RNA splicing defects in mitochondrial intron mutants can be suppressed by a high dosage of several proteins encoded by nuclear genes. In this study we report on the isolation, nucleotide sequence, and possible functions of the nuclear MRS2 gene. When present on high copy number plasmids, the MRS2 gene acts as a suppressor of various mitochondrial intron mutations, suggesting that the MRS2 protein functions as a splicing factor. This notion is supported by the observations that disruption of the single chromosomal copy of the MRS2 gene causes (i) a pet* phenotype and (ii) a block in mitochondrial RNA splicing of all four mitochondrial group II introns, some of which are efficiently self-splicing in vitro. In contrast, the five group I introns monitored here are excised from pre-mRNA in a MRS2-disrupted background although at reduced rates. So far the MRS2 gene product is unique in that it is essential for splicing of all four group II introns, but relatively unimportant for splicing of group I introns. In strains devoid of any mitochondrial introns the MRS2 gene disruption still causes a pet* phenotype and cytochrome deficiency, although the standard pattern of mitochondrial translation products is produced. Therefore, apart from RNA splicing, the absence of the MRS2 protein may disturb the assembly of mitochondrial membrane complexes.

Organelle introns have been classified as group I and group II introns according to conserved secondary structure features and group specific excision pathways (1, 2). Group II introns have been detected so far in mitochondrial and chloroplast genomes whereas group I introns are widespread in other genomes, namely in the nuclear rRNA genes of Tetrahymena and in genes of bacteriophages and cyanobacteria (1-6). Some members of each group have been shown to be catalytic in vitro; they excise themselves from pre-mRNA and ligate the exons in the absence of any protein (7-9). The splicing pathway and conserved sequence elements at the splice junctions relate group II introns with the nuclear mRNA introns; both classes of introns are excised as branched circular RNAs, the so-called lariats, with a most intriguing 2'--5' phosphodiester bond. This relatedness has led to the speculation that the autocatalytic group II introns are the evolutionary ancestors of the nuclear (non-autocatalytic) mRNA introns (10).

The finding of self-catalysis of mitochondrial introns in vitro contrasts a number of observations which have revealed that excision of group I and group II introns in vivo involves proteins (see Refs. 11 and 12 for review), encoded either within the introns themselves, the so-called maturases, or by nuclear genes. Two of these nuclear genes in yeast, MRS1 and CBP2, encode products that are needed only for the excision of group I introns (b13 and a15b; b15, respectively); yeasts lacking the introns in question are unaffected by disruption of these nuclear genes (13-15). Others encode mitochondrial proteins of dual function, like CYT18 in Neurospora crassa and NAM2 in yeast which serve as tRNA synthetases and are in addition essential splicing factors (16, 17). Finally, the yeast MSS116 gene encodes a protein with similarity to a helicase; its absence affects the excision of both group I and group II introns and other, so far unknown functions (18).

We previously described the isolation of three nuclear genes, MRS2, MRS3, and MRS4, whose products appear to be involved in the excision of group II introns from mitochondrial pre-mRNA (19). They have been detected by virtue of their ability to suppress a splice defect exerted by a group II intron mutation, when present in high copy number.

MRS3 and MRS4 were found to be closely related genes; both can be disrupted without causing any phenotypically detectable effect on splicing or any other function (20, 21). We show here that the MRS2 gene, unlike the MRS3 and MRS4 gene, is essential for the excision of all four group II introns present in yeast mitochondrial RNAs, whereas it hardly affects the excision of group I introns. MRS2 has additional, so far unknown functions since its disruption in yeast strains lacking all known introns still leads to respiratory deficiency.

**MATERIALS AND METHODS**

**Strains and Plasmids**—The genotypes and origins of the yeast strains used in this study are described in Table I. Escherichia coli strain XL1-Blue (Stratagene) and the following plasmids were used for subcloning: YEp351 (24), Bluescript (Stratagene). Rho' derivatives of yeast strains were obtained by growing cultures for about 30 generations in YPD medium containing 50 μg/ml ethidium bromide.

**Media**— Yeast strains were grown in complete medium YP (1% yeast extract, 2% peptone) enriched with either the fermentable carbon sources glucose (2% w/v) glucose, YPD), raffinose (2.5% raffinose, YPR) or with the nonfermentable carbon source glycerol (3% w/v; glycerol, YPG). YPG was occasionally supplemented with 0.05% glucose (YPG) to improve initial growth of the cells after replica plating; Synthetic minimal medium (0.67% Difco yeast nitrogen base, 2% glucose (SD) or 2.5% Raffinose (SR) were supplemented with amino acids and bases when appropriate.
Mitochondrial genomes are indicated in brackets. If not otherwise stated, rho+ strains of \textit{S. cerevisiae} contain introns b1, b2, b3, b4, and b5 of the COB gene, introns a11, a12, a13, a14, a15a, a15b, and a15c of the COX1 gene, and the 21 S rRNA intron \(\omega\). \(\Delta\)b1 and \(\Delta\)a1 indicate that the respective introns of COB or COX1 are deleted, whereas \(\Delta\)b1 and \(\Delta\)a1 indicate that all introns are deleted. The mitochondrial genome of \textit{Saccharomyces uvarum} (SU) contains the following introns: b14, b15, a13, a15c (22). EtdBr, ethidium bromide.

### TABLE 1

| Strain          | Genotype: nuclear [mitochondrial] | Origin or Reference          |
|-----------------|-----------------------------------|-----------------------------|
| DBY747          | MATa leu2-3 leu2-11 his3-1 ura3-52 trp1-289 [rho+ mit+] | ATCC 44774                  |
| DBY747/M301     | MATa leu2-3 leu2-119 his3-1 ura3-52 trp1-289 [rho+ mit-M301] | (19)                        |
| DBY747/rho*     | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho*] | (19)                        |
| GW7/gd2-21.2    | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho*] | This study; disruption of DBY747 |
| GW7/gd2-21.2/rho* | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho*] | This study; EtdBr treatment of GW7/gd2-21.2 |
| KGF177          | MATa his4 trp5 ade6 [rho+ mit+] | G. Faye* (22)               |
| KGF177/rho*     | MATa his4 trp5 ade6 [rho*] | This study; EtdBr treatment of KGF177 |
| IC8/rho*        | MATa leu2 [rho*] | J. Bousquet* (14)          |
| CK506           | MATa leu2 [rho* mit+] | G. Faye*                   |
| GF132-10A       | MATa his1 met6 [rho* mit+] | This study                  |
| GF167-7B        | MATa lye2 [rho* mit+]; \(\Delta\)b1,2,3,2a11,2,5a-c | G. Faye* (22)               |
| DBY747/CK       | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5 | This study                  |
| DBY747/SU       | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| DBY747/grII     | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| DBY747/wo       | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| GW7/gd2-21.2/CK | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| GW7/gd2-21.2/SU | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| GW7/gd2-21.2/grII | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |
| GW7/7/gd2-21.2/wo | MATa leu2-3 leu2-112 his3-1 ura3-52 trp1-289 [rho* mit+]; \(\Delta\)b11,2,3,2a11,2a5,4a5,5a-c | This study                  |

* Mitochondria were introduced in respective strains via cytoduction by the use of karl strains (23).

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**DNA Subcloning and Sequencing**—Appropriate DNA restriction fragments were subcloned into Bluescript vectors and sequencing was done with the dideoxy chain termination method using the T7 sequencing kit (Pharmacia LKB Biotechnology Inc.). Restriction enzyme sites used for subcloning are shown in Fig. 1. For sequencing of some larger fragments we used specific primers. Nucleotide sequences of both strands were determined. For DNA sequence analysis we used the MICROGENIE program (Beckman). The SWISSPROT Protein Sequence Library (release 18, May 1991) and the EMBL Nucleotide Sequence Library (release 27, May 1991) were searched by use of the FASTP program (26).

**Transformation of Yeast and \textit{E. coli}**—\textit{E. coli} strains were transformed by the CaCl2 procedure (25). Yeast transformation was done using the lithium acetate method (27).

**Southern Blot Analyses**—DNA preparation from yeast was performed following the method described in Ref. 28, whereas blotting and hybridization was done as described in Ref. 25.

**Northern Blot Analyses**—Northern blot analyses were done as described previously (21).

**Analyses of Mitochondrial Translation Products**—Labeling of mitochondrial proteins was done essentially as described by Haid et al. (29) except that cells were cultivated in YPR medium and starved for methionine prior to labeling in SR-meth medium (synthetic medium with raffinose as carbon source, lacking methionine). Cells were labeled with \([^{35}S]\)methionine (Amersham Corp.; 25 \(\mu\)Ci/ml culture) in the presence of cycloheximide (100 \(\mu\)g/ml). Small scale isolation of mitochondrial was done according to the method described by Needleman and Tzagoloff (30). Proteins were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and after fluorography the dried gels were autoradiographed (31).

**Cytchrome Spectra**—Cells were grown on YPR-plates for 2 days, suspended in water, and washed twice with water. After treatment of the cells with an excess of sodium dithionite in suspension, the pellets were applied to the window of a home-made cuvette and frozen in liquid nitrogen.

Spectra were recorded against several layers of Parafilm in a Hitachi 150-20 spectrophotometer.

### RESULTS

**Multi-copy Suppression**—In a previous communication we have reported on the isolation of three nuclear genes, MR2S, MR3S, and MR4S from a yeast gene library. When present on the high copy number vector YEp13, these three wild-type genes can suppress the phenotypic effect exerted by the mit" mutation M1301 (19). This mit" mutation is a single base pair deletion in domain 3 of the group II intron b1, the first intron of the cytochrome b gene COB; in vivo it causes a complete block in the excision of this intron and, due to a
The translation efficiency of the MRS2 gene prevented us so far from performing import assays of the MRS2 protein into isolated mitochondria as has been successfully done with the MRS3 and MRS4 proteins (21).

The DNA sequence of the MRS2 gene and the deduced amino acid sequence were compared with sequences of the available data libraries (EMBL and SWISSPROT). We did not find any convincing similarities of the MRS2 sequences with those in the libraries, nor did we detect any consensus sequence motifs which might represent RNA, DNA, or nucleotide binding elements.

Effects of the MRS2 Gene Disruption on Mitochondrial RNA Splicing—In order to inactivate the chromosomal copy of the MRS2 gene, an internal 0.5-kb SauI fragment of this gene was replaced by a 1.7-kb fragment containing the yeast HIS3 gene (Fig. 1). The haploid strain DBY747 rho" was transformed with this construct following the one-step gene disruption method (39). Stable HIS" transformants were isolated, and the replacement of the wild-type MRS2 copy by the disrupted one was confirmed by Southern hybridization (data not shown). The mrs2::HIS3 gene disrupted strain (GW7/gd2-21.2) was unable to grow on non-fermentable substrates (cf. Fig. 6). The disruption thus causes a "rho" phenotype. This indicates that the MRS2 gene product is essential for the formation of a functional respiratory chain.

To determine a possible effect of the MRS2 gene disruption on mitochondrial RNA splicing, we have performed a series of Northern hybridization experiments using mRNA of strain GW7/gd2-21.2 which contains the disrupted MRS2 gene. The patterns of the COB and COX1 transcripts of this strain are shown in Fig. 3 in parallel with those of the wild-type strain DBY747 and the intron b11 mutant DBY747/M1301, which are isogenic with strain GW7/gd2-21.2 except for the MRS2 alleles and the b11 mutation.

Probes specific for the COB exon B1 (including part of the leader) and for the COB intron b11 detect the mature mRNA and the excised b11 RNA as the major COB transcripts in the wild-type strain (Fig. 3). The strains with the nuclear MRS2 gene disruption (GW7/gd2-21.2) or with the b11 intron mutation (DBY747/M1301) lack these mature RNAs. Instead, they accumulate high molecular weight RNAs of similar sizes. These have been shown to comprise all COB exon and intron sequences, or all but the last COB intron (32).

The pleiotropic effect on intron excision may be due to a primary block in the excision of the group II intron b11 which, as a secondary effect, prevents the expression of maturases of downstream introns and hence their excision from pre-mRNA (32, 40–42). Alternatively, disruption of MRS2 may equally block excision of group I and group II introns. This latter possibility was ruled out by the following experiment. Mitochondria of strain KG177 (Saccharomyces uwarum) and of strain CK506 (Saccharomyces cerevisiae), containing only group I introns b14 and b15 or introns b13, b14 and b15, respectively (22, 14), were combined by cytoduction with the MRS2 disrupted nuclear background (strain GW7/gd2-21.2). These cytoductants accumulate mature COB mRNA (data not shown). The same result was obtained with a mrs2:: strain lacking all group II (and some of the group I) introns as shown in Fig. 5. We conclude therefore that excision of the group I introns b13, b14, and b15 from the COB precursor is only little effected by the MRS2 disruption, whereas splicing of the group II intron b11 is totally blocked in a mrs2:: strain. Additionally, we were not able to investigate the excision of the group I intron b12, since there was no strain available so far that lacks only b11; excision of b11 is necessary for splicing of b12, because this step is dependent on a functional maturase (32).

The abbreviation used is: kb, kilobase(s).
The MRS2 gene was disrupted in yeast strains. RNAs prepared from strains DBY747 (middle lanes, M1301), and from strains GW7/gd2-21.2 carrying the disrupted MRS2 (right lanes, mrs2::), were hybridized with radioactivity labeled, in vitro generated RNA probes complementary to a part of the untranslated leader and the first exon (COB-1, panel B), to the first intron (b11, panel D) of the mitochondrial COB transcript or to exon 4 of the COX1 gene (A4, panel C). Bands representing the mature COB or COX1 mRNAs or the excised intron b11 are marked by arrowheads.

In order to test whether excision of group II introns in the COX1 transcript is also affected, Northern hybridization experiments were performed with probes specific for exon A4 and for the three group II introns in the COX1 gene, a11, a12, and a15c (Figs. 3 and 4). As shown in Fig. 3 C) no mature mRNA can be detected in the mrs2:: strain. Instead, the disruption of MRS2 leads to accumulation of high molecular weight transcripts hybridizing with all group II introns. Taken together the results presented above show that the MRS2 gene product is an essential factor for the excision of all four group II introns from yeast mitochondrial COB and COX1 pre-mRNAs.
of COX1 introns but it exerts only the so-called "box" effect, an interplay between the introns b14 and a14 (44). The COX1 transcript pattern of the mrs2::HIS3 strain can be interpreted as containing various combinations of the three group I introns and the intron a14. This indicates that the other group I introns of the COX1 pre-mRNA are excised in the absence of the MRS2 gene product. A polarity effect similar to that of the non-excised intron b11 in COB pre-mRNA may not be detected with the COX1 pre-mRNA since the effect of the MRS2 disruption on excision of the first two introns a11 and a12 is somewhat leaky and thus allows for sufficient expression of maturases encoded in the downstream introns.

A more direct test for the effect of the MRS2 gene disruption on the excision of group I introns was performed with mitochondria lacking all group II introns but retaining some group I introns only. For this experiment mitochondria from strain GF132-10A (retaining group I introns b14, b15, a13, a14) were introduced into the nuclear background of the MRS2-disrupted strain GW7/gd2-21.2 via cytoduction. In Fig. 5 the effect of MRS2 disruption on processing of COB and COX1 RNAs is compared in various mitochondrial backgrounds. In mrs2:: strains containing the "long" version of mitochondrial DNA (b11–b15; a11–a15c) no mature mRNAs of both genes can be detected. In contrast, in mrs2:: cells lacking all group II and some group I introns (Δ group II) mature mRNAs are clearly present, although in a slightly lower amount than in the correspondent wild type. Further predominant bands in this figure correspond to COB mRNA+a15c (panel A) and COX1 mRNA+a14 (panel B). These bands, which are also present in the wild type lanes, together with some other weaker precursor bands occurring only in the mrs2:: background might indicate that disruption of MRS2 has also some effect on group I intron splicing. In the Δ group II strain (containing only introns a13 and a14) one would expect only three COX1 precursor bands. The A4 probe we used contains also 870 bases of intron a14, and this intron is highly homologous to the COB intron b14 (1). Additional precursor bands in a blot hybridized with the A4 probe are caused by a crosshybridization between these two introns.

When transcripts from mitochondria that lack all known group I and group II introns (22) were analyzed no difference was seen between those from a MRS2 (wild type, DBY747/wo) and mrs2:: disrupted (GW7/gd2-21.2/wo) strain (Fig. 5).

Functions of the MRS2 Gene Product aside from RNA Splicing—As shown above, deletion of group II introns or of all introns from mtDNA apparently cures MRS2-disrupted stains of their defect in RNA processing. If the only function of the MRS2 gene product were to participate in splicing, one would expect that this intron deletion would also restore growth of MRS2 disrupted cells on a non-fermentable substrate. This was not the case; the MRS2 gene disruption caused the same pet" phenotype irrespective of the mitochondrial genotype (Fig. 6). This suggests that the MRS2 gene product has another essential function in mitochondria.

A comparison of cytochrome spectra of wild-type (DBY747/wo; MRS2) and mrs2::HIS3 (GW7/gd2-21.2/wo; mrs2::) stains with intron-less mtDNAs reveals a striking absence of cytochrome a,a3, and a dramatic decrease of cytochrome b in the MRS2 disrupted cells compared with wild-type cells (Fig. 7); a cytochrome spectrum of the mrs2:: strain GW7/
**Control of Group II Intron Splicing**

The MRS2 gene was found during a search for nuclear genes that suppress a mitochondrial mutation in a group II intron when this gene was present in high copy number (19). This suppressor activity suggested that the MRS2 gene product might have a function in the excision of group II (and possibly also group I) introns from mitochondrial pre-mRNAs. Here we show that disruption of this gene indeed blocks the excision of all four known group II introns from the COB and COX1 transcripts and has little effect on the excision of some group I introns. The MRS2 gene product may have a weak effect on mitochondrial RNA splicing in general, but it is an essential factor only for the excision of group II introns.

In this respect the MRS2 gene is unique among the many known genes that affect mitochondrial RNA splicing. One of these genes, CBP2, is necessary for excision of only one single intron (bI5) (45), whereas another one, MRS1, is an indispensible factor for two group I introns (bI3 and aI5b) (13, 14). Yeasts lacking the respective introns are unaffected by the disruption of these nuclear genes. On the other hand, most of the nuclear genes involved in mitochondrial RNA splicing described so far have other functions besides their involvement in the splicing process. CYT18 in *N. crassa* and NAM2 in yeast code for proteins with dual function; they serve as tRNA synthetases and in addition are essential factors for group I intron excision (16, 17); PET54 affects translation of the mitochondrially encoded COX3 gene and also splicing of aI5b (46); NAM1 is a mitochondrial transcription factor and also seems to be involved in mitochondrial splicing (47, 48). The MSS116 gene, which turned out to encode a RNA helicase type protein, and the MSS18 gene both have a second, still unknown function besides their action on mitochondrial RNA splicing (18, 49).

Apparently the involvement in mitochondrial RNA splicing is also not the only function of the MRS2 gene product. This notion rests on the fact that disruption of the MRS2 gene causes a pet- phenotype, even when combined with a mitochondrial genome lacking the four known group II introns or all known introns (cf. Fig. 6). Furthermore, the intron-less mitochondria appear to synthesize the standard set of major mitochondrial proteins and yet lack the cytochrome a.a3 spectral bands (cf. Figs. 7 and 8). This parallels a study on the MSS116 gene (18), which is involved in both group I and group II intron excision; its disruption also resulted in a pet- phenotype, irrespective of the presence or absence of the known introns in mtDNA. Both findings could easily be explained by the assumption that not all introns in yeast mtDNA have been identified, but this seems unlikely since this genome has been sequenced almost completely. Alternatively the products of both genes MSS116 and MRS2 might be involved in other processing or modification events of mitochondrial transcripts.

Both of these explanations attribute to the MRS2 gene product the primary (and only) function in RNA processing. However, this does not easily explain the fact that cytochrome a.a3 is absent and cytochrome b is reduced in a MRS2 disrupted strain lacking all introns although the major, mitochondrially encoded proteins are synthesized. These data rather hint at the involvement of the MRS2 gene product in the formation of functional cytochrome complexes. It may fulfill a basic function in mitochondrial biogenesis, e.g. in the assembly of cytochromes as it has also been suggested for genes COX10 and COX11 (50, 51) or it may be involved in the expression or the import of some nuclear encoded subunits of the cytochrome complexes.

**DISCUSSION**

**Fig. 7. Disruption of MRS2 gene leads to a deficiency of cytochromes in an intron-less strain.** Cells of strain DBY747, GW7/gd2-12.2, and DBY747/M1301 (marked MRS, mrs:, and M1301, respectively) were grown in YPR medium and prepared for the recording of low temperature spectra as described under "Materials and Methods." cyt b, cytochrome b; aa3, cytochrome a.a3.

**Fig. 8. MRS2-disrupted strains exhibit a standard pattern of mitochondrial translation products.** Labeling and analysis of mitochondrial translation products are described under "Materials and Methods." The positions of the mitochondrially synthesized proteins are indicated by arrows: VAR1, ribosomal protein; COX1, COX2, and COX3, subunits I, II, and III of cytochrome c oxidase; ATPase 6, subunit 6 of mitochondrial oligomycin-sensitive ATPase. Strains and designations are as in Fig. 5.

gd2-21.2 containing all mitochondrial introns is identical to that of strain DBY747/M1301 (data not shown). However, it is not the absence of the mitochondrially made subunits of the cytochrome oxidase that cause this cytochrome a.a3 deficiency. As shown in Fig. 8, patterns of mitochondrial translation products are similar in MRS2 and mrs2::HIS3 strains lacking all introns whereas mrs2::HIS3 cells containing all introns lack apocytochrome b and subunit I of cytochrome oxidase, as expected from their lack of the respective mature mRNAs. The only effect which we can see is a reduced amount in the bands representing COX3 and subunit 6 of the ATPase. However, this effect is also seen in MRS2 cells with the cob- mutation M1301 and therefore may be a secondary effect of their respiratory deficiency.
We conclude from our observation that the MRS2 gene product might serve other functions besides RNA splicing and thus behave like several of the other nuclear genes involved in mitochondrial RNA splicing (12).

Yet, from our results we cannot exclude that MRS2 affects respiration and splicing fairly indirectly. We are currently investigating spontaneous suppressor mutations of MRS2 that restore growth on nonfermentable carbon sources. Preliminary results reveal the existence of a class of dominant nuclear suppressors that restore growth on glycerol of mrs2:: strains harboring the full set of mitochondrial introns (long version). Thus, the suppressor mutants are independent of the MRS2 gene product as far as basic functions in mitochondrial biogenesis are concerned. However group II intron splicing remains dependent on this product. We conclude therefore that the gene product of MRS2 is indeed involved in two different processes of mitochondrial biogenesis, directly or indirectly, and that the splicing deficiency is not simply the consequence of some yet unknown process also leading to respiratory deficiency.

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