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A single-stranded DNA binding protein required for mitochondrial DNA replication in *S.cerevisiae* is homologous to *E.coli* SSB

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It has previously been shown that the mitochondrial DNA (mtDNA) of *Saccharomyces cerevisiae* becomes thermosensitive due to the inactivation of the mitochondrial DNA helicase gene, *PIF1*. A suppressor of this thermosensitive phenotype was isolated from a wild-type plasmid library by transforming a *pif1* null strain to growth on glycerol at the non-permissive temperature. This suppressor is a nuclear gene encoding a 135 amino acid protein that is itself essential for mtDNA replication; cells lacking this gene are totally devoid of mtDNA. We therefore named this gene *RIM1* for replication in mitochondria. The primary structure of the RIM1 protein is homologous to the single-stranded DNA binding protein (SSB) from *Escherichia coli* and to the mitochondrial SSB from *Xenopus laevis*. The mature *RIM1* gene product has been purified from yeast extracts using a DNA unwinding assay dependent upon the DNA helicase activity of SV40 T-antigen. Direct amino acid sequencing of the protein reveals that RIM1 is a previously uncharacterized SSB. Antibodies against this purified protein localize RIM1 to mitochondria. The SSB encoded by *RIM1* is therefore an essential component of the yeast mtDNA replication apparatus.

Key words: DNA helicase/mitochondrial DNA replication/ single-stranded DNA binding protein/yeast

Introduction

It is generally accepted that mitochondria originated from an endosymbiotic event whereby a prokaryotic 'eubacterium-like' ancestor was incorporated into an ancestral eukaryotic cell (reviewed by Gray, 1989). According to this hypothesis, contemporary mitochondrial genomes are the remnants of a gene migration toward the nucleus. As a consequence, the organelle largely depends on nuclear information for its biogenesis. Mitochondrial DNA (mtDNA) metabolism is thus under the control of nuclear genes, and most (if not all) of the components of the mitochondrial genetic system itself are encoded in the nucleus. The identification and characterization of these proteins and the elucidation of their role still constitute a challenge.

We have previously identified a nuclear gene whose product participates in mtDNA metabolism in the yeast *Saccharomyces cerevisiae*. This gene, called *PIF1*, encodes an ATP dependent DNA helicase involved in mtDNA recombination, repair and stability (Foury and Kolodynski, 1983; Foury and Van Dyck, 1985; Foury and Lahaye, 1987; Lahaye *et al.*, 1991). In addition, the *PIF1* gene product is essential for mtDNA maintenance at an elevated temperature (Foury, Van Dyck and Delmon, unpublished). While *pif1* null mutants still retain a functional mitochondrial genome at 28°C, incubation at 36°C leads to total loss of their mtDNA, giving rise to rho^0 mutants. As a result, these mutants are unable to grow on a non-fermentable carbon source like glycerol at an elevated temperature.

To determine the role of *PIF1* in mtDNA maintenance, we have isolated suppressors that partially restore the ability of *pif1* null mutants to grow on glycerol at 36°C. In this report, one of these suppressors, called *RIM1*, is characterized. *RIM1* (replication in mitochondria) was found to encode a 135 amino acid polypeptide that is absolutely required for mtDNA replication; deletion of this nuclear gene results in the complete loss of mtDNA. The primary structure of the RIM1 protein bears homology to the prokaryotic single-stranded DNA binding (SSB) proteins from *E.coli* and its conjugative F$^+$-factor, as well as to the mitochondrial SSB from *Xenopus laevis*. To characterize the *RIM1* gene product biochemically, we purified the protein from yeast extracts using an 'unwinding' assay. This assay had been used to identify replication protein A (RPA; formerly called RFA) as the nuclear single-stranded DNA (ssDNA) binding protein in yeast. The mature *RIM1* gene product is a previously uncharacterized ssDNA binding protein and antibodies against the purified protein localize RIM1 to mitochondria. The ssDNA binding protein encoded by *RIM1* is therefore an essential component of the yeast mtDNA replication apparatus.

Results

RIM1, a partial suppressor of the mitochondrial thermosensitive defect associated with the disruption of the DNA helicase gene PIF1

pif1 null mutants, which lack the PIF1 mitochondrial DNA helicase, are unable to maintain their mitochondrial genome at 36°C, a temperature at which they become totally devoid of mtDNA (rho^0). Since the isolation of extragenic suppressors of a mutation affecting a given metabolic process has often proved useful for the identification of new factors mediating this process, the cloning of genes that could rescue the mitochondrial thermosensitive (ts) defect of a *pif1* null mutant was undertaken as a first step to determine the role of PIF1 in mtDNA maintenance.

RIM1 was isolated from a wild-type genomic library constructed in the centromeric vector YCp50 as a suppressor that partially restores the growth of the *pif1* null mutant aEVII-4b on glycerol at 36°C (Figure 1). Since the 6.35 kb cloned insert on the rescuing plasmid YCp-9A did not modify

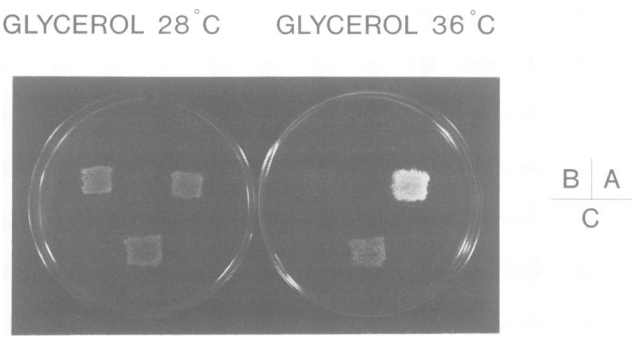


Fig. 1. *RIM1* partially suppresses the mitochondrial temperature sensitive defect exhibited by a *pif1* null mutant. Growth on glycerol at 28°C and 36°C of the following strains: (A) the wild-type control αW303-1B; (B) the *pif1* null mutant aEVII-4b; and (C) the same mutant transformed by *RIM1* on the centromeric plasmid YCp-9A. Strains were replica plated on rich medium containing glycerol and incubated at 28°C or 36°C. After 1 day, the plate at 36°C was replica plated for a second time on the same medium. Illustrated are the growth at 28°C after 32 h, and the growth at 36°C 2 days after the second replica plating.

the low copy number of the centromeric vector (data not shown), it is concluded that, in addition to the wild-type chromosomal copy, only a few additional copies of *RIM1* are required for the observed restoration. Subcloning localized the *RIM1* gene to a 1.5 kb *Hin*dIII fragment.

Structure and sequence of the RIM1 gene

The nucleotide sequence of the 1.5 kb *Hin*dIII fragment containing *RIM1* is presented in Figure 2A. It reveals an open reading frame (ORF) corresponding to *RIM1*, which is interrupted by an 83 bp intron with consensus splice site signals (Figure 2A, bottom hooks) (Pikielny *et al.*, 1983). To confirm the splicing of the message encoded by *RIM1*, a partial cDNA product was generated by combining reverse transcription and the polymerase chain reaction (PCR). First, total yeast RNA was used as a template for reverse transcription primed by an oligonucleotide primer downstream from the 3′ splice site. The reaction products were then amplified by PCR with oligonucleotide primers flanking the putative intron (Figure 2A, underlined nucleotides). While the same 3′ primer was used in all cases, two different 5′ primers were used in independent PCR reactions to ascertain the specificity of the amplification reaction. The products were then run on an agarose gel which revealed two specific bands at 322 bp or 133 bp depending on the 5′ primer used in the PCR reaction (Figure 3A, lanes 2 and 4). In each case, an additional specific band of much weaker intensity was obtained (Figure 3A, lanes 2 and 4), whose size corresponds to that predicted from the genomic sequence of *RIM1* (405 bp and 216 bp, respectively). These two bands were obtained even in the absence of reverse transcriptase (Figure 3A, lanes 3 and 5) and probably reflect contamination of the RNA preparation by genomic DNA. The bands of 322 and 133 bp correspond to the partial cDNA products and their sizes are those expected from the spliced mRNA. A 182 bp cDNA fragment generated by *Eco*RI digestion of the 322 bp band was cloned and sequenced. The sequence of this fragment confirmed the exon−intron junctions predicted by the splice site consensus sequences (Figure 3B). Genes with introns so far constitute the exception in the yeast *S.cerevisiae* and, in the vast majority of cases, the intron when present is unique and located at the extreme 5′ of the gene (reviewed by Fink, 1987). In contrast, we note that the intron in *RIM1* lies within the second half of the *RIM1* gene.

The translation product of *RIM1* is shown in Figure 2A. *RIM1* encodes a predicted polypeptide of 135 amino acids with a molecular weight of 15 386 daltons. The codon bias index (Bennetzen and Hall, 1982) (0.32) is that of a well expressed gene. The amino-terminal amino acid sequence obtained by direct amino acid sequencing of the mature protein (see below) is underlined in Figure 2A. This sequence starts either 1 or 17 amino acids downstream from the nearest potential ATG start codons. While we cannot rule out the possibility that the ATG immediately adjacent to the first residue of the mature protein serves as the start codon, we note that the 17 amino acid sequence upstream from that residue has all the hallmarks of a mitochondrial targeting presequence (reviewed by Hartl *et al.*, 1989), including a two-step cleavage signal (Hendrick *et al.*, 1989). The presence of this presequence would be consistent with the observed mitochondrial localization of RIM1 protein (see below).

The mature RIM1 protein is a 118 amino acid polypeptide, with a calculated molecular weight of 13 290 daltons (see below). It contains a rather acidic carboxy-terminus (with five acidic residues and no basic residue present in the last 14 amino acids of the protein). It has a calculated isoelectric point of 4.92.

RIM1 is essential for mtDNA maintenance

To determine whether the *RIM1* gene product performed an essential function, a construction was made in which most of its coding sequence was deleted and replaced by the selectable marker *URA3* as illustrated in Figure 2B. The deletion allele was then used to replace one of the corresponding wild-type copies in a diploid strain. Replacement was verified by Southern blot hybridization. The diploid was sporulated and the products of meiosis were dissected onto complete medium containing glucose before being analysed. In most of the tetrads all four spores were viable, indicating that *RIM1* is not essential for cell viability. However, *rim1* null cells were unable to grow in medium containing the non-fermentable substrate glycerol as the sole carbon source, suggesting that *RIM1* is essential for mitochondrial functions.

The integrity of the mtDNA in the *rim1* null strains was then analysed by genetic and physical methods. In genetic crosses, *rim1* null strains failed to restore the mitochondrial functions of a number of selected mit$^-$ mutants defective in apocytochrome *b* or subunits of cytochrome *c* oxidase (data not shown). In addition, no signal was detected when total DNA extracted from these cells was probed with mtDNA fragments in Southern blot hybridizations (data not shown). Finally, no mtDNA could be detected by DAPI staining of the *rim1* null strains (see below) which have thus become rho^0. Because the functions of the yeast mitochondrial genome are dispensable in medium containing a fermentable substrate like glucose, the loss of mtDNA in the *rim1* null strains could be due to the absence of selective pressure when the tetrads are dissected on this medium. However, when tetrads from the diploid (*RIM1/rim1*::*URA3*) were dissected directly onto a medium containing glycerol, *rim1* null spores failed to grow (data

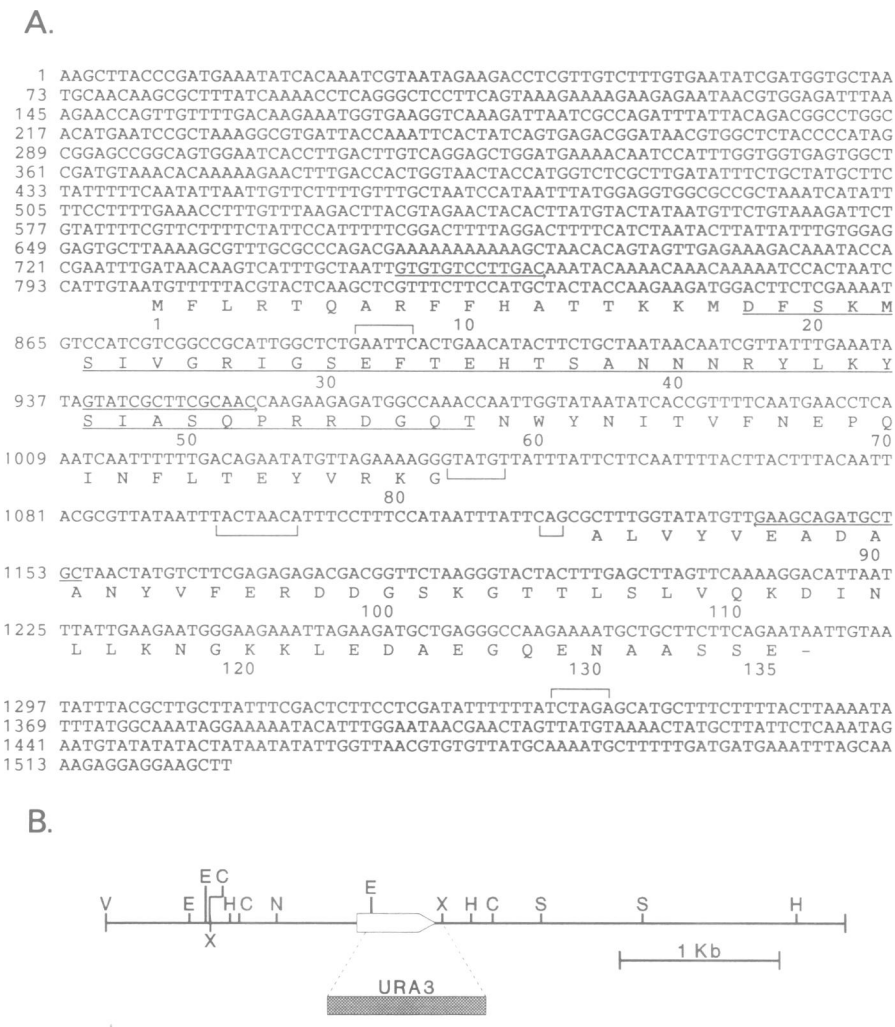


Fig. 2. (**A**) Nucleotide sequence of the *RIM1* gene and deduced amino acid sequence. The nucleotide sequence of the 1.5 kb *Hin*dIII fragment containing *RIM1* is presented. The underlined peptide corresponds to that obtained by direct amino acid sequencing of the purified RIM1 protein. Top hooks indicate the cleavage sites for *Eco*RI and *Xba*I used in the construction of the deletion allele. Bottom hooks indicate the nucleotides representing the consensus splice site sequences. The three oligonucleotide primer sequences used for the isolation and amplification of the *RIM1* partial cDNA are represented as underlined nucleotides (the direction of the primers is indicated by arrows). (**B**) Physical map of the region flanking the *RIM1* gene, and strategy used for *RIM1* gene disruption. A partial restriction map obtained from the sequence of the 4.6 kb DNA fragment encompassing *RIM1* is shown. The open arrow represents the *RIM1* ORF and indicates the direction of transcription. The construction of the deletion allele is also presented. The *URA3* marker used for the disruption is represented by a dashed box. Restriction site abbreviations: C, *Cla*I; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; N, *Nae*I; S, *Sal*I; X, *Xba*I.

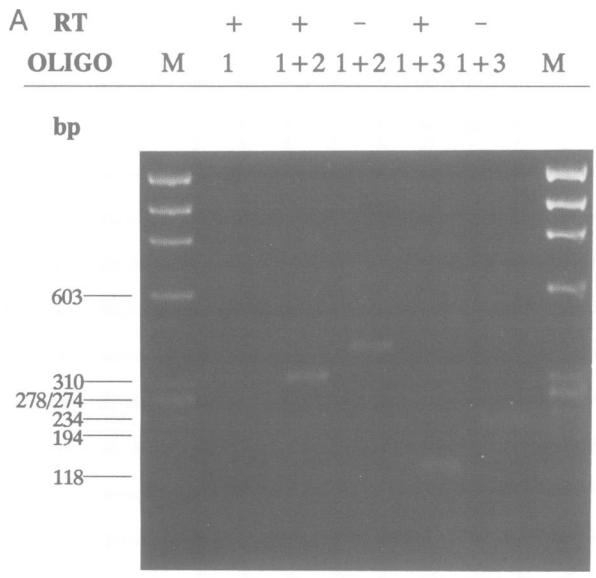
not shown). It is therefore concluded that RIM1 plays an essential role in mtDNA maintenance.

The mitochondrial defect of the *rim1* null strains could be rescued if the heterozygous disruption strain was transformed with a centromeric vector carrying *RIM1* on a 1235 bp *Nae*I−*Hin*dIII fragment (starting at position 293 on the sequence in Figure 2A) prior to sporulation. This observation indicates that the null phenotype was due to disruption of the *RIM1* gene and also suggests that all the elements required for the proper expression of the *RIM1* gene are contained in this fragment.

Analysis of the tetrads from the diploid (*RIM1/rim1*::*URA3*) also allowed the chromosomal mapping of *RIM1*. Cosegregation of the *URA3* marker and the *MAT***a** locus was observed (78 PD, 0 NPD, 7 TT in 85 tetrads analysed), indicating that *RIM1* is on chromosome III, 4.12 cM from the *MAT* locus. The perfect match between the nucleotide sequence presented in Figure 2A and the corresponding sequence obtained from the systematic sequencing of chromosome III confirmed the location of *RIM1* on the right arm of this chromosome (Agostoni Carbone *et al.*, 1992).

Similarity to single-stranded DNA binding proteins

The primary structure of RIM1 protein shows significant similarity to the prokaryotic SSBs from *E.coli* and its conjugative F^+-factor (SSB-F), as well as to the mitochondrial SSB from *X.laevis*. A comparison of the four protein sequences is presented in Figure 4. Gaps have been introduced to optimize the alignments. Overall, RIM1 protein is 24% identical and 50% similar (allowing for conservative amino acid substitutions) to *E.coli* SSB, 26% identical and 48% similar to SSB-F and 23% identical and 49% similar to the mtSSB from *X.laevis*. The similarity between the four proteins extends mainly along the amino-terminal two-thirds of *E.coli* SSB, in the region that encompasses the DNA binding domain of this protein. It should be noted that, while RIM1 and the mtSSB from *X.laevis* are approximately the



B

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GAA TTC ACT GAA CAT ACT TCT GCT AAT AAC AAT CGT TAT TTG AAA
 E   F   T   E   H   T   S   A   N   N   N   R   Y   L   K
TAT AGT ATC GCT TCG CAA CCA AGA AGA GAT GGC CAA ACC AAT TGG
 Y   S   I   A   S   Q   P   R   R   D   G   Q   T   N   W
TAT AAT ATC ACC GTT TTC AAT GAA CCT CAA ATC AAT TTT TTG ACA
 Y   N   I   T   V   F   N   E   P   Q   I   N   F   L   T
GAA TAT GTC AGA AAA GGC GCT TTG GTA TAT GTT GAA GCA GAT GCT
 E   Y   V   R   K   G   A   L   V   Y   V   E   A   D   A
GC
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Fig. 3. The *RIM1* gene contains an intron. Oligonucleotide primers flanking the putative intron were used to isolate and amplify a *RIM1* partial cDNA from yeast total RNA. (**A**) Analysis of the PCR reaction products by electrophoresis on a 1.5% agarose gel. The products of a reaction performed with (+) or without (−) reverse transcriptase (RT) were amplified by PCR. The oligonucleotide primers (OLIGO) used in the PCR reaction were: (1) the 3′ primer EV-14; (2) the 5′ primer EV-13; and (3) the 5′ primer EV-15. These primers are represented as underlined nucleotides in Figure 2A. (M) Molecular weight markers: *Hae*III digests of phiX174 RF DNA. (**B**) Nucleotide and deduced amino acid sequences from the 182 bp partial cDNA encoding *RIM1*. The *Eco*RI restriction site used to clone the partial cDNA fragment is underlined. Bold underlined nucleotides represent the oligonucleotide used as the 3′ primer for the isolation and amplification of *RIM1* partial cDNA.

same size (118 and 129 amino acids respectively), *E.coli* SSB and SSB-F are slightly larger (177 and 178 amino acids respectively). However, most of the similarity between the two prokaryotic proteins is restricted to the amino-terminus (87 out of 115 residues identical). It is the more divergent carboxy-termini of *E.coli* SSB and SSB-F that account for the difference in size between the two mitochondrial proteins and the prokaryotic SSBs (reviewed by Meyer and Laine, 1990).

A higher degree of similarity between these four proteins is found in regions that correspond to functionally important domains of *E.coli* SSB (for an extensive review on the structure of *E.coli* SSB, its interactions with DNA and other proteins, as well as the *ssb* mutations and the roles of the protein in DNA metabolism, see Meyer and Laine, 1990). Of particular interest is the conservation, in all four proteins, of residue Phe60 of *E.coli* SSB. UV-crosslinking experiments have identified Phe60 as an amino acid that interacts with the ssDNA since it provides the only site of cross-linking to ssDNA (Merrill *et al.*, 1984). Conservation of this residue in RIM1 suggests a similar involvement in ssDNA binding. Fluorescence quenching experiments have implicated residues Trp40 and Trp54 in DNA binding by *E.coli* SSB. However, only Trp54 is conserved in all SSBs. Residue Trp40 is present in all SSBs except for RIM1 where an Arg is present instead. Finally, residue His55, within the DNA binding domain of *E.coli* SSB, is conserved in all SSBs except RIM1. This residue has been shown to be important for SSB subunit interactions, because the *ssb-1* ts mutation that changes His55 into Tyr results in the dissociation of the tetrameric form of the protein into monomers (Williams *et al.*, 1984), in a temperature-dependent manner (Bujalowski and Lohman, 1991a,b). It is striking to note that while the RIM1 protein does form tetramers (see below), it is precisely a Tyr residue that occupies the corresponding position in RIM1.

At least one region important for protein−protein interactions seems to be conserved in the amino-terminus of all four SSBs. The existence of this region was suggested from the phenotype associated with *E.coli ssb-3* mutant (Schmellik-Sandage and Tessman, 1990; Meyer and Laine, 1990). The substitution of Asp for Gly15 in this mutant has

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E.coli       AS------RGVNKVILVGNLGQDPEVRYMPNGGAVANITLATSESWRD--   42
F-plasmid    AV------RGINKVILVGRLGKDPEVRYIPNGGAVANLQVATSESWRD--   42
Mt Xenopus   STDSVILERSINKVQLLGRVGQDPVMRQAEGKNPVTIFSLATNELWRSGE   50
Mt Yeast     ---------DFSKMSIVGRIGSEFTEHTSANNNRYLKYSIA-SQPRRDG-   39
                      . .*. ..*..* .   .   . .      .* .. .*.

E.coli       ----KATGEMKEQTEWHRVVLF-GKLAEVASEYLRKGSQVYIEGQLRTRK   87
F-plasmid    ----KQTGEMREQTEWHRVVLF-GKLAEVAGECLRKGAQVYIEGQLRTRS   87
Mt Xenopus   SETFHTAGDVNQKTTWHRISVFRPGLRDVAYQHVKKGARLLVEGKIDYGE  100
Mt Yeast     ------------QTNWYNITVFNEPQINFLTEYVRKGALVYVEADAANYV   77
                       .*.*... .*     .   . ..**. . .*..

E.coli       WTDQSGQDRYTTEVVVNVGGTMQMLGGRQGGGA-PAGGNIGGGQPQGGWG  136
F-plasmid    W-EDNGITRYVTEILVKTTGTMQMLVRAAGAQTQPEEGQQFSGQPQP---  133
Mt Xenopus   YTDKNNVRRQATTII----------------------------------  115
Mt Yeast     F-ERDDGSKGTTLSLVQKD--INLL--KNGKKLEDAEGQENAASSE----  118
             . . ..  . .*  .

E.coli       QPQQPQGGNQFSGGAQSRPQQSAPAAPSNEPPMD----FDDDIP--F    177
F-plasmid    EPQAEAGTKKGGAKTKGRGRKAAQPEPQPQPPEGDDYGFSDDIP--F    178
Mt Xenopus   --------------------------------ADNIIFLSDLRDKL    129
Mt Yeast     ------------------------------------------------   118
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Fig. 4. Comparison of the primary structure of the RIM1 protein with the *E.coli* SSB, the F^+-plasmid SSB and the mitochondrial SSB from *X. laevis*. The amino acid sequences of *E.coli* SSB (Sancar *et al.*, 1981), F^+-plasmid SSB (Chase *et al.*, 1983a), *X.laevis* mitochondrial SSB (Ghrir *et al.*, 1991) and the yeast mitochondrial SSB encoded by *RIM1* were aligned as described in Materials and methods. Gaps were introduced to optimize the alignments. Amino acids that are perfectly conserved between the four sequences are represented by asterisks. Dots represent well conserved positions.

a dramatic effect on DNA repair, with only a small effect on DNA replication. Since Gly15 is conserved within a stretch of highly conserved residues, it is tempting to speculate that this region represents a domain that specifically interacts with the repair machinery.

RIM1 encodes a single-stranded DNA binding protein that localizes to the mitochondrion

To identify functional ssDNA binding proteins from yeast we have used an assay that is based on the ability of SV40 T-antigen (T-Ag) to initiate DNA replication *in vitro* (Brill and Stillman, 1989). This assay requires an ssDNA binding protein to co-operate with T-Ag and DNA topoisomerase I to topologically unwind plasmids containing the SV40 origin of DNA replication (Dean *et al.*, 1987; Wold *et al.*, 1987). The product of the reaction is a highly underwound DNA called Form U, which is detected by agarose gel electrophoresis. While the nuclear ssDNA binding protein, RPA, from both yeast and human sources functions in this assay, a variety of ssDNA binding proteins from prokaryotic and eukaryotic sources are also known to function (Kenny *et al.*, 1989). We therefore expected that other ssDNA binding proteins in addition to RPA could be purified from yeast extracts using this assay.

Unfortunately, Form U production could not be detected when a crude yeast extract was used. However, when the extract was fractionated by column chromatography on Affigel-gel Blue and ssDNA–cellulose, two separate activities that could unwind plasmid DNA in a T-Ag-dependent manner were apparent. One of these activities, RPA, was quantitatively bound by the Affi-gel Blue resin and purified (Brill and Stillman, 1989). The second activity was recovered in the flow-through of this column. Following chromatography on ssDNA–cellulose and Mono-Q resin, this second activity was found to co-purify with a polypeptide of ~13 kDa as judged by SDS–PAGE analysis. The final step in the purification shows the unwinding activity co-sedimenting in a glycerol gradient with a 13 kDa polypeptide (Figure 5). Because we were unable to detect this activity in crude cell extracts, the yield during purification based upon activity could not be determined. Nevertheless, we have found the assay to be extremely reliable because the background level of unwinding is essentially zero.

Amino acid sequencing of this polypeptide revealed an unblocked amino-terminus and a 41 amino acid match to the predicted translation product of the *RIM1* gene (underlined in Figure 2). The observed size of this polypeptide is consistent with the molecular weight of the *RIM1* gene product if the putative presequence was cleaved. To determine the native molecular weight of the mature RIM1 protein, we subjected it to gel filtration and glycerol gradient sedimentation analysis (Figure 6). The protein was found to migrate as a single species with a Stokes radius of 32Å and a sedimentation coefficient of 3.7S. Taken together, these data indicate that RIM1 has a native molecular weight of 56.2 kDa and that it is a tetramer in solution (n = 3.93). [This calculation assumes a molecular weight of 13.29 kDa for the mature monomeric protein and a partial specific volume of 0.74 ml/g (Siegel and Monty, 1966).] RIM1 is

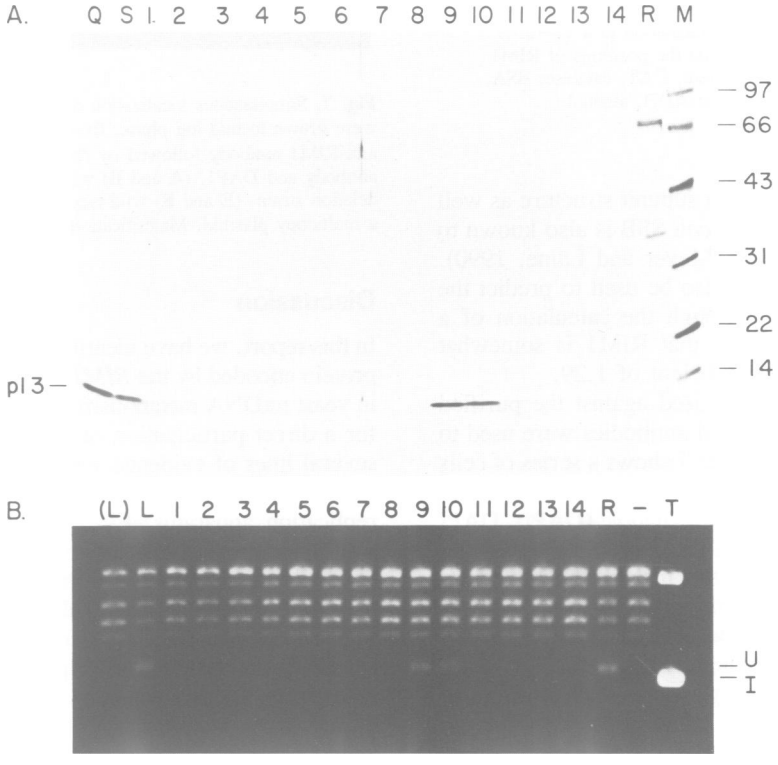

Fig. 5. RIM1 protein co-purifies with a ssDNA binding activity. RIM1 protein was purified from crude yeast extracts and subjected to glycerol gradient sedimentation. Fractions were then assayed for ssDNA binding activity using an unwinding assay. (**A**) SDS–PAGE analysis of RIM1 fractions. Lanes: Q, Mono-Q fraction; S, Superose 12 fraction; 1–14, glycerol gradient fractions (1, top of gradient; 14, bottom); R, RPA from yeast; M, marker proteins; (**B**) Agarose gel electrophoresis of the products from an unwinding assay. U, position of form U DNA; I, position of form I DNA. Lanes: L, glycerol gradient load assayed without T-Ag; L, glycerol gradient load; 1–14, glycerol gradient fractions; R, RPA from yeast; –, no additions; T, input plasmid DNA.

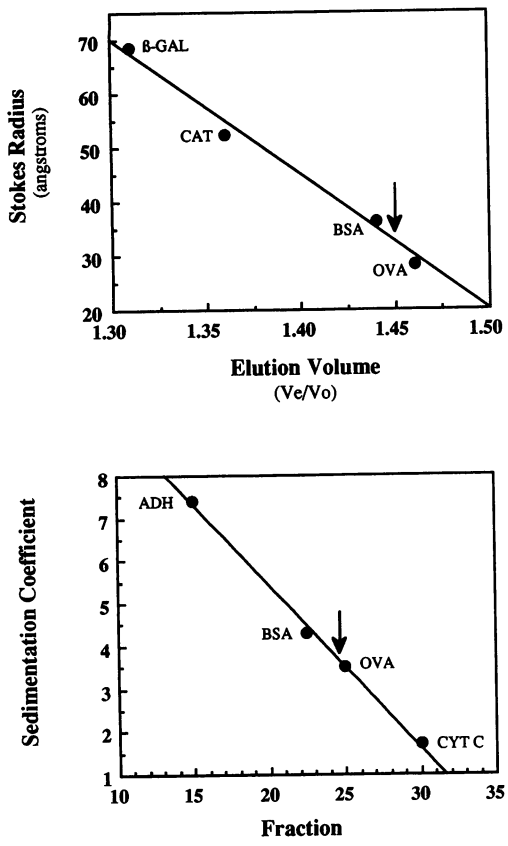

Fig. 6. RIM1 is a tetramer in solution. RIM1 protein from the Mono-Q fraction was subjected to gel filtration chromatography on a Superose 12 column (**above**) and to sedimentation in a 15–35% glycerol gradient (**below**). Arrows indicate the positions of RIM1 protein. Standards: β-GAL, β-galactosidase; CAT, catalase; BSA, bovine serum albumin; OVA, ovalbumin; ADH, alcohol dehydrogenase; CYT C, cytochrome *c*.

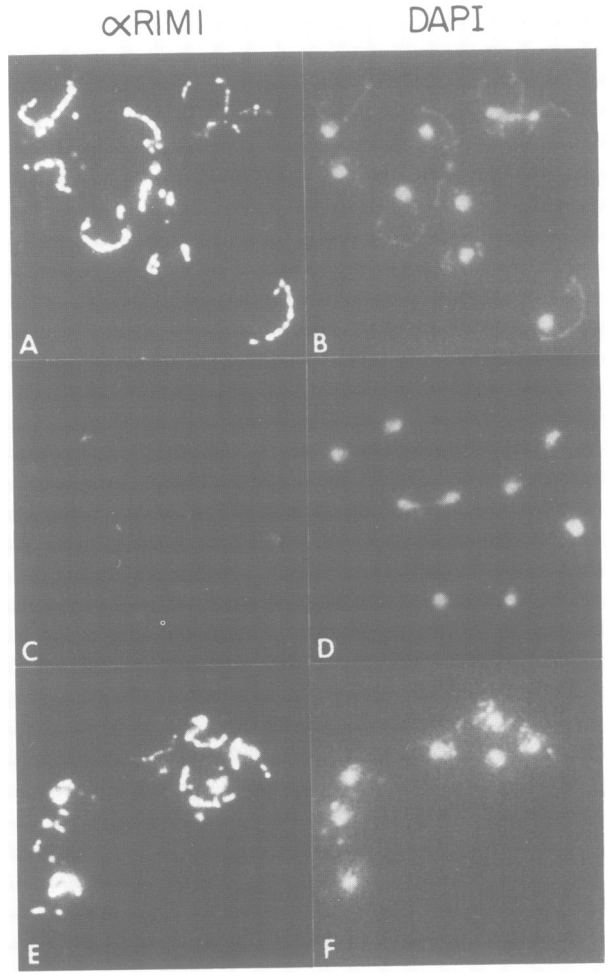

Fig. 7. Simultaneous localization of RIM1 and mtDNA. Yeast cells were grown to mid-log phase, fixed and stained with affinity purified anti-RIM1 antibody followed by rhodamine-conjugated secondary antibody and DAP1. (**A** and **B**) wild-type strain. (**C** and **D**) *rim1* deletion strain. (**E** and **F**) wild-type strain carrying the *RIM1* gene on a multicopy plasmid. Magnification: ×600.

therefore related to *E.coli* SSB in subunit structure as well as in primary sequence, since *E.coli* SSB is also known to exist as a tetramer in solution (Meyer and Laine, 1990). These physical parameters can also be used to predict the shape of the native protein through the calculation of a frictional coefficient. We found that RIM1 is somewhat aspherical with a frictional coefficient of 1.29.

A polyclonal antiserum was raised against the purified RIM1 protein and affinity purified antibodies were used to localize the antigen *in vivo*. Figure 7 shows a series of cells that have been stained with anti-RIM1 antibodies as well as the DNA stain 4,6-diamidino-2-phenylindole (DAPI). DAPI staining results in strong nuclear fluorescence as well as weaker peripheral spots representing the multiple molecules of mtDNA. The anti-RIM1 immunofluorescence localizes specifically with spots of mitochondrial DAPI staining, indicating that RIM1 is a mitochondrial protein (Figure 7A and B). Disruption of the *RIM1* genes results in the loss of immunofluorescence (Figure 7C), as well as all of the mitochondrial DNA staining (Figure 7D). Cells containing a multicopy plasmid bearing the *RIM1* gene show a small increase in fluorescence localized to mitochondria (Figure 7E). This increase in fluorescence varies from cell to cell, perhaps reflecting copy number variation in the *RIM1* plasmid. Finally, *RIM1* antigen is detected only in mitochondria and not in the nuclear or cytoplasmic compartments.

Discussion

In this report, we have identified the 13 kDa ssDNA binding protein encoded by the *RIM1* gene as a new factor involved in yeast mtDNA metabolism. Although proof is still lacking for a direct participation of RIM1 in mtDNA replication, several lines of evidence suggest that the SSB encoded by *RIM1* is an essential component of the yeast mtDNA replication apparatus. First, the *RIM1* gene product is a mitochondrial protein with ssDNA binding activity. Secondly, it is essential for mtDNA maintenance. Thirdly, it bears homology to the replicative SSB from *E.coli*, the SSB encoded by the *E.coli* conjugative F^+-factor, and the mtSSB from *X.laevis*, three proteins with which it shares other structural and functional characteristics.

The ssDNA binding protein encoded by *RIM1* was identified biochemically using an SV40 DNA unwinding assay. This assay has been used previously to identify the nuclear ssDNA binding protein complex, RPA, from *S.cerevisiae* (Brill and Stillman, 1989). It is unlikely that either RIM1 or RPA corresponds to the previously identified yeast proteins SSB-1, SSB-2 or SSB-m (Jong *et al.*, 1985). These proteins differ by their apparent molecular weights,

their elution profiles on ss DNA–cellulose (with RIM1 and RPA eluting at a much higher ionic strength) and the oligomeric form they adopt in solution (RIM1 and RPA are multimeric proteins while SSB-1, SSB-2 and SSB-m are extracted in monomeric forms). Indeed, SSB-1 has been shown to be a component of ribonuclear protein particles containing snRNAs (Clark *et al.*, 1990).

Functionally, the SSB encoded by *RIM1* appears to be the mitochondrial counterpart of the nuclear SSB, RPA. In addition to displaying ssDNA binding activity these proteins perform essential functions in mitochondrial and nuclear DNA metabolism, respectively (Heyer *et al.*, 1990; Brill and Stillman, 1991). This redundancy in SSB function raises the issue of whether these proteins may be able to substitute for each other. One trivial reason why these proteins apparently fail to complement the loss of each other is their intracellular location. This hypothesis could be tested by expressing the proteins in the alternative compartment. Moreover, these two proteins may not be able to substitute for one another due to the specific biochemical properties they have evolved in their respective intracellular compartment. For example, it has recently been demonstrated that RPA from human cells specifically binds T-Ag as well as the polymerase alpha associated primase (Dornreiter *et al.*, 1992). The striking difference in the proteins' subunit structure (RIM1 is a homotetramer while RPA is a heterotrimer) suggests that these SSBs may function and/or interact with other replication proteins by very different mechanisms. Further, an obvious difference between these two proteins is that RPA functions to replicate nuclear DNA only during S phase while mtDNA replication is unrestricted during the cell cycle. Finally, there appears to be no sequence similarity between the RIM1 or *E.coli* SSBs and RPA. A small region of similarity between the 70 kDa subunit of human RPA and *E.coli* SSB has recently been reported (16 of 37 residues identical: Dornreiter *et al.*, 1992). The corresponding region of yeast RPA, however, shows only one residue identical to *E.coli* SSB, suggesting that the similarity with human RPA may be fortuitous.

That the yeast mitochondrial ssDNA binding protein, like the mtSSB from *X.laevis*, is homologous to *E.coli* SSB and SSB-F raises interesting issues. In support of an endosymbiotic origin for mitochondria, it suggests that the mitochondrial SSBs have evolved from a prokaryotic ancestral enzyme. Moreover, the amino acid homology between the two mitochondrial SSBs and their counterparts in *E.coli* is not restricted to the region comprising the DNA binding domain of *E.coli* SSB. Also conserved in the mtSSBs is an amino-terminal region which, in *E.coli*, has been suggested to mediate specific interactions between the SSB and other proteins involved in DNA metabolism (Meyer and Laine, 1990). Thus, it is tempting to speculate that the biological function associated with this region, as well as the factors interacting with it, has also been conserved between the mitochondrial and prokaryotic systems. A severe test of this hypothesis will be to complement a *rim1* null mutant by expressing *E.coli* SSB in yeast mitochondria.

A surprising result of the sequence comparison was the identification of a tyrosine residue at amino acid 44 in RIM1. This residue corresponds precisely to the mutation seen in the *E.coli ssb-1* mutant. This mutation is known to destabilize SSB tetramers at elevated temperatures but may be tolerated in RIM1 for many reasons. For example, since the ts defect exhibited by the *ssb-1* mutant can be suppressed by overexpression of the SSB-1 variant protein (Chase *et al.*, 1983b), it may be that the abundance of RIM1 SSB in yeast mitochondria naturally compensates for the instability suggested from the sequence comparison. Similarly, it is possible that since yeast grows at a lower temperature than *E.coli*, such thermostability is not required. On the other hand, RIM1 protein appears to be relatively stable *in vitro*, suggesting that additional (unconserved) residues in RIM1 may contribute to subunit stability. Interestingly, RIM1 does not contain the carboxy-terminal tail that is found in *E.coli* SSB. This region has been suggested to be inhibitory to DNA binding since removal of this tail by partial proteolysis has been shown to increase the helix destabilizing activity of SSB (Williams *et al.*, 1983).

The *RIM1* gene was isolated as a suppressor of the mitochondrial thermosensitivity associated with the disruption of the mtDNA helicase gene, *PIF1*. The reason for this thermosensitivity is unclear. It is possible that some other component of mtDNA metabolism, possibly another mtDNA helicase, substitutes for PIF1 at lower temperatures. This model suggests that the activity of this component may be unstable, or unable to function efficiently at elevated temperatures. Alternatively, PIF1 protein could be involved in a process whose function becomes essential only when mtDNA is exposed to stress. The observation that *pif1* null mutants are impaired in mtDNA repair after UV irradiation or ethidium bromide treatment (Foury and Kolodynski, 1983; Foury, 1990) supports this hypothesis. A similar situation could occur when the temperature of the culture is elevated. Because it is rich in A and T residues (82%; reviewed by Dujon, 1981), yeast mtDNA could undergo some local melting and/or formation of secondary structures at 36°C. The DNA helicase encoded by *PIF1* could perform an essential function in controlling these events. Since the ts defect observed in mtDNA maintenance is only one of the pleiotropic effects of *PIF1* disruption, it will be interesting to test the ability of *RIM1* to suppress the defects exhibited by *pif1* null mutants in mtDNA repair and recombination.

That overexpression of RIM1 SSB compensates for the absence of the DNA helicase PIF1 at an elevated temperature is not too surprising. SSBs and DNA helicases are often associated in the unwinding of duplex DNA where they perform complementary functions. Because of their ssDNA stabilizing activity, SSBs prevent the renaturation of the single strands created during the unwinding of the DNA helix and thus enhance the reaction catalysed by DNA helicases. In this regard, it is worth mentioning that a related, though opposite, situation was reported in *E.coli* where the temperature and UV sensitivity of the *ssb-1* and *ssb-113* mutants could be suppressed to different extents by a variant form of the DNA helicase Rep (Tessman and Peterson, 1982). Similarly, the UV sensitivity of the DNA helicase II *uvrD* mutant was partially suppressed upon introduction of the *ssb-1* or *ssb-113* alleles in the *uvrD* strain (Quinones and Piechochi, 1987). In each case, these observations were taken as indirect evidence for interactions between *E.coli* SSB and the DNA helicases. In higher cells, a DNA helicase has recently been identified that is absolutely dependent upon RPA for its function (Seo *et al.*, 1991).

Genetic and biochemical studies of SSBs from both prokaryotic and eukaryotic organisms suggest that the SSB encoded by *RIM1* should perform a central role in yeast mtDNA metabolism. In addition to its essential function in mtDNA replication, a role for RIM1 can be anticipated both

in mtDNA repair and recombination. While it has recently been postulated that yeast mitochondria replicate their DNA by a rolling circle mechanism (Maleszka *et al.*, 1991), yeast mtDNA metabolism remains poorly characterized at the biochemical level. Thus, we are still limited in our effort to assign precise functions to RIM1. From our knowledge of SSB−DNA polymerase interactions, we would predict that the RIM1 protein will specifically stimulate the mitochondrial DNA polymerase (DNA polymerase γ). In *X.laevis*, the stimulation of DNA polymerase γ by the mtSSB has been reported to occur *in vitro* under certain conditions (Mignotte *et al.*, 1988). The development of appropriate assays should render our hypothesis testable in yeast as well. The recent identification of a yeast homologue (Fisher *et al.*, 1991; Diffley and Stillman, 1991) of the human mitochondrial transcription factor (mtTF1; Parisi and Clayton, 1991) has given additional weight to numerous indications suggesting that some fundamental features of mtDNA replication priming by transcription are conserved between the two systems (reviewed by: Schinkel and Tabak, 1989; Clayton, 1991). Roles for the *X.laevis* mtSSB have been proposed in the switchover from RNA primer to mtDNA synthesis (Mignotte *et al.*, 1988) as well in modulating DNA transcription by its mtRNA polymerase *in vitro* (Barat-Gueride *et al.*, 1989). Such functions for the RIM1 SSB in yeast mitochondria have yet to be determined.

The cloning of the gene encoding the yeast mitochondrial SSB offers new opportunities to study the replication and maintenance of mtDNA. Combined with a growing list of mitochondrial DNA replication factors, the yeast genetic system should provide a powerful tool for probing the functions of the RIM1 SSB as well as identifying new factors involved in mtDNA metabolism.

Materials and methods

Yeast strains and growth media

All yeast strains are derivatives from the standard wild-type W303-1B (*MATα ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* [rho$^+$]) and W303-1A (*MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* [rho$^+$]). The *pif1* null mutants used for the cloning experiments are aEVII-4b (*MATa pif1::LEU2 ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* [rho$^+$]) and αEVI-1a (*MATα pif1::LEU2 ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can-100* [rho$^+$]). These two mutants are isogenic. The diploid strain EVW used for the disruption experiments issued from a cross between W303-1B and W303-1A. Disruption was carried out by the one-step gene replacement method of Rothstein (1983). The resulting diploid EVRIM1 (*2n RIM1/rim1::URA3* [rho$^+$]) was used for tetrad dissection and analysis. The genetic linkage between *RIM1* and the *MAT* locus was calculated from the following equation: $Xp = (100/2) \times [T + 6NPD)//PD + NPD + T)]$, where Xp is expressed in centimorgans (cM). The following strains were used for immunofluorescence experiments: αEVRIM1-8a (*MATα RIM1 ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* [rho$^+$]), αEVRIM1-8b (*MATα rim1::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100* [rho^0]), and αEVRIM1-2a (*MATα RIM1 ade2-1 his3-11,15 leu2-3,112 ura3-1, trp1-1 can1-100* [rho$^+$], carrying pFL45*RIM1*: 2 μ micron, *TRP1*). The strains were grown on the following media: YD (2% yeast extract, 2% glucose); YG (2% glycerol); minimal medium (2% glucose, 0.7% yeast nitrogen base) supplemented with the auxotrophic requirements (40 mg/l).

DNA manipulations

Standard protocols were used for *E.coli* and yeast transformations, plasmid DNA preparations, yeast total DNA isolation, gel electrophoresis, nick translations and Southern blotting hybridizations using nitrocellulose membranes (Maniatis *et al.*, 1982). Dideoxy sequencing was achieved on dsDNA or ssDNA by the method of Sanger *et al.* (1977) using T7 DNA polymerase (Pharmacia). DNA fragments to be sequenced were generated at random by sonication or by directional deletions using exonuclease III and mung bean nuclease.

Cloning the RIM1 gene

Yeast genomic libraries constructed in the shuttle vectors YCp50, YEp24 and pHCG3 (Gerbaud *et al.*, 1981) were used to transform the *pif1* null mutants aEVII-4b or αEVI-1a to uracil prototrophy. Ura3$^+$ transformants were gridded on minimal medium supplemented with the required amino acids before being replicated on glycerol medium and incubated at 36°C for two days. This second replica plating, which eliminates all unrestored *pif1* null clones, allowed the selection of two clones whose growth on glycerol medium at 36°C was partially restored upon transformation. *RIM1* was isolated from one of these clones on the rescuing centrometric plasmid YCp-9A. Portions of the 6.35 kb insert containing *RIM1* were then subcloned into the multicopy vector pFL44 (kindly given by F.Lacroute). A. 4.6 kb rescuing DNA fragment was first obtained, and its sequence was determined. The size of the fragment containing *RIM1* could be further reduced to a 1.5 kb *Hind*III fragment.

Cloning of the RIM1 partial cDNA

The reverse transcription reaction was carried out as follows: 5 μg of a preparation of total yeast RNA (Foury and Lahaye, 1987) were used as a template with 60 U of avian myelobastosis virus (AMV) reverse transcriptase (Pharmacia) in a 20 μl reaction containing 50 mM Tris−HCl, pH 8.15; 6 mM $MgCl_2$; 40 mM KCl; 5 mM dithiothreitol; 2 mM dNTPs; 40 U of RNasin; and 15 ng of the 14mer 3′ oligonucleotide primer EV-14 (5′-GCAGCATCTGCTTC-3′). After 90 min of incubation at 42°C, the reaction mix was subjected to phenol−chloroform and chloroform extractions before the nucleic acids were ethanol precipitated in the presence of 0.3 M sodium acetate. The pellet was washed once with 70% ethanol, dried and resuspended in 25 μl TE. Five microlitres of the reverse transcription reaction were then subjected to PCR (94°C for 30 s/40°C for 1 min/72°C for 1 min/30 cycles, followed by a last run of 10 min at 72°C) with EV-14 as the 3′ oligonucleotide primer and either the 13mer (5′-GTGTGTCC-TTGAC-3′) or the 15mer EV-15 (5′-GTATCGCTTCGCAAC-3′) as the 5′ primers (all oligonucleotide primers were used at a concentration of 1 ng/ml). The 322 bp band resulting from the reaction performed with primers EV-14 and EV-13 was isolated from a 1.4% agarose gel and digested with *Eco*RI. A 182 bp DNA fragment produced by this digestion was then cloned into PTZ19U cut by *Eco*RI and *Sma*I and sequenced.

Plasmid constructions and RIM1 disruption

Plasmid pFL45RIM1 was constructed by cloning the 1.5 kb *Hind*III fragment containing *RIM1* into the multi-copy vector pFL45 (kindly given by F.Lacroute). Plasmid PTZs*RIM1::URA3*, which contains the *rim1::URA3* allele, was constructed in three steps. The *Xba*I site present in the polylinker of PTZ18U was first removed by digestion of the plasmid with the two flanking enzymes *Hind*III and *Eco*RI. The *Eco*RI ends were rendered blunt by treatment with the Klenow fragment of the DNA polymerase I and the plasmid was religated onto itself. A 1.5 kb *Hind*III fragment (whose sequence is presented in Figure 2A) containing *RIM1* was then inserted in the resulting plasmid cut by *Hind*III to create PTZs*RIM1*. Finally, a 450 bp *Eco*RI−*Xba*I fragment from PTZs*RIM1* comprising most of the *RIM1* ORF was replaced by a 1.17 kb *Eco*RI−*Xba*I fragment containing the *URA3* marker. The resulting plasmid, PTZs*RIM1::URA3*, was digested by *Hind*IIII, and the 2.25 kb DNA fragment containing the *rim1::URA3* allele was then used to replace one of the corresponding wild-type copies in the diploid strain EVW. The disruption was verified by Southern blotting, using the 1.5 kb *Hind*III fragment containing *RIM1* as a probe.

Amino acid sequence comparisons

Protein primary structures were aligned by the CLUSTAL program of the PC/GENE software for nucleotide and protein sequences analysis IntelliGenetics, Inc.) using the method of Higgins and Sharp (1988, 1989). Gaps were introduced to optimize the alignments. For the determination of the percentage of homology between RIM1 protein and the SSBs from *E.coli*, the F$^+$-plasmid and *X.laevis* mitochondria, alignments of respectively 116, 117 and 91 residues were taken into consideration. Similarities were evaluated according to Dayhoff *et al.* (1983).

Purification and analysis of RIM1 protein

A crude extract from 10 l mid-log yeast cells was prepared, adjusted to 0.5 M NaCl, and applied to a 60 ml Affi-gel Blue (Bio-Rad) column essentially as described by Brill and Stillman (1989). The flow-through (14 g protein) was applied to a 10 ml ssDNA cellulose (USB) column and washed consecutively with 5 vol buffer A (25 mM Tris−HCl pH 7.5, 1 mM EDTA, 0.01% NP-40, 10% glycerol, 1 mM phenylmethylsufonyl fluoride)

containing 0.5 M NaCl, 5 vol buffer A containing 0.75 M NaCl, and 2 vol buffer A containing 1.5 M NaCl and 50% ethylene glycol. Peak protein fractions from the 1.5 M NaCl eluate were pooled and dialyzed against 50 vol buffer A containing 0.2 M NaCl and 20% sucrose. The dialysate was adjusted to 0.1 M NaCl with buffer A and loaded onto an FPLC Mono-Q column (HR5/5). The column was then washed with 3 ml buffer A containing 100 mM NaCl and eluted with a 10 ml gradient from 100–500 mM NaCl in buffer A followed by a 3 ml wash with buffer A containing 1 M NaCl. Fractions containing RIM1 (peak: 300 mM NaCl) were identified using an unwinding assay (Dean *et al.*, 1987; Wold *et al.*, 1987; Tsurimoto *et al.*, 1989), pooled and dialysed against 50 vol buffer A containing 100 mM NaCl. This highly purified RIM1 protein was concentrated by binding the protein to a 0.15 ml Q-Sepharose column and eluting it with buffer A containing 0.5 M NaCl. Peak protein fractions were pooled and dialysed on a micro-dialyser (BRL) against buffer A containing 25 mM NaCl and 5% sucrose (Mono-Q pool, 140 μg). Eighty micrograms of RIM1 from the Mono-Q pool was adjusted to 150 mM NaCl, applied to an FPLC Superose 12 column equilibrated in buffer A containing 150 mM NaCl, and eluted at a flow rate of 0.25 ml/min. Fractions (110 μl) containing RIM1 protein were detected by SDS–PAGE followed by silver staining and pooled (Superose 12 pool). Molecular weight standards (10 μg each) were chromatographed, collected and analysed separately under identical conditions. Twenty micrograms of RIM1 from the Superose 12 pool was then layered onto a 5 ml, 15–35% glycerol gradient in buffer A containing 100 mM NaCl. For analytical purposes, an additional gradient was layered with 15 μg RIM1 protein (mono-Q pool) and molecular weight standards (15 μg each). Both gradients were centrifuged in an SW50.1 rotor at 49 000 r.p.m. for 21 h. Fractions (130 μl) were collected from both gradients, assayed for unwinding activity and subjected to SDS–PAGE followed by silver staining.

Immunolocalization

Rabbit polyclonal antibodies were raised against purified RIM1 protein by injecting 50 μg purified protein into the lymph node followed by boosts with 20 μg protein at 4 and 6 weeks. The serum was found to be positive by immunoblotting and the animal killed and bled. Antibodies were affinity purified from the serum as described by Diffley and Stillman (1989). Yeast cells were grown in YPD or SD-TRP (Sherman *et al.*, 1986) to select for the plasmid and then fixed and processed for immunofluorescence as described by Sherman *et al.* (1986). Primary antibody was incubated overnight at a 1:300 dilution followed by a 2 h incubation with rhodamine-conjugated donkey anti-rabbit secondary antibody (Jackson Immunologicals) at 1:300 dilution.

Amino acid sequencing

RIM1 protein (Mono-Q pool) was prepared for sequencing by subjecting 8 μg protein to reversed phase chromatography on a Hewlett-Packard 1090 liquid chromatograph with a 2.1 × 220 mm Aquapore RP-300 column (C_8 resin). This column was run at room temperature with a flow rate of 0.4 ml/min under the following conditions: isocratic at 0% B for 5 min; a linear gradient from 0 to 50% B in 35 min; and a linear gradient from 50 to 100% B in 20 min. Solvent A is 0.1% TFA and solvent B is 70% acetonitrile and 0.06% TFA. A single peak of protein was detected at 44 min by absorbance at 215 nm. This protein was collected, lyophilized and sequenced on an Applied Biosystems model 475 automated sequencer with an on-line 120A HPLC PTH analyser. The initial yield was >500 pmol.

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Note added in proof

The *RIM1* gene designated as YCR29C-a has the coordinates 172169 to 171682 on chromosome III and is proximal to YCR29C [Oliver *et al.* (1992) *Nature*, **357**, 38–46].