Mitochondrial Mg$^{2+}$ homeostasis is critical for group II intron splicing in vivo

Juraj Gregan,1 Martin Kolisek, and Rudolf J. Schweyen2

Vienna Biocenter, Department of Microbiology and Genetics, University of Vienna, A-1030 Vienna, Austria

The product of the nuclear MRS2 gene, Mrs2p, is the only candidate splicing factor essential for all group II introns in mitochondria of the yeast Saccharomyces cerevisiae. It has been shown to be an integral protein of the inner mitochondrial membrane, structurally and functionally related to the bacterial CorA Mg$^{2+}$ transporter. Here we show that mutant alleles of the MRS2 gene as well as overexpression of this gene both increase intramitochondrial Mg$^{2+}$ concentrations and compensate for splicing defects of group II introns in mit$^{-}$ mutants M1301 and B-loop. Yet, covariation of Mg$^{2+}$ concentrations and splicing is similarly seen when some other genes affecting mitochondrial Mg$^{2+}$ concentrations are overexpressed in an mrs2Δ mutant, indicating that not the Mrs2 protein per se but certain Mg$^{2+}$ concentrations are essential for group II intron splicing. This critical role of Mg$^{2+}$ concentrations for splicing is further documented by our observation that pre-mRNAs, accumulated in mitochondria isolated from mutants, efficiently undergo splicing in organello when these mitochondria are incubated in the presence of 10 mM external Mg$^{2+}$ (mit$^{-}$ M1301) and an ionophore (mrs2Δ). This finding of an exceptional sensitivity of group II intron splicing toward Mg$^{2+}$ concentrations in vivo is unprecedented and raises the question of the role of Mg$^{2+}$ in other RNA-catalyzed reactions in vivo. It explains finally why protein factors modulating Mg$^{2+}$ homeostasis had been identified in genetic screens for bona fide RNA splicing factors.

[Key Words: Group II introns; RNA splicing; Mg$^{2+}$; yeast; mitochondria; Mrs2p]

Received February 21, 2001; revised version accepted July 6, 2001.

Group II intron RNAs are distinct from group I intron RNAs by their secondary and tertiary structures as well as by their mechanisms of splicing. RNAs of several group II introns have been shown to undergo self-splicing reactions in vitro via a lariat intermediate (for review, see Michel and Ferat 1995), and they are widely believed to be ancestors of nuclear pre-mRNA introns (Hetzer et al. 1997; Sontheimer et al. 1999). Standard in vitro assay conditions are elevated temperatures, high salt, and 50–100 mM Mg$^{2+}$. These unphysiological in vitro conditions are likely to reflect the absence of factors that facilitate RNA splicing in vivo (for review, see Saldanha et al. 1993; Grivell 1995). Two of these factors are the DEAD box proteins involved in RNA-associated functions and DNA endonucleases in intron mobility (for review, see Curcio and Belfort 1996; Eickbush 2000). As revealed by studies on a bacterial group II intron and its open reading frame, binding of the intron-encoded protein to its cognate RNA is a prerequisite for its splicing (Wank et al. 1999).

Mitochondrial transcripts of the yeast Saccharomyces cerevisiae contain a total of four group II introns—aI1, aI2 and aI5c in the COX1 gene, and bI1 in the COB gene, all of which have been shown to catalyze their own splicing in vitro (for review, see Michel and Ferat 1995). Two of them (aI1, aI2) contain open reading frames whose products function as so-called RNA maturases of the cognate introns, as first revealed by genetic analyses (Carignani et al. 1983; Kennell et al. 1993), and as reverse transcripts and DNA endonucleases in intron mobility (for review, see Curcio and Belfort 1996; Eickbush 2000). As revealed by studies on a bacterial group II intron and its open reading frame, binding of the intron-encoded protein to its cognate RNA is a prerequisite for its splicing (Wank et al. 1999).

Genetic screens have been instrumental in identifying nuclear genes whose products affect mitochondrial group II intron splicing. However, most of them proved to be involved in other mitochondrial functions as well (for reviews, see Saldanha et al. 1993; Grivell 1995). The yeast MSS116 gene encodes a protein related to the DEAD box proteins involved in RNA-associated functions. Its overexpression promotes ATP-dependent splicing of a yeast group II intron in mitochondrial extracts. However, its function is not restricted to group II introns (Seraphin et al. 1989; Niemer et al. 1995). In algae and plants a series of nuclear gene products has been shown to affect group II intron trans-splicing in chloroplasts, among them the Mia2 and Csr2 gene products, related to pseudouridine synthases and peptidyl tRNA hydrolase, respectively (Perron et al. 1999; Jenkins and Barkan 2001).

We selected several nuclear genes that are able to suppress the RNA splicing defect of a mit$^{-}$ mutation (M1301) in the group II intron bI1 when they are ex-
pressed from a multicopy plasmid. One of them, MRS2, proved to be essential for the excision of all four group II introns in yeast mitochondrial transcripts, but not for the splicing of group I introns or other mitochondrial RNA processing events (Wiesenberger et al. 1992). In a different search for suppressors of group II intron splicing defects the MRS2 gene has been isolated once more (Schmidt et al. 1996, 1998), indicating that its suppressor effect on RNA splicing is of high significance. So far MRS2 is the only gene whose product is known to be involved in splicing of all introns of a given type in yeast mitochondria. However, its role is not restricted to RNA splicing, as revealed by the fact that mitochondrial functions of yeast strains with intronless mitochondria are also affected by the absence of the MRS2 protein, resulting in the so-called petite (pet) growth phenotype (Wiesenberger et al. 1992). It has been hypothesized, therefore, that MRS2 might be bifunctional, being involved in group II intron splicing and in some other mitochondrial function. Alternatively, the effect of MRS2 might be secondary to a more general mitochondrial function (Wiesenberger et al. 1992, Schmidt et al. 1998). In fact, we have recently shown that the MRS2 protein (Mrs2p) is an integral protein of the inner mitochondrial membrane, structurally and functionally related to CorA, the Mg2+ transporter of the bacterial plasma membrane (Bui et al. 1999).

Other multicopy suppressors were selected that could compensate both for the splicing defects of the mit− mutation M1301 and an mrs2 deletion mutant (mrs2Δ). Of those suppressor genes, MRS3, MRS4, and MRS12/RIM2 encode integral proteins of the inner mitochondrial membrane, belonging to the large family of mitochondrial solute carriers. Although the function of these three carrier proteins is still unknown, it had been speculated previously that their overproduction may alter solute concentrations in mitochondria, which in turn may compensate for RNA splicing and DNA replication defects (Wiesenberger et al. 1991; Van Dyck et al. 1995). Furthermore, two genes of this series, MRS5 and MRS11, have been shown to encode proteins of the mitochondrial intermembrane space. Mrs5p and Mrs11p (renamed Tim12p and Tim10p, respectively) have been found to be key components of a specific import pathway for solute carrier proteins and other multimembrane-spanning proteins (Koehler et al. 1998). Taken together then, the MRS series of multicopy suppressor genes studied so far either code for putative members of the mitochondrial ion or solute transporters, or mediate the import of these into the inner mitochondrial membrane.

Here we present evidence for a prominent role of the intramitochondrial Mg2+ concentration in supporting group II intron splicing. The increase of Mg2+ concentration by a factor of 1.5, mediated by either overexpression or by certain mutations of the putative Mg2+ transporter Mrs2p, can suppress RNA splice defects resulting from mit− point mutations in group II introns a15c and b11. A decrease of the mitochondrial Mg2+ concentration to about half of the wild type, as observed in mrs2Δ mutants, blocks RNA splicing of all four mitochondrial group II introns. This block can be overcome to a considerable degree by the overexpression of other proteins raising Mg2+ concentrations to near wild-type levels. Moreover, incubation of isolated mitochondria of mit− M1301 mutant mitochondria in 10 mM external Mg2+ or of mitochondria from an mrs2Δ mutant in 10 mM Mg2+ in the presence of an ionophore partially restored splicing of accumulated precursor RNAs. These results are indicative of a particular sensitivity of group II intron RNA splicing in vivo toward changes in Mg2+ concentrations.

Results

Gain-of-function mutations in the MRS2 gene suppress RNA splicing defects

The MRS2 gene has been selected as a multicopy suppressor of the mitochondrial mit− mutation M1301, a single base deletion in domain III of the first intron of the COB gene [b11], which causes a splicing defect of this intron in vivo and in vitro (Schmelzer and Schweyen 1986; Koll et al. 1987). The suppressor phenotype has been assumed to arise from a high dose of the MRS2+ gene and its product, Mrs2p (Wiesenberger et al. 1992). We have now transformed M1301 mutant yeast cells with the MRS2 gene on a low-copy-number, centromeric plasmid (YCp). Indeed, this gene dose leads to a very weak suppressor effect only (Fig. 1A). This offered the possibility to select for mutations in the plasmid-bound MRS2 gene that would suppress the effect of the intron mutation M1301 efficiently even when the gene was present on a low copy vector.

Upon random in vitro mutagenesis of the MRS2 gene by hydroxylamine, the mutagenized plasmid (YCplac22–MRS2+) was transformed into mit− mutant M1301 yeast cells, and the resulting transformants were replica-plated onto a nonfermentable substrate (YPdG) that does not support growth of mutant M1301 cells, and the resulting transformants were replica-plated onto a nonfermentable substrate (YPdG) that does not support growth of mutant M1301 cells, except for a weak initial growth. YPdG positive transformants, which restored growth to levels similar to mit− cells, were detected at a frequency of 10−4. Plasmid DNAs of 20 transformants were extracted, amplified in Escherichia coli, and used to retransform mit− mutant M1301 cells to confirm their suppressor activity. Nucleotide sequences of four inserts of the suppressing plasmids (alleles MRS2-M1, MRS2-M2, MRS2-M7, and MRS2-M9) were found each to carry one or two neighboring base substitutions (Fig. 2), leading to amino acid substitutions in the middle of the protein (positions 222, 260, 250, and 174/175, respectively). Three other gain-of-function mutations in the MRS2 gene (cf. Fig. 2), which previously had been identified by a different approach, also affected this central amino acid stretch of the Mrs2 protein (Schmidt et al. 1998).

In order to exclude any copy-number effects, these gain-of-function MRS2+ alleles were integrated into the yeast chromosome of two group II intron mutants, mit− M1301, defective in group II intron b11 (Schmelzer and Schweyen 1986), and in mit− B-loop, defective in group II

Gegen et al.
intron ale [Schmidt et al. 1996]. Mutant B-loop [Fig. 1B] as well as mutant M1301 [data not shown] regained growth on nonfermentable substrate. This indicated that the MRS2* alleles were efficient suppressors even when present in single copies and, furthermore, that they were not allele- or intron-specific.

RT–PCR was performed to analyze the extent to which the gain-of-function mutations restored splicing of group II intron-containing RNAs of the M1301 mutant. As shown in Figure 3, mutant M1301 transformed with the gain-of-function alleles MRS2-M1, MRS2-M2, MRS2-M7, or MRS2-M9 had splicing of intron bI1 restored to a considerable extent. The wild-type MRS2* allele on a low-copy plasmid [YCp] did not restore splicing to a significant extent, whereas this allele on a multicopy plasmid [YEp] did, but much less efficiently than the gain-of-function MRS2* alleles. Interestingly, growth rates of M1301 cells transformed with YEp-MRS2* wild-type and with YCp-MRS2* gain-of-function alleles were similar on nonfermentable substrate, indicating that a small fraction of mature COB mRNA is sufficient to sustain growth.

Levels of mutant MRS2 transcripts and proteins

These dominant effects of the four mutant alleles MRS2-M1, MRS2-M2, MRS2-M7, and MRS2-M9 may be owing to either increased expression or stability of the mutant Mrs2 proteins or to changes in their activity and specificity. Steady-state mRNA levels transcribed from the wild type and from the gain-of-function mutant MRS2* alleles integrated into the chromosome were not significantly different when tested by RT–PCR [Fig. 4A], excluding major effects of the mutations on the expression of the MRS2 gene.

Mutant protein levels, however, were somewhat increased as compared to the wild-type protein level [Fig. 4B]. This parallels findings of Schmidt et al. [1998], who also found elevated levels of Mrs2 proteins in their three gain-of-function mutants. Interestingly, overexpression...
Suppression of group II intron splice defects by overexpression of proteins other than Mrs2p

Suppression of growth defects of mrs2-1A mutant strains has been shown to be exerted by overexpression of other genes implicated in metal ion transport or homeostasis [Wiesenberger et al. 1992; Van Dyck et al. 1995]. We have now asked whether this suppression is correlated with a restoration of Mg$^{2+}$ concentrations in mitochondria.

Overexpression of Mrs3p or Mrs4p, two members of the mitochondrial carrier family, has been shown previously to suppress growth defects of mrs2-1A mutant cells efficiently and to restore RNA splicing [Waldherr et al. 1993]. As shown in Table 2, overexpression of these proteins in mrs2-1A strains also raised mitochondrial Mg$^{2+}$ concentrations by a factor of 2 from a low mutant to a standard wild-type level.

Similarly, overexpression of Alr1p, the plasma membrane Mg$^{2+}$ transporter [Graschopf et al. 2001], raised mitochondrial Mg$^{2+}$ concentrations in an mrs2-1A strain.

| Allele    | Mg$^{2+}$ [nmol/mg protein] |
|-----------|-----------------------------|
| YEp-MRS2+ | 284.2 ± 30.7                |
| YEp-M1    | 401.6 ± 30.9                |
| YEp-M7    | 409.2 ± 22.5                |
| YEp-M9    | 398.1 ± 24.8                |
| YEp-MRS2+ | 390.3 ± 51.7                |

Mitochondrial extracts were obtained from strain DBY747 mitM1301 transformed with the wild-type MRS2 gene in the multi-copy vector YEp351 (YEp-MRS2+) or in the low-copy vector YCplac22 (YCp-MRS2+) or with MRS2* mutant alleles in this low-copy vector (YCp-M1, YCp-M7, YCp-M9). Mg$^{2+}$ concentrations were determined by use of the metallochromic indicator eriochrome blue (Gregan et al. 2001). The values represent averages of at least four independent experiments.
Table 2. Overexpression of mitochondrial carrier-type proteins Mrs3p or Mrs4p, as well as overexpression of plasma-membrane Mg^{2+} transporter Alr1p, restore mitochondrial Mg^{2+} levels of strain DBY747 mrs2-1Δ

|          | Mg^{2+} [nmol/mg protein] |
|----------|--------------------------|
| wt       | 278.3 ± 25.2             |
| mrs2Δ    | 155.3 ± 38.2             |
| mrs2Δ/MRS3| 280.1 ± 30.3            |
| mrs2Δ/MRS4| 283.1 ± 29.8            |
| mrs2Δ/ALR1| 269.0 ± 32.5            |

Mitochondrial extracts were obtained from strain DBY747 transformed with an empty vector [wt] or from strain DBY747 mrs2-1Δ transformed either with an empty vector [mrs2Δ] or a multi-copy vector carrying the MRS3 [mrs2Δ/MRS3], the MRS4 [mrs2Δ/MRS4], or the ALR1 [mrs2Δ/ALR1] gene. Mg^{2+} concentrations were determined by atomic absorption spectrometry. The values represent averages of at least four experiments.

COB RNA of mutant mrs2-1Δ mitochondria was not processed upon addition of 10–50 mM Mg^{2+}, unless ionophore A23187, which is known to facilitate transport of divalent metal ions across membranes (Reed and Lardy 1972), and an uncoupler (DNP) were added [Fig. 6B]. Incubation of these mitochondria with other divalent ions [Ca^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}] in the presence of the ionophore A23187 again did not lead to the maturation of the transcripts in a detectable amount (data not shown). The need for an ionophore to raise Mg^{2+} concentrations in mitochondria of mrs2-1Δ cells is consistent with the notion that these mitochondria lack an efficient Mg^{2+} transport system.

Using the Mg^{2+}-specific mag-fura 2 indicator, we attempted to measure free ionized Mg^{2+} in yeast mitochondria, essentially following the protocol of Rodriguez-Zavala and Moreno-Sanchez (1998). Although precise Mg^{2+} determinations await further calibration of the method to be used with yeast mitochondria, we observed a significant increase in free intramitochondrial Mg^{2+} concentrations upon addition of 10 mM Mg^{2+} to mitochondria of mit− M1301 cells and mrs2-1Δ cells without and with added ionophore, respectively. We estimated that at the end of the incubation time [prior to harvesting mitochondria for RNA preparation] intramitochondrial free Mg^{2+} concentrations reached less than half of the extramitochondrial concentration of 10 mM.
It should be stressed here that effects observed in these experiments do not just reflect self-splicing of group II introns as observed in vitro. Concentrations of Mg$^{2+}$, concentrations of other salts, and the incubation temperature stayed far below those of in vitro splicing assays (Michel and Ferat 1995). Furthermore, disruption of mitochondria by sonication or by the addition of chaotropic salts before the addition of 10 mM Mg$^{2+}$ completely prevented the RNAs from splicing (Fig. 6). This treatment might not be expected to prevent in vitro RNA self-splicing because the precursor RNAs apparently stayed intact as it served as well as a template for PCR, as did the RNA of mitochondria not disrupted by sonication or chaotropic salts. Mg$^{2+}$-stimulated RNA splicing in vivo therefore appears to depend on certain Mg$^{2+}$ concentrations and the intactness of mitochondrial structures.

**Discussion**

Several attempts have been made to identify products of nuclear genes that affect splicing of group II introns in yeast mitochondria. Of the factors described so far, Mrs2p only has been shown to be imported into mitochondria and to be essential for the splicing of group II introns, but not of group I introns. The fact that this is not its only role in mitochondria (Wiesenberger et al. 1992) raised a question whether Mrs2p might be bifunctional, involved in RNA splicing and in other functions, or if its effect on splicing might be indirect, resulting from some other, vital function in mitochondria. Data presented here indicate that the intramitochondrial Mg$^{2+}$ concentration plays a critical role in group II intron splicing in vivo. The effect of Mrs2p on group II intron RNA splicing is shown to be essentially indirect, through providing mitochondria with suitable Mg$^{2+}$ concentrations.

Whereas *MRS2* is known to act as a suppressor of group II intron mutations when present in high copy number, we have obtained mutant *MRS2* alleles that can exert the suppressor effect even when present in single copy. The four gain-of-function mutations characterized so far cause amino acid substitutions in a small region in the N-terminal half of the Mrs2 protein, which we assume to be oriented toward the mitochondrial matrix space (Bui et al. 1999). Three further gain-of-function *MRS2* suppressor alleles of a group II intron mutation were found independently in the same region of the Mrs2 protein by Schmidt et al. (1998), confirming the prominent involvement of Mrs2p in group II intron splicing and defining a small region of the protein as being particularly important for this activity. Consistent with the findings of Schmidt et al. (1998), we observe a slight increase in Mrs2 protein levels in all four gain-of-function mutants. A similar increase in wild-type Mrs2 protein levels (obtained by expression from a centromeric vector) leads neither to a reconstitution of splicing nor to an increase in intramitochondrial Mg$^{2+}$ concentration as observed with the Mrs2* mutant proteins. These effects therefore appear to reflect enhanced activities of the mutant proteins in establishing mitochondrial Mg$^{2+}$ concentrations, which in turn suppress splicing defects.

The correlation between elevated Mg$^{2+}$ concentrations and enhanced splicing of mutant intron RNA (this work) and between reduced Mg$^{2+}$ concentrations as found in *mrs2A* cells and a block in splicing of wild-type RNAs (Bui et al. 1999) suggest a major role of Mg$^{2+}$ concentrations in group II intron splicing. Accordingly, we conclude here that Mrs2p is mediating suitable Mg$^{2+}$ concentrations in mitochondria but is otherwise dispensable for splicing, or, in other words, that group II introns splice in the absence of Mrs2p if appropriate Mg$^{2+}$ concentrations are provided by other means.
Several observations support this conclusion and highlight the prominent role of Mg$^{2+}$ in group II intron splicing. (1) Expression of Mrs3p and Mrs4p, two members of the mitochondrial carrier family, in high copy number raises total mitochondrial Mg$^{2+}$ concentrations in an mrs2Δ mutant to wild-type levels and suppresses splicing defects of mrs2Δ cells. When overexpressed in MRS2+ cells these proteins also suppress splicing defects resulting from mit+mutation M1301 in group II intron bI1 [Wiesenberger et al. 1992]. (2) Splicing of wild-type group II introns in mrs2-1Δ cells is restored when mitochondrial Mg$^{2+}$ concentrations are normalized by overexpression and targeting to yeast mitochondria of Mrs2p homologs from bacteria (CorA Mg$^{2+}$ transporter, Bui et al. 1999) or from human (hsaMrs2p, Zsurka et al. 2001), which both come from organisms lacking group II introns. (3) Overexpression of the plasma membrane Mg$^{2+}$ transporter Alr1p [Graschopf et al. 2001], leading to increased total cellular and normalized intramitochondrial Mg$^{2+}$ concentrations, restores group II intron splicing as well. (4) Most significantly, precursor RNAs accumulated in mitochondria isolated from mit+ M1301 cells or mrs2-1Δ cells undergo splicing to a considerable extent in organello upon addition of 10 mM Mg$^{2+}$. Concentrations of other metal ions are neither significantly affected by gain-of-function mutations of Mrs2p nor do they have any stimulating effect on splicing in organello, even when added in concentrations similar to those of Mg$^{2+}$ and thus exceeding their physiological concentrations of other metal ions are neither significantly affected by gain-of-function mutations of Mrs2p nor do they have any stimulating effect on splicing in organello, even when added in concentrations similar to those of Mg$^{2+}$ and thus exceeding their physiological concentrations. This underscores the specific role of Mg$^{2+}$ in group II intron splicing in vivo.

This particular dependence of group II intron RNA splicing on Mg$^{2+}$ concentrations in vivo and in organello parallels results on in vitro self-splicing of these introns (as opposed to self-splicing group I introns). For optimal activity they require 50–100 mM Mg$^{2+}$ in high salt buffers and at elevated temperatures [for review, see Michel and Ferat 1995]. Furthermore, the in vitro self-splicing defect of the bI1 intron RNA with mutation M1301 under standard Mg$^{2+}$ concentrations is partly alleviated by an increase in Mg$^{2+}$ concentrations (M.W. Mueller, pers. comm.). Obviously, physiological interference in vivo in mitochondria in mitochondria are just one of many factors that make up the environment of group II introns in vivo. These may include certain other ions, proteins like helicases [Seraphin et al. 1989], as well as proteins tethering mRNAs to membrane complexes [Costanzo et al. 2000], to name a few possible factors. The importance of intact mitochondrial structures, and not just certain Mg$^{2+}$ concentrations, is illustrated by our observation that restoration of splicing by an increase in Mg$^{2+}$ concentrations is no longer detected when mitochondria are disrupted by chaotropic salts or sonication.

The particular sensitivity of group II intron splicing to changes in Mg$^{2+}$ concentrations is not an intron-specific phenomenon but a common feature of all four group II introns in yeast mitochondria, and we may raise the issue of Mg$^{2+}$ concentrations possibly coordinating splicing activities of these introns. It will be of particular interest to test whether other group II introns, for example, in bacteria, and other RNA-catalyzed reactions are similarly sensitive to changes in Mg$^{2+}$ concentrations. Folding of these RNAs as well as their catalytic reactions involve Mg$^{2+}$ bound to particular sites of the RNAs [Sontheimer et al. 1999]. It remains to be shown whether one of these functions is particularly sensitive to Mg$^{2+}$ concentrations in vivo. Alternatively, Mg$^{2+}$ concentrations may be critical for cellular factors that promote the RNA-catalyzed splicing reactions, for example, a helicase involved in structural transitions of intron RNA. Although this possibility cannot be excluded, no proteins have been found so far (except Mrs2p) that specifically promote group II intron splicing in yeast, although many attempts have been made. Functions of all factors characterized to date, particularly a DEAD box helicase, are not restricted to group II introns [Seraphin et al. 1989; Niemer et al. 1995].

A more direct role of Mrs2p in mitochondrial RNA splicing (e.g., binding of the protein to intron RNA as invoked previously by Schmidt et al. 1998) cannot be excluded, but if it exists, it is not essential for splicing of group II intron RNA with wild-type sequences or with mit+ mutation M1301. There remains the possibility of an enhancement of splicing by the Mrs2 protein beyond rates attained by suitable Mg$^{2+}$ concentrations. Several experiments presented here or previously led to the restoration of wild-type levels of Mg$^{2+}$ in mrs2-1Δ cells, but not to full restoration of wild-type levels of splicing (e.g., overexpression of Mrs3p or of bacterial, human, or plant MRS2 homolog, Bui et al. 1999; Schock et al. 2000; Zsurka et al. 2001). Also, high copy-numbers of yeast MRS2 and low copy-numbers of the gain-of-function mutants MRS2-M1, MRS2-M2, MRS2-M7, and MRS2-M9 raise Mg$^{2+}$ concentrations similarly, but suppression of M1301 or B-Loop RNA splicing defects by the gain-of-function mutations is superior to overexpression of Mrs2p.

These differences in splicing efficiency may be accidental, but they are consistent with a putative function of Mrs2p in RNA splicing aside from its effect via modulation of Mg$^{2+}$ concentrations. As our data reveal, this more direct interference of Mrs2p with group II intron RNA is not essential for splicing and therefore, if it exists at all, will be more difficult to document than the interference of factors that have been shown to be essential for splicing in vivo, namely, the RNA maturases encoded by some group II introns, particularly yeast introns aI1 and aI2 [but not aI5c and bI1 studied here] [Groudinsky et al. 1981; Wank et al. 1999] or the nuclear gene products Maa2 and Crs2 identified in algae and plants, respectively [Perron et al. 1999; Jenkins and Barkan 2001].

**Material and methods**

*Strains, plasmids, and growth media*

Plasmids, genotypes, and origins of the yeast strains as well as media for their growth have been described previously [Wiesenberger et al. 1992; Jarosch et al. 1996; Bui et al. 1999]. The origin of mit+ B-loop has been given in Schmidt et al. [1996].
In vitro mutagenesis of the MRS2 gene and vector constructions

A SacI–PstI fragment containing the entire MRS2 gene was cloned into the low-copy vector YCplac22. The resulting plasmid YCplac22-MRS2 was incubated with hydroxyamine at 37°C for 20 h according to the protocol of Rose et al. (1987). The mutagenized plasmid DNA was transformed into the yeast strain DBY747 MRS2/mit+ M1301 (Wiesenberger et al. 1992). Upon growth on selective media, transformants were replicated onto YPD plates. Gain-of-function mutations in the MRS2 gene (MRS2Δ), suppressing the splice defect of mit- mutant M1301, were expected to be among YPD-positive transformants of strain DBY747 MRS2Δ/M1301. To identify plasmid-borne mutations in the MRS2 gene, plasmids were recovered from transformants, amplified in E. coli, and retransformed into the strain DBY747 MRS2Δ/M1301. To exclude mutations in the YCp vector, a SacI–PstI fragment containing the MRS2 gene was recloned into the YCplac33 vector digested with SacI–PstI. The mutated MRS2 genes of four plasmids that retained a suppressor activity after retransformation (MRS2-M1, MRS2-M2, MRS2-M7, MRS2-MP) were sequenced. The mutant MRS2 alleles M1, M7, and M9 were PCR-amplified from the plasmid YCplac33 using oligonucleotide primers MRS2(BHI): 5′-ccggatctcattttttgtctttc-3′ and MRS2(PstI): 5′-tttctgagatttttcttgtcttc-3′. The PCR products were digested with BamHI and PstI restriction enzymes and cloned into the BamHI–PstI sites of the plasmid pBS(SK+), creating plasmids pBS-M1, pBS-M7, and pBS-M9.

A cassette coding for the triple hemagglutinin (HA) epitope tag (Tyers et al. 1993) was cloned into the PstI– HindIII sites of the plasmid Ylp-lac211, resulting in the Ylp-lac211–HA construct. Plasmids pBS-M1, pBS-M7, and pBS-M9 were digested with SacI–PstI restriction enzymes and cloned into the SacI–PstI sites of the plasmid Ylp-lac211–HA, creating plasmids Ylp-M1–HA, Ylp-M7–HA, and Ylp-M9–HA. These plasmids were linearized by Apal digestion and transformed into strains DBY747 mrs2Δ–1A, DBY747 MRS2Δ/M1301, and DBY947 MRS2Δ–1A. Loading of mitochondria with metal ions

Mitochondrial suspensions of strain DBY747/M1301 were supplemented with up to 50 mM metal ions (final concentrations), whereas mitochondrial suspensions of strain DBY747 mrs2Δ–1A additionally were preincubated with the ionophore A23187 (Molecular Probes) at final concentrations of 5 mM for 5 min before the uncoupler 2,4-dinitrophenol (JCN) at a final concentration of 2.5 mM and metal ions were added. After incubation for 50 min at 20°C, mitochondria were pelleted (10,000g for 10 min) and washed twice with 1 mL of the breaking buffer. RNA from the treated mitochondria was isolated by use of the SV Total RNA Isolation System (Promega). Mg2+ loading of mitochondria was determined by mag-fura 2 measurements of free ionized Mg2+ (Rodriguez-Zavala and Moreno-Sanchez 1998) using an LS55 luminescence spectrophotometer (Perkin Elmer Instruments).

Determination of Mg2+ concentrations in mitochondrial extracts

Mitochondria isolated from cells grown in the YPD medium to A600 = 1.0 were resuspended in water and sonified with an Elma sonificator TRANSSONIC TS540 five times for 1 min. To obtain blanks, empty tubes were rinsed with same amounts of water, which then were submitted to sonication, as were the mitochondria samples. Ion concentrations of the supernatant obtained after centrifugation (40,000g for 10 min) were determined by atomic absorption spectrometry (Perkin Elmer S100 PC), or Mg2+ concentrations were determined using an Mg2+-specific metallochromic indicator, eriochrome blue, as described previously (Bui et al. 1999). Relative Mg2+ values obtained for the blank stayed below 5% of the values of the samples from wild-type mitochondria. Sample values were corrected by subtracting blank values before calculating the Mg2+ concentrations given in Tables 1 and 2.

Acknowledgments

We are grateful to Gerlinde Wiesenberger (Vienna) and Maria Hoellerer (Vienna) for helpful criticism, to Udo Schmidt (Berlin) for sending us the B-loop mutant strain, to D.R. Pfeiffer (Columbus, Ohio) for advice concerning the loading of mitochondria with Mg2+, and to M. Schweigel (Berlin) for introducing us to mag-fura 2 measurements of Mg2+. This work was supported by the Austrian Science Foundation (FWF).

The publication costs of this article were defrayed in part by page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References

Bonen, L. and Vogel, J. 2001. The ins and outs of group II introns. Trends Genet. 17: 322–331.

Bui, D.M., Gregan, J., Jarosch, E., Ragnini, A., and Schweyen, R.J. 1999. The bacterial Mg2+ transporter CorA can functionally substitute for its putative homologue Mrs2p in the yeast inner
Role of Mg\(^{2+}\) in RNA splicing in vivo

Reed, P.W. and Lardy, H.A. 1972. A23187: A divalent cation ionophore. J. Biol. Chem. 247: 6970–6977.

Rodriguez-Zavala, J.S. and Moreno-Sanchez, B. 1998. Modulation of oxidative phosphorylation by Mg\(^{2+}\) in rat heart mitochondria. J. Biol. Chem. 273: 7850–7855.

Rose, M.D. and Fink, G.R. 1987. Kar1, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell 48: 1047–1060.

Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A.M. 1993. Group I and group II introns. FASEB J. 7: 15–24.

Schmelzer, C. and Schweyen, R.J. 1986. Self-splicing of group II introns in vitro: Mapping of the branch point and mutational inhibition of lariat formation. Cell 46: 557–565.

Schmidt, U., Podar, M., Stahl, U., and Perlman, P.S. 1996. Mutations of the two nucleotide bulge of D5 of a group II intron block splicing in vitro and in vivo: Phenotypes and suppressor mutations. RNA 2: 1161–1172.

Schmidt, U., Maue, I., Lehmann, K., Belcher, S.M., Stahl, U., and Perlman, P.S. 1998. Mutant alleles of the MRS2 gene of yeast nuclear DNA suppress mutations in the catalytic core of a mitochondrial group II intron. J. Mol. Biol. 282: 525–541.

Schock, I., Gregan, J., Steinhauer, S., Schweyen, R.J., Brennicke, A., and Knoop, V. 2000. A member of a novel Arabidopsis thaliana gene family of candidate Mg\(^{2+}\) ion transporters complements a yeast mitochondrial group II intron splicing mutant. Plant J. 24: 489–501.

Seraphin, B., Simon, M., Boulet, A., and Faye, G. 1989. Mitochondrial splicing requires a protein from a novel helicase family. Nature 337: 84–87.

Sontheimer, E.J., Gordon, P.M., and Piccirilli, J.A. 1999. Metal ion catalysis during group II intron self-splicing: Parallels with the spliceosome. Genetics & Dev. 13: 1729–1741.

Tyers, M., Tokiwa, G., and Futcher, B. 1993. Comparison of the Saccharomyces cerevisiae Gt cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. 12: 1955–1968.

Van Dyck, E., Jank, B., Ragnini, A., Schweyen, R.J., Duyckaerts, C., Sluse, F., and Fours, F. 1995. Overexpression of a novel member of the mitochondrial carrier family rescues defects in both DNA and RNA metabolism in yeast mitochondria. Mol. Gen. Genet. 246: 426–436.

Waldherr, M., Ragnini, A., Jank, B., Teply, R., Wiesenberger, G., and Schweyen, R.J. 1993. A multitude of suppressors of group II intron-splicing defects in yeast. Curr. Genet. 24: 301–306.

Wank, H., SanFilippo, J., Singh, R.N., Matsuura, M., and Lambowitz, A.M. 1999. A reverse transcriptase/maturase protein is essential for the excision of group II introns from yeast mitochondrial transcripts in vivo. J. Biol. Chem. 274: 6963–6969.

Zsuzka, G., Gregan, J., and Schweyen, R.J. 2001. The human mitochondrial Mts2 protein functionally substitutes for its yeast homologue; a candidate magnesium transporter. Genomics 72: 158–168.