suspension to reach the following concentrations: 6, 4, 2, 1, 0.5, 0.2 and 0.0 mM. These subcultures were incubated overnight at 28°C. After incubation, cultures were centrifuged, cell pellets were resuspended in 0.1 ml of Ringer solution, then plated onto lactate plates. Revertants appeared after 5−10 days (the reversion rate was between 2 and 5 × 10^{-7}). Each of them was subcloned on a lactate plate and then underwent the following genetic tests. (i) They were crossed with BS111-3B/2 (rho^0). Only when the revertants are mitochondrial or nuclear dominant do the resulting diploids grow on lactate; if they are nuclear recessive they do not. (ii) Revertants were made rho^0 by ethidium bromide treatment, then crossed wtih BS111-3B (rho$^+$). If the revertants are mitochondrial, the diploids cannot utilize lactate; if they are nuclear the results are as above. So these two tests allow one to distinguish between nuclear recessive, nuclear dominant and mitochondrial revertants.

Southern and Northern transfers

MtRNA of yeast cells grown in 1% yeast extract, 1% bacto peptone, 2% raffinose and 0.1% glucose was extracted as described (Faye and Simon, 1983a). Total RNA was extracted from strain AB320 and poly(A)$^+$ mRNA were purified on an oligo(dT)-cellulose column.

Southen transfers were carried out as described by Maniatis *et al.* (1982). RNA transfers to nitrocellulose filter and RNA−DNA hybridization were performed according to the Southern method as modified by Thomas (1980).

In vivo labelling of mitochondrial proteins

Cells were grown in galactose or in raffinose complete media. Cells were labelled with L-[^{35}S]methionine in the presence of cycloheximide as described by McKee *et al.* (1984). Radiolabelled mitochondrial translation products were prepared and then analyzed by SDS−PAGE according to Douglas *et al.* (1979).

S1 mapping

Two pTZ18R recombinants, named plasmid C18 and D3, were prepared for DNA sequencing of intron aI5β of strains BS108-8 and BS108-8/R19 and contained mtDNA fragments extending from the *Sac*I site to nucleotide 258 and 337 respectively [cf. Figure 7(2)], were used to prepare single-stranded radiolabelled DNA probes (Simon *et al.*, 1986). A 22 nt long primer, hybridized to the single-stranded form of these plasmids, was extended towards the *Sac*I site with Klenow polymerase in the presence of [α-^{32}P]dCTP (600 Ci/mmol). The dsDNA obtained was cleaved with *Sac*I. About 8.0 × 10^4 c.p.m. of probe S18 were hybridized wtih 20 μg of mtRNA from strains BS104-8, BS108-8 or O_1P_2. The S3 probe was hybridized with 20 μg of BS108-8/R19 mtRNA. Hybridization was done at 55°C as described (Simon and Faye, 1984). After an overnight incubation, hybridization mixtures were treated with nuclease S1 (Sharp *et al.*, 1980). S1 protected DNA segments were analyzed on an 8% acrylamide/urea sequencing gel.

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References

Akins,R. and Lambowitz,A. (1987) *Cell*, **50**, 331−345.
Baldari,C. and Cesareni,G. (1985) *Gene*, **35**, 27−32.
Been,M. and Cech,T. (1986) *Cell*, **47**, 207−216.
Bennetzen,J. and Hall,B. (1982) *J. Biol. Chem.*, **257**, 3026−3031.
Bolotin-Fukuhara,M., Faye,G. and Fukuhara,H. (1977) *Mol. Gen. Genet.*, **152**, 295−305.
Bonitz,S., Coruzzi,G., Thalenfeld,B., Tzagoloff,A. and Macino,G. (1980) *J. Biol. Chem.*, **255**, 11927−11941.
Broach,J., Strathern,J. and Hicks,J. (1979) *Gene*, **8**, 121−133.
Cech,T. (1986) *Cell*, **44**, 207−210.
Cech,T. and Bass,B. (1986) *Annu. Rev. Biochem.*, **55**, 599−629.
Conde,J. and Fink,G. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 3651−3655.
Davis,R., Thomas,M., Cameron,J., St John,T., Scherer,S. and Padgett,R. (1980) *Methods Enzymol.*, **65**, 404−411.
De la Salle,H., Jacq,C. and Slonimski,P. (1982) *Cell*, **28**, 721−732.
Donahue,T.F., Daves,R., Lucchini,G. and Fink,G. (1983) *Cell*, **32**, 89−98.
Douglas,M., Finkelstein,D. and Butow,R. (1979) *Methods Enzymol.*, **56**, 58−66.
Faye,G and Simon,M. (1983a) *Cell*, **32**, 77−87.
Faye,G. and Simon,M. (1983b) In Schweyen,R., Wolf,K. and Kaudewitz,F. (eds), *Mitochondria 1983*. Walter de Gruyter, Berlin, pp. 433−439.
Grivell,L. (1984) In O'Brien,S. (ed.), *Genetic Maps 1984*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 234−247.
Hensgens,L., Bonen,L., de Haan,M., van der Horst,G. and Grivell,L. (1983a) *Cell*, **32**, 379−389.
Hensgens,L., Arnberg,A., Roosendaal,E., van der Horst,G., van der Veen,R., Van Ommen,G. and Grivell,L. (1983b) *J. Mol. Biol.*, **164**, 35−38.
Hill,J., McGraw,P. and Tzagoloff,A. (1985) *J. Biol. Chem.*, **260**, 3235−3238.
Hu,N. and Messing,J. (1982) *Gene*, **17**, 271−277.
Kotylak,Z., Lazowska,J., Hawthorne,D. and Slonimski,P. (1985) In Quagliariello,E., Slater,E.C., Palmieri,F., Saccone,C. and Kroon,A. (eds), *Achievements and Perspectives of Mitochondrial Research*. Elsevier, Amsterdam, Vol. II, pp. 1−20.
Kreike,J., Schulze,M., Pillar,T., Körte,A. and Rödel,G. (1986) *Current Genet.*, **11**, 185−191.
Labouesse,M., Dujardin,G. and Slonimski,P. (1985) *Cell*, **41**, 133−143.
Lancashire,W. and Mattoon,J. (1979) *Mol. Gen. Genet.*, **170**, 333−344.
Lang,F. (1984) *EMBO J.*, **3**, 2129−2136.
Lazowska,J., Jacq,C. and Slonimski,P. (1980) *Cell*, **22**, 333−348.
Lin,H.C., Lei,S.P. and Wilex,G. (1983) *Anal. Biochem.*, **147**, 114−119.
Maniatis,T., Fritsch,E. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McKee,E., McEwen,J. and Poyton,R. (1984) *J. Biol. Chem.*, **259**, 9332−9338.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20−78.
Michel,F. and Dujon,B. (1983) *EMBO J.*, **2**, 33−38.
Michel,F. and Lang,B. (1985) *Nature*, **316**, 641−643.

Michel,F., Jacquier,A. and Dujon,B. (1982) *Biochimie*, **64**, 867–881.
Nasmyth,K. and Thatchell,K. (1980) *Cell*, **19**, 753–764.
Pape,L., Koerner,T. and Tzagoloff,A. (1985) *J. Biol. Chem.*, **260**, 15362–15370.
Peebles,C., Perlman,P., Mecklenburg,K., Petrillo,M., Tabor,J., Jarrel,K. and Cheng,H. (1986) *Cell*, **44**, 213–223.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202–211.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schmelzer,C. and Schweyen,R. (1986) *Cell*, **46**, 557–565.
Séraphin,B., Simon,M. and Faye,G. (1985) *Nucleic Acids Res.*, **13**, 3005–3014.
Séraphin,B., Boulet,A., Simon,M. and Faye,G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6810–6814.
Sharp,P., Berk,Z. and Berget,S. (1980) *Methods Enzymol.*, **65**, 750–768.
Sherman,F., Fink,G. and Hicks,J. (1983) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Simon,M. and Faye,G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 8–12.
Simon,M., Séraphin,B. and Faye,G. (1986) *EMBO J.*, **5**, 2697–2701.
Tabak,H., van der Horst,G., Kamps,A. and Arnberg,A. (1987) *Cell*, **48**, 101–110.
Thomas,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
van der Horst,G. and Tabak,H. (1985) *Cell*, **40**, 759–766.
van der Veen,R., Arnberg,A., van der Horst,C., Bonen,L., Tabak,H. and Grivell,L. (1986) *Cell*, **44**, 225–234.
Waring,R. and Davies,R. (1984) *Gene*, **28**, 277–291.
Waring,R., Davies,W., Scazzocchio,C. and Brown,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6332–6336.
Waring,T., Towner,P., Minter,S. and Davies,W. (1986) *Nature*, **321**, 133–139.
Yanisch-Perron,C., Vieira,J. and Messing,T. (1985) *Gene*, **33**, 101–119.

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