Introduction

Mitochondria have evolved a crucial dependency clear gene products for the replication and exp their genomes. Selection of recombinant clone suppress defects by overexpression has been u yeast Saccharomyces cerevisiae to analyze rat steps in biological pathways and to understand molecular interactions and cell structure (rev Rine 1991). Suppressor genes that rescue defects mitochondrial (mt) DNA or mtRNA metab involved in mitochondrial replication, trans splicing and translation (reviewed by Griv Costanzo and Fox 1990; Pon and Schatz 1991 al. 1993).

Among the nuclear genes whose products a to take part in mtDNA metabolism or mito gene expression in yeast are PIF1 and MRS2 gene encodes a DNA helicase involved in mt combination, repair and stability (Lahaye et references therein). Although its function pensive under normal growth condition (Foury and Lahaye 1987), the PIF1 gene pro pears essential for mtDNA metabolism at 36° perature at which pipf1 null mutants lose all rho° and thus become unable to utilize mentable carbon sources like glycerol (Van E 1992; and references therein).

The MRS2 gene product is required for tron splicing and is involved in the assembly of ponents of the respiratory chain. Even in a stra of mitochondrial introns, MRS2 gene disrupt ciated with the absence of cytochrome aa3 s in cytochrome b spectral bands. When a multicytoplasmid it acts as a suppressor of NA splicing defect caused by a single base pair in domain 3 of the mitochondrial group II at the first intron of the cytochrome b gene (K 1987).

We have taken advantage of the condition type exhibited by the pipf1 null mutants to is
The genotypes and origins of the yeast strains used for analysis of the suppression of the mrs2 respiratory defect, DBY747, DBY747/M1301, GW/gd2-21.2 and GW/gd7-21.2 have all been described before (Wiesenberger et al. 1992). Stains from the strain DBY747 (MATa leu2-112 his3-1 ura3-52 trpl [rho⁺ mit-]) ATCC 44774) strains DBY747/M1301 (isogenic the nuclear markers but carrying a single-base deletion in the mt group II intron B11]) and GW/gd2-21.2 (with a null mrs2::HIS3 disruption, [rho⁺ mit⁺]) have been constructed this work. In a similar way, the strain GW/gd2-21.2 (mrs2::HIS3 [rho⁺ mit⁺ ΔΔα1 ΔΔβ1 ΔΔα] was derived from DBY747/wo (lacking all known mitochondrial introns, i.e., ΔΔα1 ΔΔβ1 ΔΔα; a gift from I. Bousquet, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette).

The following media were used to grow the strains: gluc rich medium [2% glucose, with 2% yeast extract (Kat) or yeast extract (Difco) and 2% bactopeptone (Difco)]; glycerol medium [2% glycerol, with 2% yeast extract (Kat) or 1% extract (Difco), 2% bactopeptone (Difco), 2% glycerol]; minimal medium [2% glucose, 0.7% yeast nitrogen base (Difco)] supplemented with the auxotrophic requirements (40 mg/l); galactose medium [2% galactose, 0.7% yeast nitrogen base (Difco)] supplemented with the auxotrophic requirements (40 mg/l). Solid media were prepared with 2% Difco agar (minimal media) or 2% agar (rich media).

**DAPI staining and visualization of DNA**

Total cell DNA was stained with the DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI) according to Williamson Fennel (1979), using ethanol as fixative. A 100 μl portion of culture were mixed with 200 μl of ethanol and incubated at room temperature for 20 min. Cells were washed once with 400 μl H2O and resuspended in 100 μl of H2O. Then 10 μl of D [0.5 μg/ml] were added to 3 μl of this suspension and the cells examined by fluorescent microscopy. Module UV of a Pol microscope (Reichert-Jung) equipped with a 200 W mercury lamp was used for this purpose.

**DNA and RNA manipulations**

Standard protocols were used for *Escherichia coli* and yeast transformations, plasmid DNA preparations, yeast total DNA isolation, gel electrophoresis, nick translation, Southern and Northern hybridizations using nitrocellulose membranes (Mati et al., 1982). Dideoxy sequencing was achieved on one-stranded (ds) DNA or single-stranded (ss) DNA by the method of Sanger et al. (1977) using T7 DNA polymerase (Pharmacia). E fragments to be sequenced were obtained by unidirectional deletions using exonuclease III and mung bean nuclease.

**Cloning and subcloning of the RIM2/MRS2 gene**

Yeast genomic libraries constructed in the shuttle vectors YC YE24 and pHC3G were used to transform the pff1 null mutant aEVII-4b or aEVI-1a to uracil prototrophy. Ura3⁺ transformants were gridded on minimal medium supplemented with the required amino acids before being replica-plated onto glycerol medium incubated at 36°C for 1 day. They were then replicated 2nd and 3rd time onto the same medium and incubated at 36°C for 4 days. This second replica-plating, which eliminates all nonpressed pff1 null clones, allowed the selection of two clone which growth on glycerol medium at 36°C was partially rest upon transformation. Approximately 8000 Ura⁺ transformants were screened. RIM2 was uncovered on the rescuing plasmid from a DNA library constructed in vector pHC3G (a cos vector that contains the yeast selectable marker URA3) and
Detection of RIM2/MSRS2 in mitochondria

The strains aEV7-7a (RIM2) and aEV7-8a (RIM2), the plasmid RIM2/PFL45-8-1 were grown in glycerol while the strain aEV7-7b (rim2::URA3) was grown in medium. Cells were harvested in the exponential phase and mitochondria were isolated by a combination of the reported by Daum et al. (1982) and Yaffe (1991). Mitoplast prepared by incubation of the mitochondria at 0°C for the presence of 0.3 mg digitoitin per mg protein in a containing 0.6 M mannitol, 10 mM Tris-HCl, 2 mM 0.1% BSA, pH 6.8. The reaction was stopped by a tenfold centrifugation. The mitoplasts were frozen, thawed, ed in 50 mM sodium carbonate, pH 9.6. Inner membra isolated by centrifugation at 178000 x g for 10 min in Airfuge microcentrifuge. Inner membrane proteins iso- the same volume of mitoplasts (volume accessible to triter) were subjected to SDS-polyacrylamide gel electrophoresis. For western blotting, the proteins were transferred onto bilon-P (Millipore) membrane. Immunodetection was using an antibody raised against a synthetic peptide (1:50 dilution) that includes residues 2-12 of the NH2 part of RIM2/MSRS2. The preparation of this antibody described elsewhere (Hillen and Sluse, unpublished res). The reaction was visualized by 35S-labeled protein A (Amer was used as host for plasmid con-

Results

Independent approaches have led to the conclus the same gene, named RIM2/MSRS2, can act a ticy suppressor of two types of defect in mito- nuc acid metabolism. The RIM2 gene was ered as a suppressor of a DNA helicase defect chondria, and is allelic to MSRS2, which is as a suppressor of a mitochondrial RNA splicin

RIM2, a partial suppressor of a DNA helicase in yeast mitochondria

RIM2 was originally isolated from a wild-type library constructed in the cosmid vector pHC suppressor rescuing the temperature sensi growth defect of the pif1 null strain aEV7-4b erol (Fig. 1). A restriction enzyme analysis of tling plasmid cosS indicated that neither PIF1 i the previously uncovered suppressor RIM present on this plasmid (data not shown). It w fore concluded that the rescuing activity as is with a novel suppressor gene. This gene was RIM2.
mid cos8 rescues the temperature sensitivity of the aEVII-4b on glycerol. The pif1 null strain aEVII-
transformed with a yeast genomic library based on the
(URA3) and Ura" transformants displaying a
phenotype at the restrictive temperature were selected.
It is illustrated that the URA3 marker and the growth phenotype
at 36°C in mitotic segregants issued from transform-
cos8 is illustrated. Transformants aEVII-4b/cos8
overnight in glucose-rich medium at 28°C before
individual colonies on glucose-rich medium. Individ-
were then gridded on glucose plates and replica-plat-
rich medium, (C) glucose-rich medium and
medium lacking uracil. Plates A, C and D were incu-
Plate B was incubated at 36°C for 1 day before
cluded for a second time on the same medium. The
shows the growth at 28°C after 2 days and the
mates at 36°C 2 days after the second replica-plating. As illus-
3 marker and the Glyco" phenotype at 36°C C
mitotic segregants

Fig. 2 Physical map of RIM2/MRS12 and flanking sequen-
The top line represents a partial restriction map of the RI1-
MRS12 gene and surrounding sequences. The arrow repres-
tion at 36°C. YEpM12 derivatives were tested for t1
ability to rescue the mitochondrial defect displayed by
The null strain aEVII-4b on glycerol at 36°C. YEpM12 derivat-
were tested for their ability to restore glycerol growth in the st
DBY747/mrs2. (E EcoRI, II HindIII, S Sall, K KpnI, P Fstl,
Note that not all of these restriction enzymes were used
independent analyses of pFL45-8-1 and YEpM12)

tives of this plasmid were then constructed and tes-
t for their ability to rescue the ts growth defect of str-
aEVII-4b on glycerol (Fig. 2). From these experimen-
tes it was concluded that the RIM2/MRS12 gene resides
2.35 kb Sall fragment on pFL45-8-1 (one of the 5
sites lies in the polynucleotide of pFL45).

In a similar way, the RIM2/MRS12 gene was lo-
ized on the 9 kb insert of plasmid YEpM12 by con-
structing differentially deleted clones that were tes-
t for mrs2 suppressor activity (Fig. 2). The gene was fo-
to reside on a 2.9 kb XbaI fragment.

Sequencing of the rescuing regions of pFL45-8-1:
YEpM12 revealed that the inserts of these plasmid
overlapped, with their non-overlapping segments o-
taining sequences upstream and downstream, respec-
tively, of an open reading frame (ORF) correspond-
t to the RIM2/MRS12 gene (Fig. 2). This fragment of
S. cerevisiae genome has already been characterized
detail during the systematic sequencing of chromos-
II (Demolits et al. 1993) and its localization confirmed
the physical mapping of RIM2/MRS12 between sn1
and CDC28 on the right arm of this chromosome (d
not shown, L. Riles, personal communication).

Sequence and expression of the RIM2/MRS12 gene
The RIM2/MRS12 gene is identical to ORF YBR1

compressor of a mitochondrial RNA splicing

MRS2

null strain GW/gd21.2 (a

DBY747 that contains mitochondrial intron-containing
expression of MRS12 restores the glycerol
The mrs2 mutant both in intron-containing
cells but surprisingly Northern analyses of
mitochondrial transcripts have revealed only a
transcription of the group II intron splicing defect in
his strain (data not shown). In contrast to the
MRS4 genes, overexpression of MRS12
to the defect caused by the mit' mutation
on b11.

to the
ntron b11.

to the
ntron b11.

1 kb

SS E H

RIM2

X H

KXPPP

HK

MRS12

YEpM12

Plasmid R

pFL45-8-1 +

pFL45-H

pFL45-E

pFL45-S

pFL45-S1 +

DBY747/mrs2. (E EcoRI, II HindIII, S Sall, K KpnI, P Fstl,
Note that not all of these restriction enzymes were used
independent analyses of pFL45-8-1 and YEpM12)
The RIM2/MRS12 gene is essential for mtDNA maintenance and proper cell growth.

In order to determine whether the RIM2/MRS product performs an essential function, a construct made in which a 418 bp EcoRV-HindIII DNA 

internal to the RIM2 ORF was replaced by an selectable marker URA3, as illustrated in Fig. 2. A deletion allele was then used to replace one of the corresponding wild-type RIM2 copies in a diploid strain. Placement was verified by Southern blot hybridization (Fig. 3B). The resulting diploid, called EVR, sporulated, and the products of meiosis were dissected to complete medium containing glucose before analyzed.

All four spores were found to be viable in no tetrads, indicating that RIM2 is not essential for viability. However, rim2 null colonies displayed growth phenotype (Fig. 4A, see below). In addition, they were unable to grow on medium containing the fermentable substrate glycerol as the sole carbon source.

The nucleotide sequence is available in EMBL, EMBL and DDBJ nucleotide sequence databases under accession number Z21487 (partial sequence of EMBL X69839). The RIM2/MRS12 gene encodes a 197 amino acids protein with a predicted molecular weight of 24 kDa and a calculated isoelectric point of 10.15. The nucleotide distribution around the ATG start codon (5'-aaagATGcct-3') represents a good match to the yeast consensus sequence (5'-a/taagatat-3') (reviewed by Cigan and Donahue 1987).

RIM2/MRS12 gene is transcribed into an mRNA.
RIM2 encodes a new member of the MCF

The predicted primary structure of the RIM2/MRS12 protein was compared with sequence databases using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) search programs. Significant matches were identified with members of a protein family that span the inner mitochondrial membrane, MCF (reviewed by Aquila et al. 1987; Klingenberg 1989; Kuan and Saier 1993). While the best stc member of the MCF is the ADP/ATP carrier A1 from S. cerevisiae (Nelson et al. 1993), numerous sequences of proteins belonging to this family have been identified in various organisms. In addition, homol of the MCF have been identified in amyloplasts (Stoll 1992) and peroxisomes (Jank et al. 1993) multiple sequence alignment of RIM2/MRS12 and one of these carriers is presented in Fig. 6 (top).

Members of the MCF consist of three repeating sequences, each containing two putative membrane-spanning helices separated by a hydrophilic region. In addition, the threefold presence of the energy trans protein signature P × (DE) × (LIVAT) (RK) × (LIVMFY) (PROSITE PD0080189 ; Bairoch 1993) is hallmarks of this protein family. This sequence is
Alignment of the deduced amino acid sequence of the MRS12 protein with members of the mitochondrial carrier family.

Multiple sequence alignment of RIM2/MRS12 (RIM2-21847) with the peroxisomal membrane protein PMP47 (CANBO, McCammon et al. 1990) and mitochondrial carriers from the two isoforms of the MR34 (MRS3/4_YEAST, Wiesenberger et al. 1991) have been previously involved in tRNA splicing of the mitochondrial carrier family (MCF) that have been assigned to a transport substrate are the ADP/ATP carrier (MCF1/2_YEAST, Adrian et al. 1986; Lawson and Douglas 1992) and phosphate carrier (MCF1_YEAST, Phelps et al. 1991). The mitochondrial respiration and mitochondrial shuttle (M20M, Bovin, Runswick et al. 1992) and the uncoupler protein (UCP, Boulaud et al. 1993) are the central and C-terminal repeats of the sequences of the same repeat as well as between the repeats. Transmembrane (TM-) domains are boxed according to the yeast ADP/ATP carrier (Nelson et al. 1993).

The alignment of the first hydrophilic loop of RIM2/MRS12 and PMP47. The alignment was done using the computer program MACAW (Schuler et al. 1993). Note the similarity of the matrices used RIM2 between 10 and 35 for the other carriers. The similarity of the matrices used RIM2 between 10 and 35 for the other carriers. In the internal hydrophilic loop of RIM2, the MRS12 protein is much longer (66 residues) than the repeats (44 residues) and III (42 residues), all repeats of the other carriers (about 40 residues). Finally, with a size of about 35 residues, the
Fig. 7A-F Dot plot comparisons of the amino acid sequence of RIM2/MRS12 against itself and against sequences of other members of the carrier family. The RIM2/MRS12 sequence was compared with itself, PMP47 from Candida boidinii, the yeast ADP/ATP carrier ACC2, the yeast phosphate carrier MPAC, the bovine oxoglutarate malate shuttle M2OM and the rat uncoupler protein UCP. The sequences for the carriers were taken from the references listed in Fig. 6.

Connecting repeats II and III in RIM2/MRS12 is larger than in any other member of this family. It is tempting to speculate that these observations reflect the functional specialization of RIM2/MRS12 during evolution. Comparison of RIM2/MRS12 with PMP47, a peroxisomal homolog of the MCF from the methylotrophic yeast Candida boidinii (McCannon et al. 1990; Jank et al. 1993), might be interesting in this regard. The internal hydrophilic loops in repeat I of RIM2/MRS12 and PMP47 are indeed of similar length and share a homologous stretch of nine residues that reads GSxP-KxN (Fig. 7B). The significance of this observation is still unknown. This conserved region does not represent any known PROSITE motif. Besides, PMP47 also contains an extended loop between TM domains V and VI, whereas RIM2/MRS12 does not. Finally, there is no N-terminal extension preceding the first repeat of PMP47 (Fig. 7A).

Mitochondrial localization of the RIM2/MRS12 protein

All the data presented above suggest that the RIM2/MRS12 protein functions in the mitochondria. As a first step to determine the cellular location of the RIM2/MRS12 gene product, a polyclonal antiserum was raised against a synthetic peptide (pep1) corresponding to amino acids 2–12 of the deduced RIM2/MRS12 protein, and affinity-purified antibodies were used in western blotting experiments to probe mitochondrial proteins from the strains EVRT-7a (RIM2), EVRT-7b (rim2::URA3) and EVRT-8a/pFL45-8-1 (a RIM2 strain carrying the plasmid pFL45-8-1). As illustrated in Fig. 8 (lanes 3, 4), antibodies to pep1 antiserum show the presence of a major protein of about 42 kDa, consistent with the predicted molecular weight of the RIM2/MRS12 protein. That this protein indeed corresponds to RIM2/MRS12 is further confirmed by the observation that it is not detected in the rim2 null strain (lanes 5, 6) while it is present at an increased level in the strain carrying the RIM2 gene on a multicopy plasmid (lanes 1, 2). Finally, our observation that antibodies directed against the N-terminus of the RIM2/MRS12 protein are able to detect RIM2/MRS12 in mitochondria suggests that this protein is imported into the organelle without any eukaryotic amino-terminal presequence. The lack of a N-terminal presequence in the RIM2/MRS12 protein is further supported by the acidic character of its N-terminus (with 11 acidic residues and only 5 basic
Fig. 8 The RIM2/MRS12 protein is present in mitochondria. Western blot of mitochondrial preparations obtained from various yeast strains shows the presence of the RIM2/MRS12 protein in the inner mitochondrial membrane from a wild-type RIM2 strain (EVRT-7a, lanes 3, 4) and a strain carrying RIM2 on a multicycopy plasmid (EVRT-8a/Pl45-9-1, lanes 1, 2). RIM2/MRS12 is not detected in a rim2 deletion mutant (EVRT-7b, lanes 5, 6). Inner membrane proteins from 0.18 µg (lanes 1, 3, 5) and 0.36 µg (lanes 2, 4, 6) mitoplasts (mitoplast volume that is accessible to H 2O 2) were subjected to SDS-polyacrylamide gel electrophoresis. The RIM2/MRS12 protein is indicated by an arrow.

residues in the first 45 amino acids of the protein). The absence of N-terminal targeting sequence is a characteristic feature of the MCF (reviewed by Planer and Neupert 1990).

Discussion

Overexpression of a novel gene, RIM2/MRS12, rescues both a DNA helicase and a RNA splicing defect in yeast mitochondria. The notion that RIM2/MRS12 is involved in mitochondrial metabolism is further supported by the two following observations. First, inactivation of the RIM2/MRS12 gene not only impairs mitochondrial functions but also causes the total loss of mtDNA. Second, antibodies directed against an N-terminal peptide of the protein detect RIM2/MRS12 in mitochondria. Yet, far from encoding a component of the machinery of mtDNA replication or mtRNA splicing, the RIM2/MRS12 gene identifies a new member of the MCF, suggesting that the control of mitochondrial nucleic acid metabolism by RIM2/MRS12 is mediated at the level of mitochondrial transport.

Mitochondrial carriers constitute important transport systems that ensure the traffic of solutes through the inner mitochondrial membrane, therefore maintaining an essential connection between mitochondrial and cytosolic functions (Aquila et al. 1987; Klingenberg 1989; 1990). A large variety of substrates (including adenine nucleotides, inorganic anions and cations, mono-, di-, and tricarboxylic acids, amino acids, fatty acids; Kramer and Palmeri 1989) are known to be specifically transported across the inner mitochondrial membrane by these proteins. The mitochondrial solute carriers sequenced to date form an homologous protein

While the ligand(s) transported by RIM2/MR are at present not known, RIM2/MRS12 is so far only member of the MCF whose elimination causes loss of mtDNA. Although we cannot rule out the possibility that such a severe phenotype is due to a perturbation of the integrity of the inner mitochondrial membrane, our data suggest that this phenotype reflects direct and important involvement of the RIM2/MR protein in mitochondrial nucleic acid metabolism. In particular, it is tempting to speculate that RIM2/MRS12 transports mtDNA precursors or components necessary for the mtDNA synthesis machinery. It has been shown that isolated yeast mitochondria incorporate externally added deoxynucleotides to synthesize mtDNA de novo (Mattick and Hall 1977). RIM2/MRS12 could encode a translocator for deoxynucleotides. Assuming a limiting pool of deoxynucleotides in yeast mitochondria, such a hypothesis would provide an attractive explanation to our observations that, when present on a multicopy plasmid, the RIM2/MRS12 gene rescues the temperature-sensitive loss of mtDNA exhibited by cells lacking the D helicase PIF1.

On the other hand, overexpression of RIM2/MR also rescues defects in mtRNA group II intron splicing and mitochondrial assembly/expression caused by MRS2 gene disruption. This additional role for RIM2/MRS12 in mitochondrial biogenesis suggests that factors controlling both cytochrome oxidase assembly (or expression) mitochondrial group II intron splicing and mtDNA synthesis make better candidates for our search for a substrate transported by the RIM2/MRS12 protein. In this regard, an attractive (although not exclusive) candidate is Mg 2+, for which mitochondrial DNA is important stores. Although the adenine nucleotide carrier has been proposed to participate in Mg 2+ transport in rat liver mitochondria under certain conditions (Romani et al. 1991), the major translocator of Mg 2+ remains to be characterized. Finally, the suppression phenotype displayed by RIM2/MRS12, shared by two previously uncovered carrier proteins with unknown functions, MRS3 and MRS4 (Wald et al. 1993 and references therein). Unlike RIM2/MRS12, however, disruption of the MRS3 and MRS4 genes has no apparent effect on mitochondrial functions or cell viability (Wiesenberger et al. 1991). With regard to a possible role in mtDNA metabolism, it will therefore be interesting to learn whether overexpression of MRS3 and MRS4 is able to rescue the mitochondrial thermosensitivity of a pif1 null mutant.

Several hypotheses could explain the growth defects of the rim2 null strains. One could consider that these substrate transported by RIM2/MRS12 from the cytosol to mitochondria or vice versa is important for proper cellular function. This defect could also result...
the accumulation of untransported substrates in the cytosol. Finally, in addition to its presumed transport function, RIM2/MRS12 might also be involved in the mitochondrial structure, the maintenance of which appears necessary to eukaryotic life.

This work has shown that overexpression of the same gene product can suppress defects in several mitochondrial pathways. Another puzzling observation is the large number of genes whose overexpression can rescue mrs2 mutants, since a dozen complementation groups have thus far been discovered. Some of these suppressors may be auxiliary factors that interact with the mRNA splicing machinery. Many, however, are certainly modulators of the intramitochondrial environment, which facilitate group II intron splicing, in agreement with the observation that these introns are capable of efficient self-splicing in vitro. That this view also holds for suppressors of mtDNA metabolism defects is illustrated by the RIM1 and RIM2 genes. Thus, while it is not surprising that the RIM1 gene is a partial suppressor of a DNA helicase defect since it encodes an ssDNA binding protein that is an essential component of the mtDNA replication apparatus, and plays a role in the maintenance of unwound DNA, it is unlikely that RIM2, a mitochondrial carrier, interacts directly with the mtDNA. The transport function of RIM2, MRS3 and MRS4 suppressors suggests that several solutes are rate-limiting in mitochondrial metabolism even though the loss of mtDNA in rim2/mrs2 null mutants argues in favor of a more direct involvement of this gene in nucleic acid metabolism. Thus, search for suppressors of defects in mitochondrial nucleic acid metabolism has revealed the existence of gene products and metabolites that are rate-limiting in mitochondrial metabolism and suggests the existence of alternative pathways that can compensate for nucleic acid metabolism defects.

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