The EMBO Journal vol.6 no.7 pp.2123-2129, 1987

A yeast nuclear gene, MRS1, involved in mitochondrial RNA splicing: nucleotide sequence and mutational analysis of two overlapping open reading frames on opposite strands

Jan Kreike[1], Marion Schulze, Fred Ahne and B.Franz Lang[2]

Institut für Genetik und Mikrobiologie, Universität München, Maria Ward Strasse 1a, D-8000 München 19, FRG

[1]Present address: Institut für Mikrobiologie und Genetik, Universität Wien, Althanstrasse 14, A-1090 Wien, Austria

[2]Present address: Département de Biochimie, Université de Montreal, CP 6128, Succursale A, Montreal, Quebec, Canada H3C 3J7

Communicated by L.A.Grivell

We have cloned a 1.6-kb fragment of yeast nuclear DNA, which complements *pet*⁻ mutant MK3 (*mrs1*). This mutant was shown to be defective in mitochondrial RNA splicing: the excision of intron 3 from the mitochondrial *COB* pre-RNA is blocked. The DNA sequence of the nuclear DNA fragment revealed two open reading frames (ORF1 with 1092 bp; ORF2 with 735 bp) on opposite strands, which overlap by 656 bp. As shown by *in vitro* mutagenesis, ORF1, but not ORF2, is responsible for complementation of the splice defect. Hence, ORF1 represents the nuclear *MRS1* gene. Disruption of the gene (both ORFs) in the chromosomal DNA of the respiratory competent yeast strain DBY747 (long form *COB* gene) leads to a stable *pet*⁻ phenotype and to the accumulation of the same mitochondrial RNA precursors as in strain MK3. The amino acid sequence of the putative ORF1 product does not exhibit any homology with other known proteins, except for a small region of homology with the gene product of another nuclear yeast gene involved in mitochondrial RNA splicing, *CBP2*. The function of the *MRS1* (ORF1) gene in mitochondrial RNA splicing and the significance of the overlapping ORFs in this gene are discussed.

Key words: DNA sequence/mitochondrial RNA splicing/*in vitro* mutagenesis/overlapping open reading frames/yeast

Introduction

Introns in genes of cell organelles have been assigned to two groups according to conserved nucleotide sequences and potential secondary structure (Michel and Dujon, 1983; Waring and Davies, 1984). Predominant in fungal mitochondria are introns of group I. Also the intron in the pre-rRNA encoded in *Tetrahymena* nuclear DNA belongs to this group. This RNA precursor was the first transcript shown to be capable of autocatalytic splicing (self-splicing) *in vitro* (Kruger *et al.*, 1982), an activity essentially attributed to the intron. Meanwhile, several mitochondrial transcript precursors in *Neurospora* and yeast, containing either group I or group II introns, have been shown to exert this activity *in vitro* (Garriga and Lambowitz, 1984; Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986; Van der Veen *et al.*, 1986; see Cech, 1986, for a review).

This suggests that self-splicing is a basic phenomenon of group I and group II introns. Yet, genetic studies in fungi suggest that for mitochondrial RNA splicing *in vivo* proteins are needed; this holds true even for *in vitro* self-splicing introns (Schmelzer *et al.*, 1983; Garriga and Lambowitz, 1984; Schmelzer and Schweyen, 1986). The function of these proteins may be accessory to the basically intron-catalyzed splicing reaction, but nevertheless essential for the process *in vivo*.

Proteins involved in splicing of the yeast mitochondrial *COB* mRNA, encoding apocytochrome *b*, may be encoded in the introns themselves or in nuclear genes. There is ample genetic evidence for such intron-encoded proteins, the so-called RNA maturases, encoded in group I introns bI2 and bI4 (Lazowska *et al.*, 1980; Weiss-Brummer *et al.*, 1982). Nuclear gene products appear to be involved in the excision of introns bI1 (group II) and bI5 (group I) (McGraw and Tzagoloff, 1983; Schmelzer *et al.*, 1983; Hill *et al.*, 1985). As shown by McGraw and Tzagoloff, intron bI5 cannot be excised in yeast mutants lacking a functional copy of the nuclear gene *CBP2*. For group I intron bI3 the situation may be more complex. For the excision of this intron the gene product of a nuclear gene *MRS1* is required (Kreike *et al.*, 1986) and probably also the product of the bI3 intronic ORF (Jacq *et al.*, 1982; Holl *et al.*, 1985).

Here we report the complete nucleotide sequence of the nuclear DNA fragment which complements the *pet*⁻ mutant MK3 (*mrs1*). We have identified two overlapping open reading frames (ORF1 and ORF2) on opposite strands. Only ORF1 is responsible for complementation of the *mrs1* mutant and is therefore thought to correspond to the *MRS1* gene. Our results furthermore suggest that this gene plays an essential role in the excision of a single group I intron from mitochondrial RNA.

Results

The DNA fragment complementing the mrs1 mutation contains two overlapping open reading frames on opposite strands

The effect of the nuclear *mrs1* mutation on the excision of the single group I intron bI3 from the mitochondrial *COB* transcript in *Saccharomyces cerevisiae* and the isolation of a yeast nuclear DNA fragment complementing this mutation have been described before (Pillar *et al.*, 1983a; Kreike *et al.*, 1986). The com-

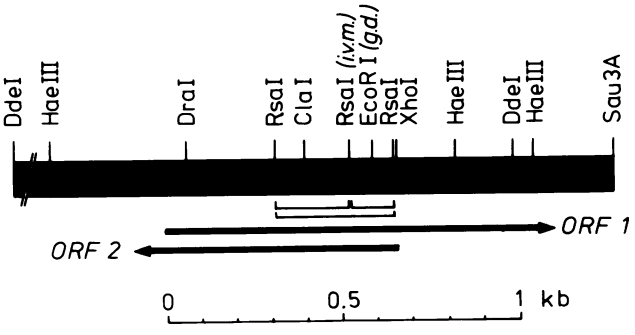

Fig. 1. Physical map of the cloned nuclear DNA fragment, which complements the *mrs1* mutation. The positions of ORF1 and ORF2 are shown, as well as the positions of the *Rsa*I fragments, described in the text. The site of the gene disruption is marked by 'g.d.' and the site of the *in vitro* mutagenesis by 'i.v.m.'. Only the essential *Rsa*I and *Sau*3A sites are shown.

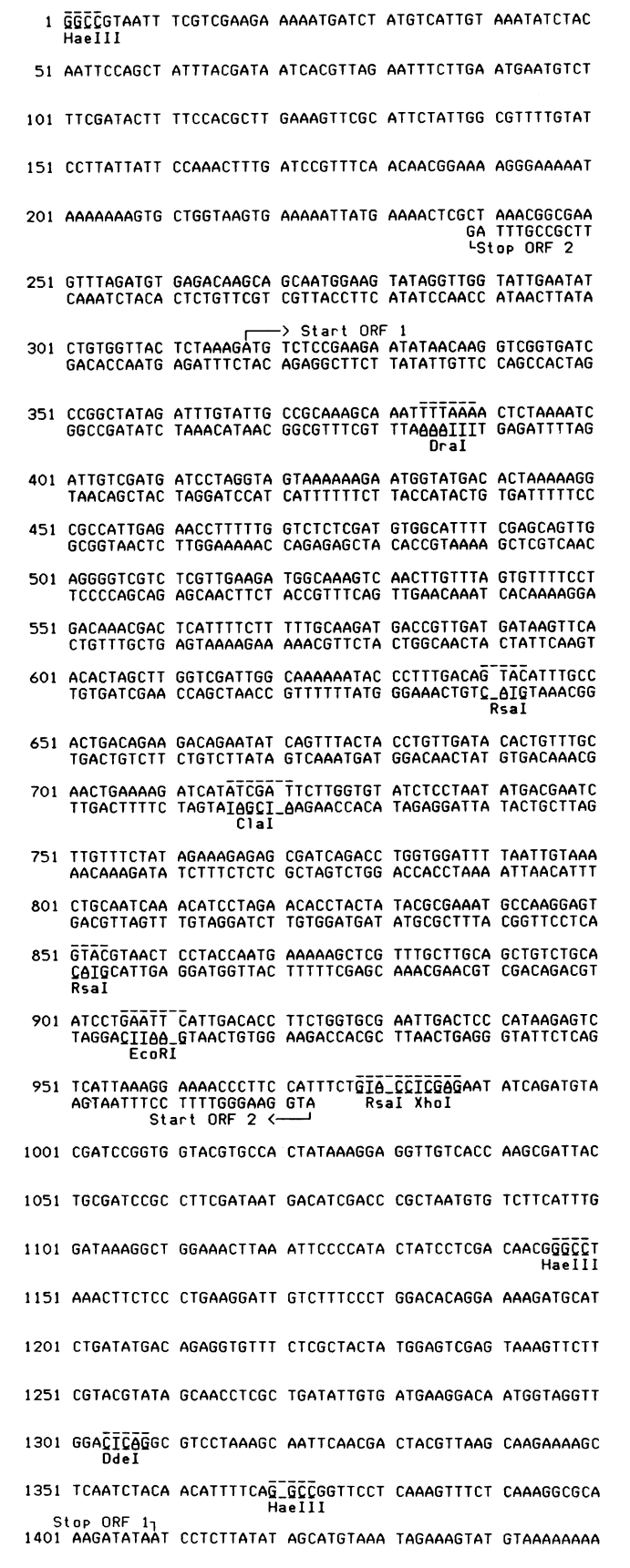

Fig. 2. DNA sequence of the complementing 1.6-kb *Hae*III−*Sau*3A fragment. The complete sequence is shown with the exception of ~100 bp near the *Sau*3A site at the end of the DNA insert. ORF2 is indicated by the double-stranded representation. Restriction sites are over- and underlined. Start and stop codons of both ORFs are indicated.

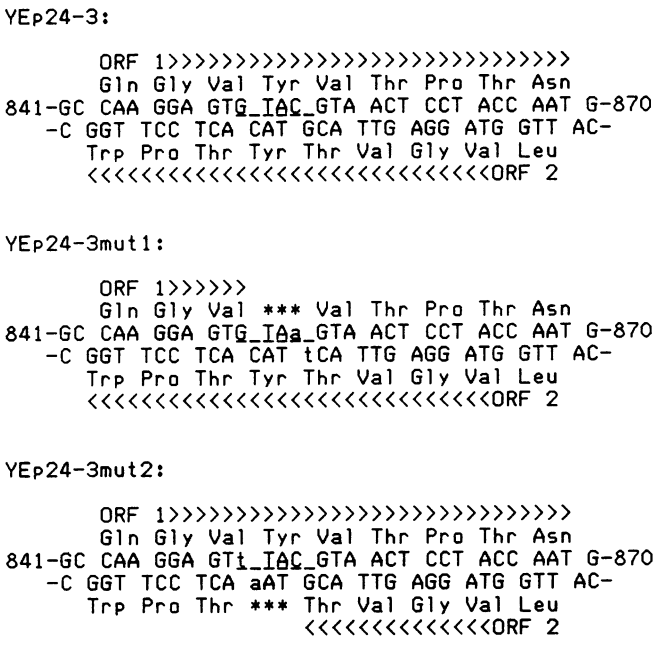

Fig. 3. *In vitro* mutagenesis of ORF1 and ORF2. Only a 30-bp region around the *Rsa*I site (underlined) at position 851−854 of the DNA sequence is shown. The DNA sequence and the deduced amino acid sequence of both ORFs are shown, the direction of them is indicated with the arrowheads. The introduced base pair changes are shown in lower case and the stop codons introduced are marked by ***.

plementing 1.6-kb *Hae*III−*Sau*3A fragment is thought to contain the functional *MRS1* gene. A restriction map of this fragment is presented in Figure 1, its DNA sequence (with the exception of ~100 bp near the *Sau*3A site) in Figure 2. This sequence shows two open reading frames (ORF1 and ORF2, 1092 and 735 bp respectively) in opposite direction, which overlap each other by 656 bp.

We searched in this DNA sequence for motifs which are frequently found at the 5′ and 3′ ends of yeast nuclear genes (Kozak, 1981). Such motifs are absent, with the exception of TAG and TATGT sequences present at the 3′ end of ORF1 (Zaret and Shermann, 1982) and a TATATAA sequence 422 bp upstream of ORF2. However, evidence for a role of these sequences in initiation or termination of transcription is so far lacking. We also searched for consensus sequences involved in the excision of introns from transcripts of yeast nuclear genes (Langford and Gallwitz, 1983). Although the 5′ end consensus sequence GTANGT is found four times in ORF1 and once in ORF2, the highly conserved TACTAAC box, which is found invariably in the 3′ region in yeast introns (Langford and Gallwitz, 1983), is completely absent from our DNA sequence (both strands). Therefore, we consider it most unlikely that the *MRS1* gene is a mosaic gene and that ORF1 or ORF2 represents only one of its exons.

We have isolated cytoplasmic RNA from respiratory competent strains and performed Northern hybridization experiments using *in vitro* synthesized, highly labeled RNA as single-strand-specific probes. However, we were not successful in identifying transcripts of ORF1 or ORF2, neither in untransformed *mrs1*$^{+}$ or *mrs1*$^{-}$ strains, nor in strains transformed with high copy plasmids carrying the functional *MRS1* gene. Apparently the *MRS1* gene is very poorly expressed (*vide infra*) or its transcript is highly unstable.

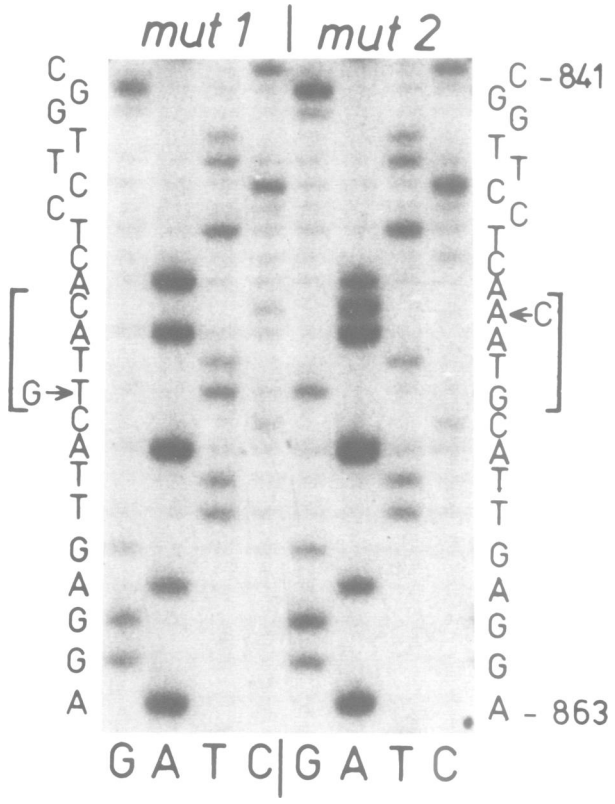

Fig. 4. Partial DNA sequence of the 180-bp *ClaI*−*Eco*RI fragment in M13mp9rev after the introduction of the mut1 and mut2 mutations. The base pair changes are indicated by the arrows.

ORF1 is responsible for complementation of the mrs1 mutation

To investigate which one of the two ORFs is responsible for complementation of the *mrs1*$^-$ mutation and therefore represents the *MRS1* gene, we performed *in vitro* mutagenesis using the procedure of Kramer *et al.* (1984). The 180-bp *ClaI*−*Eco*RI fragment (located completely within both ORFs) was isolated from plasmid YEp24-3 and subcloned in M13mp9 (see Materials and methods). Two mutations were introduced in this fragment with the help of synthetic oligonucleotides. The first (mut1) leads to a stop codon in ORF1, without amino acid substitution in the product of ORF2. The other (mut2) leads to the reverse situation: a stop codon in ORF 2, without amino acid substitution in the product of ORF1 (Figure 3). Both mutations were introduced into the single *Rsa*I site within the 180-bp fragment (position 851−854 in the DNA sequence of Figure 2). This enabled us to verify the mutations in M13 ssDNA by DNA sequence analysis and by *Rsa*I digestion and Southern hybridization after recloning the mutated fragment into YEp24-3. The DNA sequence showed the desired and no other base pair changes in 50% of the clones. Examples are shown in Figure 4. The DNA sequences of the other clones were all identical to the wild-type sequence.

The mutant sequences were recloned into the autonomous plasmid YEp24-3, in a way that with the exception of the two mutations, both ORFs would be fully intact. Digestion of the resulting plasmid DNAs with *Rsa*I and Southern hybridization with the 180-bp *ClaI*−*Eco*RI fragment as a probe was used to check the absence of the *Rsa*I site after this step. In the plasmids YEp24-3 mut1 and YEp24-3mut2, a novel 340-bp *Rsa*I fragment was expected to hybridize to the probe instead of two *Rsa*I fragments (127 bp and 211 bp) in plasmid YEp24-3 (Figure 1).

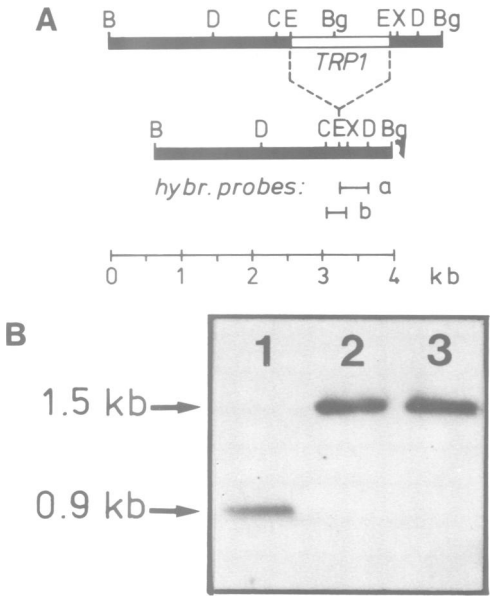

Fig. 5. (**A**) Restriction map of the chromosomal DNA of strain DBY747 (black bar) and the position of the inserted *TRP1* gene (open bar) after disruption of the *MRS1* gene. The hybridization probes are indicated by (**a**) and (**b**) and are described in the text. (**B**) Southern hybridization of chromosomal DNAs from respiratory deficient (RD) and competent (RC) strains, after digestion with *Cla*I and *Bgl*II. Hybridization probe was the 400-bp *Eco*RI−*Dde*I fragment from plasmid YEp24-3 (probe a of Figure 1A). **Lane 1**: strain DBY747 (*trp1*$^-$, RC); **lanes 2, 3**: two transformants of DBY747, in which the *MRS1* gene is disrupted (*trp1*$^+$, RD).

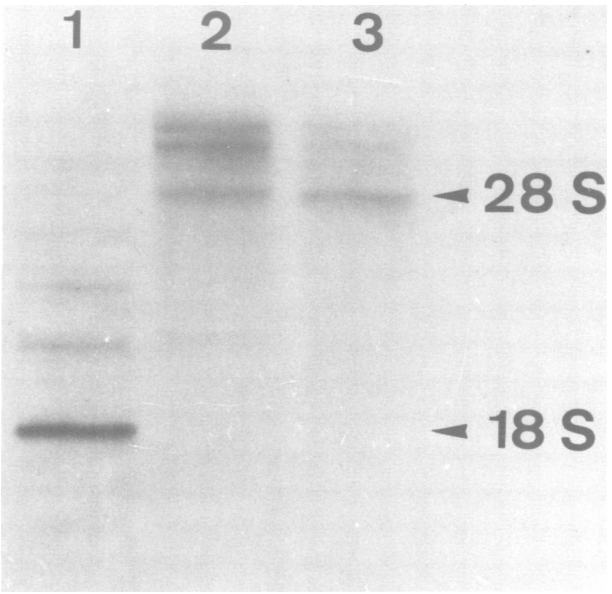

Fig. 6. Northern hybridization of mitochondrial RNAs isolated from the respiratory competent strain DBY747 (**lane 1**), from mutant MK3 (**lane 2**) and from the respiratory deficient strain DBY747 (*MRS1::TRP1*) after disruption of the *MRS* gene (**lane 3**). The DNA probe used is specific for the *COB* gene (leader-region up to intron bI1).

This was indeed the case (data not shown).

The *mrs1*$^-$ mutant MK3 as well as strain DBY747 (*MRS1::TRP1*) after disruption of the *MRS1* gene (*vide infra*) were transformed with these plasmids. The *ura3*$^+$ transformants were all respiratory competent, when YEp24-3mut2 (premature termination of the ORF2 product) or the unmodified YEp24-3

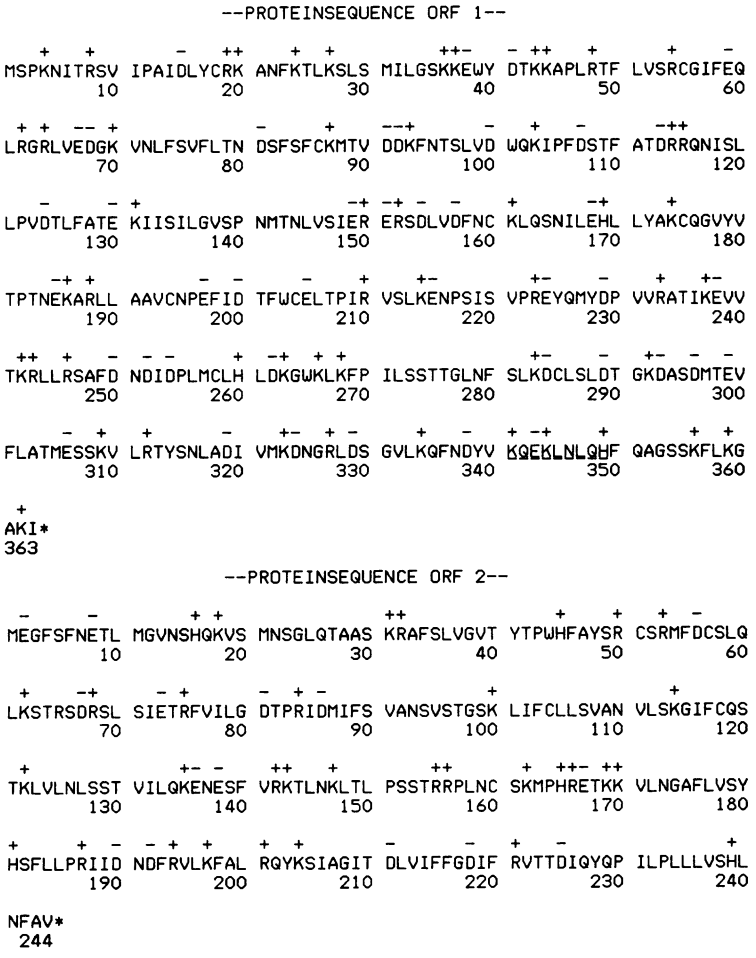

Fig. 7. Amino acid sequence of the putative proteins coded by ORF1 and ORF2. Positively (basic) and negatively charged (acidic) residues are indicated by (+) and (−).

--CODON USAGE ORF1 AND ORF2--

	ORF	1:	2:			1:	2:			1:	2:			1:	2:
Phe	TTT	12	12	Ser	TCT	7	7	Tyr	TAT	4	1	Cys	TGT	4	2
	TTC	9	6		TCC	6	2		TAC	4	4		TGC	5	3
Leu	TTA	3	7		TCA	7	12	Ter	TAA	1	0	Ter	TGA	0	0
	TTG	11	8		TCG	5	4		TAG	0	1	Trp	TGG	4	1
Leu	CTT	7	6	Pro	CCT	6	3	His	CAT	2	4	Arg	CGT	4	0
	CTC	5	2		CCC	3	1		CAC	1	1		CGC	1	1
	CTA	11	4		CCA	1	4	Gln	CAA	6	6		CGA	5	3
	CTG	7	4		CCG	4	0		CAG	4	2		CGG	0	1
Ile	ATT	5	2	Thr	ACT	9	3	Asn	AAT	11	7	Ser	AGT	5	4
	ATC	6	7		ACC	5	5		AAC	8	5		AGC	2	1
	ATA	9	6		ACA	7	6	Lys	AAA	17	9	Arg	AGA	5	5
Met	ATG	9	6		ACG	3	3		AAG	15	5		AGG	3	5
Val	GTT	8	6	Ala	GCT	5	3	Asp	GAT	14	6	Gly	GGT	5	2
	GTC	7	5		GCC	4	3		GAC	12	3		GGC	6	1
	GTA	4	4		GCA	5	3	Glu	GAA	11	3		GGA	2	5
	GTG	6	4		GCG	2	1		GAG	5	3		GGG	0	2

Fig. 8. Codon usage in ORF1 and ORF2.

were used in the transformation. YEp24-3mut1 (premature termination of the ORF1 product), however, leads to respiratory deficient *ura3*$^+$ transformants. We concluded from this result that only the ORF1 encoded protein is needed directly for complementation of the *mrs1* mutation.

Disruption of the functional MRS1 gene in the respiratory competent strain DBY747

Since the *mrs1* mutation in strain MK3 is probably a point mutation it may not prohibit the function of the gene product com-

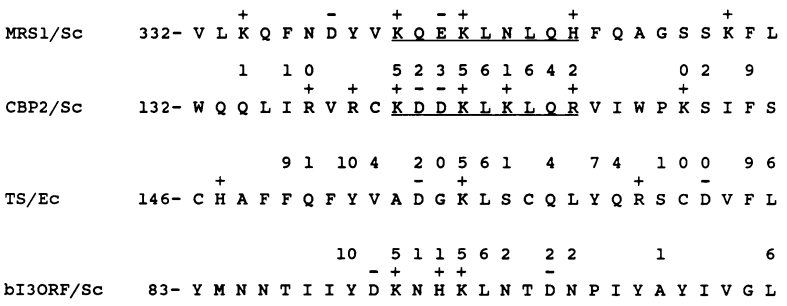

Fig. 9. Region of homology between the putative *MRS1* protein, the *CBP2* protein from *S. cerevisiae* (Sc), the thymidylate synthase from *E. coli* (TS/Ec) and the product of the open reading frame in intron bI3 (bI30RF/Sc). The position of these protein regions within the complete protein sequence is indicated, for bI30RF/Sc the first residue encoded in intron bI3 was taken as reference. Only zero and positive values according to Dayhoff (1979) are shown. The block of nine residues homologous in the *MRS1* and *CBP2* gene products is underlined.

pletely. Therefore, we cannot conclude from the splice defect in the mutant that the *MRS1* gene product is exclusively involved in intron bI3 excision. To investigate this we disrupted the chromosomal *MRS1* gene essentially as described by Rothstein (1983). In order to do this we first constructed a derivative of plasmid YEp24-3 in which the *Eco*RI site within the *MRS1* gene is the only site for this enzyme. Into this site the 1.4-kb *Eco*RI fragment from plasmid YRp7, containing the *TRP1* gene, was inserted (Figure 5A). Then the resulting plasmid was digested with *Bam*HI and *Sal*I and used for transformation of respiratory competent (RC) strain DBY747 (*trp1*$^-$; having the long form of the *COB* gene including intron bI3, data not shown). Five transformants with a stable *trp1*$^+$ phenotype were obtained which were all respiratory deficient (RD) and of a stable *pet*$^-$/*rho*$^+$ phenotype. The disruption was verified by Southern hybridization and the result is shown in Figure 5B. Chromosomal DNAs from two of the transformants (RD) and from strain DBY747 (RC) were digested with *Cla*I and *Bgl*II, transferred to nitrocellulose filters and hybridized with the 400-bp *Eco*RI–*Dde*I fragment from plasmid YEp24-3 (probe a in Figure 5A). In the DNA from strain DBY747 a band of ~900 bp hybridizes to this probe, whereas DNA from the two *trp1*$^+$ transformants showed a fragment of ~1.5 kb (Figure 5B). Digestion of the DNAs with *Cla*I and *Xho*I, followed by hybridization with the 300-bp *Cla*I–*Xho*I fragment from plasmid YEp24-3 (probe b in Figure 5A) gave similar results: in DNA from strain DBY747 only the 300-bp fragment characteristic for the functional *MRS1* gene hybridized. In DNA from the RD, *trp1*$^+$ transformants a fragment that was 1.4 kb longer, was found instead, due to the insertion of the *TRP1* gene (data not shown).

The effect of the disruption of the *MRS1* gene on mitochondrial RNA splicing was studied in two ways. First, we isolated mitochondrial RNA from strain DBY747, from strain DBY747 (*MRS1::TRP1*) having the disrupted *MRS1* gene and from mutant MK3. Northern hybridization using a DNA probe specific for the *COB* gene (leader-region up to intron bI1) gave the same transcript patterns in both RD strains: the mature 18S transcript is absent and a 28S precursor and larger ones accumulate (Figure 6) (see also Pillar *et al.*, 1983b). Hybridization of the RNAs isolated from the RD strains with an *OXI3*-specific DNA probe (exon A4 and intron aI4) showed the 26S RNA precursor as the shortest *OXI3* transcript and no mature RNA (data not shown). Second, we combined the disrupted *MRS1* nuclear gene with the 'short form' mitochondrial genome; this combination has a respiratory competent phenotype. The experiment was done by crossing a *rho*^{0} strain derived from DBY747 with the disrupted *MRS1* gene (*MRS1::TRP1*/*rho*^{0}) with strain D273-10B (*mrs1*$^+$/*rho*$^+$, short form *COB* gene lacking introns bI1-bI3). Sporulation of the resulting diploids gave only tetrads with four respiratory competent spores. This result clearly shows that in a strain with a disrupted *MRS1* gene the mitochondrial introns bI4 and aI4 are excised correctly. From these two experiments we conclude that the disruption of the *MRS1* gene and the mutation in mutant MK3 lead to identical defects in mitochondrial RNA splicing.

Properties of the putative ORF1 and ORF2 encoded proteins

The amino acid sequences of the putative ORF1 and ORF2 encoded proteins deduced from the DNA sequence and their codon usage are given in Figures 7 and 8. If no post-translational modifications occur *in vivo*, the ORF1 product is 363 residues long and has a mol. wt of 41 224 daltons. It contains 14.5% basic (positively charged) and 11.6% acidic (negatively charged) residues. The putative ORF2 product has 244 residues (27 446 daltons) and an excess of basic residues (15%) over acidic residues (6%).

The DNA sequences of ORF1 and ORF2 and the deduced amino acid sequences were compared with sequences present in DNA and protein sequence collections. No extensive homologies exist. Only short regions of homology were found with several eukaryotic and prokaryotic proteins, e.g. between ORF1 and thymidylate synthase genes from *Escherichia coli* (Belfort *et al.*, 1983) and from *Lactobacillus casei* (Bellisario *et al.*, 1979). Furthermore, we compared the *MRS1* (ORF1) amino acid sequence with the sequence of the *CBP2* gene product (McGraw and Tzagoloff, 1983), since up to now these two are the only sequenced yeast genes which are specific for the excision of single (group I) mitochondrial introns. The results are shown in Figure 9. There is no extensive homology between the two proteins, except for a region of nine amino acids in which five residues and four charges are conserved. Interestingly, this region also shows some homology with the putative bI3 maturase (Holl *et al.*, 1985). The region of homology in the *MRS1* and *CBP2* proteins is the same region which is homologous with the prokaryotic thymidine synthase proteins (Figure 9). This region of the *E. coli* protein may be functionally important, since a temperature-sensitive mutation has been mapped in it (Belfort and Pederson-Lane, 1984) and a functional site (Cys-146), responsible for the binding of the inhibitor 5-fluoro-2′-deoxyuridine, is located at only three residues distance. At present it is difficult to say what is the significance of these homologies (if any).

Discussion

In this paper we presented the DNA sequence of a fragment of yeast nuclear DNA, which complements a respiratory deficient *mrs1* mutant to respiratory competence. It has previously been

shown that this mutant is defective in mitochondrial RNA splicing and that the nuclear *MRS1* gene is involved in the excision of group I intron bI3 from the mitochondrial *COB* transcript (Kreike *et al.*, 1986). The effect of the disruption of the nuclear *MRS1* gene, as presented in this paper, on the accumulation of mitochondrial RNA precursors confirmed and extended this conclusion. The *mrs1* mutant and a strain with the disrupted *MRS1* gene have completely identical splice defects. This means that the excision of intron bI3 from the *COB* RNA is the only function of the *MRS1* gene in mitochondrial RNA splicing. The excision of this intron therefore seems to be dependent on the function of at least two proteins: the nuclear coded *MRS1* gene product and the bI3 encoded maturase (Jacq *et al.*, 1982; Holl *et al.*, 1985). The functional cooperation of proteins of different origin in the excision of a single mitochondrial intron is a very interesting aspect in the study of mitochondrial RNA splicing. Both proteins may be directly involved in the splicing process, e.g. by stabilization of the secondary structure of the bI3 intron. Or, alternatively, only one of them may interact with the RNA precursor but may require to be modified first by the other. Proteolytic cleavage of the pre-maturase, as has been shown to occur with the bI4 maturase (Weiss-Brummer *et al.*, 1982), would be one possible example of activation of the bI3 maturase by the *MRS1* gene product. Discrimination between these possibilities is currently under investigation.

The DNA sequence of the complementing DNA fragment revealed two open reading frames (ORF1 and ORF2) on opposite strands overlapping by 656 base pairs. *In vitro* mutagenesis showed that ORF1 alone is responsible for complementation of the defect in the *mrs1* mutant and in the strain with the disrupted *MRS1* gene. Therefore, we suggest that this ORF corresponds to the *MRS1* gene. From the codon usage we calculated the codon bias index for both ORFs. This codon bias index has been correlated with the efficiency of RNA synthesis in yeast: a value of 1 is found for highly expressed genes and a lower value is found for more poorly expressed genes. For instance the transcript of a gene with codon bias index 0.15 is approximately only 0.003% of the total mRNA in the cell (Bennetzen and Hall, 1982). The values calculated for ORF1 and ORF2 are even much lower: 0.045 and 0.007, respectively. This would mean that if both ORFs are expressed *in vivo*, they are probably expressed very poorly. This would explain why we have not yet been able to identify their transcripts by Northern hybridization, in spite of the use of highly labeled RNA as strand-specific probes.

The overlapping configuration of ORF1 and ORF2 is a very unusual situation for eukaryotic nuclear genes. Overlapping genes are common only in bacteriophages, animal viruses, bacteria and in organelles of eukaryotic cells (Normark *et al.*, 1983). Does ORF2 represent a regular gene or not? Statistically, one would expect only short open reading frames in coding DNA strands (which are complementary to the transcript of a gene) as is the case in random DNA. However, computer analysis of the sequences of such coding DNA strands revealed that open reading frames longer than 300 bp are not uncommon (Casino *et al.*, 1981). Numerous yeast nuclear genes have now been sequenced, but no example of such antiparallel overlapping open reading frames has been discussed. At the moment we do not know whether ORF2 is expressed and codes for a functional protein or not. We have not been able to identify its transcript and have no data to indicate what the function of its product might be.

One possibility, which deserves some comment, is that ORF2 may represent a gene or pseudogene, which has lost its protein-coding function during evolution. But why then should the open reading frame be maintained? The following explanation seems plausible: that part of ORF2, which overlaps with ORF1, could not accumulate mutations because of selection for functional ORF1. For instance, all 1310 possible frame shift mutations (+1 or −1 bp) would probably lead to a loss of the ORF1 function. In addition we have identified 103 possible single base pair changes leading to a premature termination of the ORF2 protein. Of these changes, ~60% lead to a premature stop in ORF1 too, or result in amino acid substitutions with strong negative values according to Dayhoff (1979), and are presumably drastic changes in the ORF1 product. In other words, selection for ORF1 function dramatically slows down the evolutionary mutation rate of ORF2 and probably its promotor sequences.

Disruption of the *MRS1* gene, which also leads to disrupted ORF2, results in the same phenotype as the original *mrs1* mutation. The constructed strain grows normally on glucose-containing media and is able to sporulate giving four viable spores. Obviously, ORF2 has neither a direct effect on mitochondrial RNA splicing, nor is it involved in essential cellular functions. We cannot exclude the possibility that ORF2 has an indirect regulatory function on ORF1 expression, effective only when ORF1 and ORF2 are located in the normal configuration in the chromosomal DNA. In that case it would have escaped our attention, as we have studied cells with multiple episomal copies of ORF1 and ORF2.

Materials and methods

Strains and plasmids

The respiratory deficient strain NP1/MK3 (*α mrs1 leu2-3 leu2-112 his3-11 his3-15 ura3-251 ura3-372 ura3-228*) has been described before (Kreike *et al.*, 1986). Strain DBY747 (*a his3-1 leu2-3 leu2-112 ura3-52 trp1-289*) was a gift from D.Botstein. *Escherichia coli* strains 490 (r_k r_m *met*$^-$ *thr*$^-$ *leu*$^-$ *recA*$^-$ *lac*$^-$ *su*$^+$, a gift from G.Hobom) and JM101 (Messing, 1983) were routinely used for transformations. *E. coli* strains BMH71-18 and WK6 and the M13mp9 and M13mp9rev were kind gifts from H.-J.Fritz.

The yeast gene bank used for isolation of the MRS1 gene was a gift from F.Lacroute (Fasiolo *et al.*, 1981), pAT153 (Twigg and Sherratt, 1983) was used for the recloning of the *in vitro* mutagenized *Cla*I−*Eco*RI fragment from M13 RF-DNA into YEp24-3. Plasmid YRp7 has been described by Stinchcomb *et al.* (1979).

DNA sequence determination

For the determination of the DNA sequence of the 1.6-kb *Hae*III−*Sau*3A fragment, containing the complete functional *MRS1* gene, the procedure of Maxam and Gilbert (1979) was used with the modifications described elsewhere (Lang, 1984). Overlapping fragments were always sequenced in both directions. The sequence has been confirmed and extended with the method of Messing (1983).

In vitro mutagenesis

For *in vitro* mutagenesis of the 180-bp *Cla*I−*Eco*RI fragment the method of Kramer *et al.* (1984) was used. From a subclone we isolated an *Eco*RI fragment consisting of the 180-bp fragment from YEp24-3 plus the 25 bp pBR322 sequence between the *Eco*RI and *Cla*I restriction sites. This fragment was subcloned into the *Eco*RI site of RF-DNA of M13mp9, having two mutations in essential M13 genes. Gapped duplex DNA was made by hybridization of single-stranded DNA from this construct with *Eco*RI digested linear double-stranded DNA of M13mp9rev, lacking these two mutations. Synthetic oligonucleotides 5′-GGAGTTACTTACACTCC-3′ (mut1) and 5′-GGAGTTACGTAAACTCCTTG-3′ (mut2) (the introduced base changes are underlined) were a kind gift of H.Domdey (Munich). They were hybridized to the gapped duplex DNA and filled in with *E. coli* DNA-polymerase (Klenow fragment) and ligated with T4 DNA-ligase. The resulting DNA was transformed into strain BMH18-71 and white plaques were selected for phage-isolation and dot-blot hybridization to the 180-bp fragment. Phage positive in this test were used for infection of strain WK6. Normally, >80% of the plaques in this strain are formed by phage with the desired mutation in the single-stranded DNA (Kramer *et al.*, 1984). From RF-DNA the 180-bp *Eco*RI−*Cla*I fragment was reisolated and recloned in the autonomous plasmid YEp24-3. In order to do this a derivative of YEp24-3 was constructed with single sites for these enzymes in the DNA insert.

Miscellaneous

Published procedures have been used for the isolation and hybridization of mitochondrial DNA and RNA (Pillar *et al.*, 1983b), the isolation of plasmid DNA

and *E. coli* and yeast transformations (Kreike *et al.*, 1986). The 1.2-kb *Hae*III fragment from Yep24-3 was subcloned in plasmid pSP64 and *in vitro* transcribed RNA was used as a radioactive probe (Green *et al.*, 1983). Evaluation of the DNA sequence data was performed on a Commodore personal computer PC10 using programs described by Lang and Burger (1986).

Acknowledgements

The helpful and critical comments of G.Rödel and R.J.Schweyen on the manuscript are acknowledged. We thank H.-J.Fritz and colleagues (Munich) for hospitality and help with the *in vitro* mutagenesis experiments, H.Domdey (Munich) for the gift of the oligonucleotides and E.Praetzel for expert technical assistance. J.K. was the recipient of a grant from the Commission of the European Communities. This work was supported by the Deutsche Forschungsgemeinschaft.

References

Belfort,M. and Pedersen-Lane,J. (1984) *J. Bacteriol.*, **160**, 571–378.
Belfort,M., Maley,G., Pedersen-Lane,J. and Maley,F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4914–4918.
Bellisario,R.L., Maley,G.F., Guarino,D.U. and Maley,F. (1979) *J. Biol. Chem.*, **254**, 1296–1300.
Bennetzen,J.L. and Hall,B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
Casino,A., Cipollaro,M., Guerrini,A.M., Mastrocinque,G., Spena,A. and Scarlato,V. (1981) *Nucleic Acids Res.*, **9**, 1499–1518.
Cech,T.R. (1986) *Cell*, **44**, 207–210.
Dayhoff,M.O. (1979) *Atlas of Protein Sequence and Structure*. NBRF, Washington, DC, Vol. 5.
Delahodde,A., Banroques,J., Becam,A.M., Goguel,V., Perea,J., Schroeder,R. and Jacq,C. (1985) In Quagliariello,E., Slater,E.C., Palmieri,F., Saccone,C. and Kroon,A.M., (eds), *Achievements and Perspectives of Mitochondrial Research, Vol. II, Biogenesis*. Elsevier, Amsterdam, pp. 79–88.
Fasiolo,F., Bonnet,J. and Lacroute,F. (1981) *J. Biol. Chem.*, **256**, 2324–2328.
Garriga,G. and Lambowitz,A.M. (1984) *Cell*, **39**, 631–641.
Green,M.R., Maniatis,T. and Melton,D.A. (1983) *Cell*, **32**, 681–694.
Hill,J., McGraw,P. and Tzagoloff,A. (1985) *J. Biol. Chem.*, **260**, 3235–3238.
Holl,J., Schmidt,C. and Schweyen,R.J. (1985) In Quagliariello,E., Slater,E.C., Palmieri,F., Saccone,C. and Kroon,A.M. (eds), *Achievements and Perspectives of Mitochondrial Research, Vol. II, Biogenesis*. Elsevier, Amsterdam, pp. 227–236.
Jacq,C., Pajot,P., Lazowska,J., Dujardin,G., Claisse,M., Groudinski,O., De la Salle,H., Grandchamp,C., Labouesse,M., Gargouri,A., Guiard,B., Spyridakis,A., Dreyfus,M. and Slonimski,P.P. (1982) In Slonimski,P.P., Borst,P. and Attardi,G. (eds), *Mitochondrial Genes*. Cold Spring Harbour Laboratory Press, New York, pp. 155–183.
Kozak,M. (1981) *Nucleic Acids Res.*, **9**, 5233–5252.
Kramer,W., Drutsa,V., Jansen,H.-W., Kramer,B., Pflugfelder,M. and Fritz,H.-J. (1984) *Nucleic Acids Res.*, **12**, 9441–9456.
Kreike,J., Schulze,M., Pillar,T., Körte,A. and Rödel,G. (1986) *Curr. Genet.*, **11**, 185–191.
Kruger,K., Grabowski,P.J., Zaug,A.J., Sands,J., Gottschling,D.E. and Cech,T.R. (1982) *Cell*, **31**, 147–157.
Lang,B.F. (1984) *EMBO J.*, **3**, 2129–2136.
Lang,B.F. and Burger,G. (1986) *Nucleic Acids Res.*, **14**, 455–465.
Langford,C.J. and Gallwitz,D. (1983) *Cell*, **33**, 519–527.
Lazowska,J., Jacq,C. and Slonimski,P.P. (1980) *Cell*, **22**, 333–348.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
McGraw,P. and Tzagoloff,A. (1983) *J. Biol. Chem.*, **258**, 9459–9468.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20–79.
Michel,F. and Dujon,B. (1983) *EMBO J.*, **2**, 33–38.
Myers,A.M. and Tzagoloff,A. (1985) *J. Biol. Chem.*, **260**, 15371–15377.
Myers,A.M., Pape,L.K. and Tzagoloff,A. (1985) *EMBO J.*, **4**, 2087–2092.
Normarck,S., Bergström,S., Edlund,T., Grundström,T., Jaurin,B., Lindberg,F.P. and Olsson,O. (1983) *Annu. Rev. Genet.*, **17**, 499–525.
Peebles,C.L., Perlman,P.S., Mecklenburg,K.L., Petrillo,M.L., Tabor,J.H., Jarrell,K.A. and Cheng,H.-L. (1986) *Cell*, **44**, 213–223.
Pillar,T., Kreike,J., Lang,B.F. and Kaudewitz,F. (1983a) In Schweyen,R.J., Wolf,K. and Kaudewitz,F. (eds), *Mitochondria 1983*. Walter de Gruyter, Berlin, pp. 411–423.
Pillar,T., Lang,B.F., Steinberger,I., Vogt,B. and Kaudewitz,F. (1983b) *J. Biol. Chem.*, **258**, 7954–7959.
Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
Schmelzer,C., Schmidt,C., May,K. and Schweyen,R.J. (1983) *EMBO J.*, **2**, 2047–2052.
Schmelzer,C. and Schweyen,R.J. (1986) *Cell*, **46**, 557–565.
Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Nature*, **282**, 39–43.
Twigg,A.J. and Sherratt,D. (1983) *Nature*, **283**, 216–218.
Tzagoloff,A., Crivellone,M., Gampel,A., Hill,J., Koerner,T.J., Myers,A. and Pape,L. (1985) In Quagliariello,E., Slater,E.C., Palmieri,F., Saccone,C. and Kroon,A.M. (eds), *Achievements and Perspectives of Mitochondrial Research. Vol. II, Biogenesis*. Elsevier, Amsterdam, pp. 371–378.
Van Der Veen,R., Arnberg,A.C., Van Der Horst,G., Bonen,L., Tabak,H.F. and Grivell,L.A. (1986) *Cell*, **44**, 225–234.
Waring,R.B. and Davies,R.W. (1984) *Gene*, **28**, 277–291.
Weiss-Brummer,B., Rödel,G., Schweyen,R.J. and Kaudewitz,F. (1982) *Cell*, **29**, 527–536.
Zaret,K.S. and Shermann,F. (1982) *Cell*, **28**, 563–573.

Received on January 12, 1987; revised on April 13, 1987