Molecular Biology of the Cell
Vol. 5, 529–538, May 1994

SMS1, a High-Copy Suppressor of the Yeast *mas6* Mutant, Encodes an Essential Inner Membrane Protein Required for Mitochondrial Protein Import

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Submitted November 23, 1993; Accepted March 15, 1994
Monitoring Editor: Randy W. Schekman

MAS6 encodes an essential inner membrane protein required for mitochondrial protein import in the yeast *Saccharomyces cerevisiae* (Emtage and Jensen, 1993). To identify new inner membrane import components, we isolated a high-copy suppressor (*SMS1*) of the *mas6-1* mutant. *SMS1* encodes a 16.5-kDa protein that contains several potential membrane-spanning domains. The Sms1 protein is homologous to the carboxyl-terminal domain of the Mas6 protein. Like Mas6p, Sms1p is located in the mitochondrial inner membrane and is an essential protein. Depletion of Sms1p from cells causes defects in the import of several mitochondrial precursor proteins, suggesting that Sms1p is a new inner membrane import component. Our observations raise the possibility that Sms1p and Mas6p act together to translocate proteins across the inner membrane.

INTRODUCTION

Because most mitochondrial proteins are encoded on nuclear genes, mitochondrial biogenesis depends on the import of proteins synthesized in the cytosol (for review see Attardi and Schatz, 1988; Pfanner and Neupert, 1990; Kiebler *et al.*, 1993a). Most imported proteins are initially synthesized as precursors, carrying amino-terminal targeting sequences called presequences (Hurt *et al.*, 1984; Horwich *et al.*, 1985; van Loon *et al.*, 1986). Precursors specifically interact with receptor proteins on the mitochondrial surface (Söllner *et al.*, 1989, 1990; Hines *et al.*, 1990; Moczko *et al.*, 1993; Ramage *et al.*, 1993). After import, presequences are removed by a two-subunit protease located in the matrix (Hawlitschek *et al.*, 1988; Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988; Yang *et al.*, 1988). Folding of imported proteins to their native conformations is assisted by matrix-localized chaperones (Cheng *et al.*, 1989; Ostermann *et al.*, 1989; Kang *et al.*, 1990). Proteins imported into the matrix are translocated across both mitochondrial membranes by a mechanism that requires ATP and an inner membrane potential (Schleyer *et al.*, 1982; Chen and Douglas, 1987; Eilers *et al.*, 1987; Pfanner *et al.*, 1987; Hwang and Schatz, 1989). Import of precursors occurs initially through contact sites between the inner and outer membranes (Schleyer and Neupert, 1985; Pon *et al.*, 1989), but each membrane appears to have its own independent translocation machinery (Ohba and Schatz, 1987; Hwang *et al.*, 1989; Mayer *et al.*, 1993). Ssc1p, a matrix-localized member of the hsp70 family, is thought to drive the completion of transport into the mitochondrial matrix (Kang *et al.*, 1990; Gambill *et al.*, 1993).

At least six proteins, MOM72, MOM38, MOM22, MOM19, MOM8, and MOM7, are proposed to mediate translocation across the mitochondrial outer membrane in *Neurospora crassa* (Söllner *et al.*, 1989, 1990, 1992; Kiebler *et al.*, 1990, 1993b; Keil and Pfanner, 1993), and homologues of several of these proteins have also been identified in *Saccharomyces cerevisiae* (Hase *et al.*, 1983; Baker *et al.*, 1990; Hines *et al.*, 1990; Moczko *et al.*, 1993; Ramage *et al.*, 1993). In contrast, only two inner membrane import components have been identified, both in *S. cerevisiae*. One of these proteins is Isp45p, a 45-kDa integral membrane protein (Maarse *et al.*, 1992; Scherer *et al.*, 1992; Horst *et al.*, 1993). Isp45p can be crosslinked to a precursor during import (Scherer *et al.*, 1992), and mutants in Isp45p are defective in the import of a mitochondrial fusion protein (Maarse *et al.*, 1992). The

yeast *MAS6* gene encodes another inner membrane import component, a 23-kDa integral membrane protein with several potential membrane-spanning domains (Emtage and Jensen, 1993). Antibodies to Mas6p inhibit import into isolated mitochondria, but only after the outer membrane has been disrupted. Depletion of Mas6p from cells causes the accumulation of unprocessed precursors. Furthermore, mitochondria isolated from temperature-sensitive lethal *mas6* mutants are defective for the import of several different precursor proteins in vitro. We recently showed that Mas6p can be cross-linked to a precursor arrested in transit across the inner membrane (Ryan and Jensen, 1993), suggesting that Mas6p directly interacts with precursors during import. An allele of *mas6* has recently been identified in a genetic selection for mitochondrial protein import mutants (Dekker *et al.*, 1993).

To identify new inner membrane proteins required for mitochondrial protein import, we screened for genes that, when overexpressed in the yeast cell, rescued the temperature-sensitive growth defect of a *mas6* mutant. High-copy suppression has often been used to identify proteins that act at the same step in a cellular pathway (Brizuela *et al.*, 1987; Deshaies and Schekman, 1990; Kurihara and Silver, 1993). We report here the identification of *SMS1*, a high-copy suppressor of *mas6-1*. *SMS1* encodes an essential protein located in the inner membrane, and Sms1p is homologous to the carboxyl-terminal domain of the Mas6 protein. Depletion of Sms1p from cells results in a defect in mitochondrial protein import. Our results suggest that Sms1p is a new import component that may act together with Mas6p to translocate proteins across the inner membrane.

MATERIALS AND METHODS

Yeast Strains and Relevant Genotypes

MATa mas6-1 leu2 his3 ura3-52 trp1Δ1 strain JE14-5b (Emtage and Jensen, 1993), strain AH216 (Yaffe and Schatz, 1984), and *MATa/MATα trp1Δ2/trp1Δ2* strain YPH501 (Sikorski and Hieter, 1989) have been described. *MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ2/trp1Δ2* strain 410 was obtained by mating strains YPH857 and YPH858 (Spencer *et al.*, 1994). Standard yeast media (Sherman *et al.*, 1982) and genetic techniques (Rose *et al.*, 1988) were used.

Isolation of the SMS1 Gene

A yeast genomic library in the 2μ-*LEU2* vector YEp13 (Nasmyth and Tatchell, 1980), a gift from K. Nasmyth (IMP, Vienna, Austria), was transformed (Schiestl and Gietz, 1989) into the temperature-sensitive *mas6-1* strain JE14-5b. We selected Leu^+ transformants at 22°C and screened them for growth at 34°C by replica plating to YP medium containing 2% lactate. Of 5500 Leu^+ transformants, 32 grew at 34°C. Rescue of the *mas6-1* defect was plasmid-dependent for four transformants. Plasmids were isolated from each of the transformants as described (Hoffman and Winston, 1987) and electroporated into bacterial cells using a BioRad Gene Pulser (Richmond, CA) with settings of 2.5 kV, 25 μF, 200 Ω. All four plasmids, pKR3, pKR4, pKR5, and pKR6, rescued the *mas6* defect when reintroduced into JE14-5b cells. Restriction endonuclease mapping showed that none of these plasmids carried the *MAS6* gene. In addition, no DNA fragment of the correct size was obtained from these plasmids using the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) with oligonucleotides specific to the *MAS6* open reading frame (ORF). Restriction endonuclease digestion of the four plasmids indicated that they all carried overlapping genomic DNA inserts.

To localize the gene that suppresses the *mas6-1* temperature-sensitive phenotype, we digested pKR3 partially with *Sau3A*. We collected DNA fragments 500–5000 basepairs (bp) in length and ligated them into the *Bam*HI site of the 2μ-*URA3* vector pRS426 (Sikorski and Hieter, 1989). We transformed this collection of ~500 plasmids into strain JE14-5b and selected ~1000 Ura^+ transformants at 22°C. Five transformants able to grow at the nonpermissive temperature were chosen, and the plasmid was recovered from each transformant as described above. One plasmid, pKR7, with an insert of ~1750 bp, was selected for further study (Figure 2).

DNA Sequence of SMS1

Limited DNA sequencing of pKR7 showed that it contained sequences adjacent to the 3′ end of the *YAK1* gene (Garrett and Broach, 1989). The complete sequence of *SMS1* was determined as follows. Plasmid pGS191 was constructed by inserting a 3.5-kilobase (kb) *Sal*I-*Bgl*II fragment (Figure 2) containing *SMS1* into *Bam*HI-*Sal*I–digested Bluescript SK^+ (Stratagene, La Jolla, CA). pGS195 carries a 2.4-kb *Hin*dIII fragment containing *SMS1* in Bluescript SK^+. Overlapping deletions of the *SMS1* gene were made by digesting pGS191 and pGS195 with *Spe*I, followed by sequential exonuclease III and S1 nuclease digestion according to manufacturer's instructions (Promega, Madison, WI). To prevent exonuclease digestion of vector sequences, pGS191 and pGS195 were also digested with *Sac*I before exonuclease treatment. Using a collection of plasmids each carrying a different deletion of the *SMS1* region, both strands of the *SMS1* gene were completely sequenced (Garrett and Broach, 1989). Sequence comparison using the BLAST program (Altschul *et al.*, 1990) was performed at the National Center for Biotechnology Information using the BLAST network service. Comparisons using the FASTA algorithm (Lipman and Pearson, 1985) were carried out at the European Molecular Biology Laboratories, Heidelberg, Germany, using the Mail-FASTA network service.

SMS1 Gene Disruption

We disrupted the *SMS1* ORF by inserting the yeast *TRP1* gene as follows. We inserted a 1750-bp *Xba*I-*Eco*RI fragment from pKR7 into *Xba*I-*Eco*RI–digested Bluescript II SK^+ (Stratagene) to form pKR8. We digested pKR8 with *Nco*I, which removes nucleotides 68–262 from the *SMS1* open reading frame, and filled in the DNA ends with DNA polymerase. We isolated a 1700-bp *Bgl*II fragment containing the *TRP1* gene from plasmid p112 (obtained from P. Hieter, Johns Hopkins University School of Medicine) and filled in the DNA ends with DNA polymerase. We then blunt-end ligated the *TRP1* fragment into the *Nco*I-cut pKR8 vector to form pKR9.

For gene disruptions in yeast, we isolated a 3150-bp *Sal*I-*Not*I fragment containing *sms1::TRP1* from pKR9 and transformed it into two different *trp1/trp1* diploids, strain 410 or strain YPH501. Stable Trp^+ transformants were isolated, and the meiotic products of three independent transformants were analyzed at 22 or 30°C on YPD medium. Of 36 tetrads, none gave rise to more than two viable spores, even after 2 wk of incubation. All viable spores were Trp^-. Southern analysis of the diploids confirmed that one of the two copies of *SMS1* had been replaced by *sms1::TRP1*.

To verify that the *SMS1* ORF was responsible for the suppression of *mas6-1*, we ligated the above *Sal*I-*Not*I fragment from pKR9 into *Sal*I-*Not*I–digested pRS426 to form pKR12. We transformed pKR12 into *mas6-1* cells and tested Ura^+ transformants for growth at 34°C on YP lactate medium as above.

Construction of an Epitope-tagged Sms1 Protein

pKR10, which contains a unique *Not*I site immediately preceding the termination codon of *SMS1*, was constructed as follows. Using

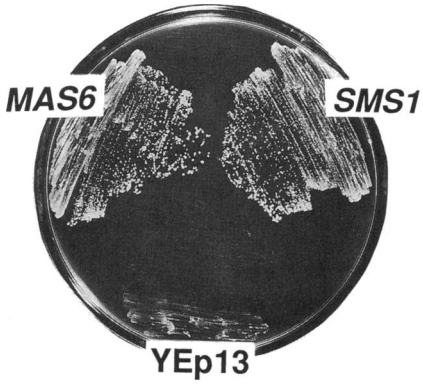

Figure 1. *SMS1* encodes a high-copy suppressor of *mas6-1*. *mas6-1 leu2* strain JE14-5b was transformed with one of three different plasmids: pJE7, a wild-type *MAS6* gene carried on a *LEU2-CEN6* plasmid (Emtage and Jensen, 1993); pKR3, a 2-μm-*LEU2* library plasmid, carrying *SMS1*, which suppressed the temperature-sensitive growth defect of *mas6* mutants; or YEp13, the 2μ-*LEU2* plasmid lacking a genomic DNA insert. Leu$^+$ transformants were streaked onto YP medium containing lactate as the sole carbon source and incubated at 34°C for 3 d.

pKR7, oligonucleotide No. 108 (5'-CTTGCGGCCGCCAGCTTGCAGAGGTTGAG-3'), and oligonucleotide No. 21 (5'-ATTAACCCTCACTAAAG-3'), we isolated a 1.1-kb PCR fragment. We digested this fragment at one end with *Xba*I, filled in the DNA end with DNA polymerase, and then digested the other end with *Not*I. pJE7 is a *LEU2-CEN6* vector that carries a 2172-bp *Sac*I-*Bam*HI DNA fragment containing the *MAS6* gene with a unique *Not*I site immediately preceding the *MAS6* termination codon (Emtage and Jensen, 1993). We digested pJE7 with *Sac*I, filled in the ends with DNA polymerase, and digested it with *Not*I. We then ligated the *SMS1*-containing PCR product into pJE7 to form pKR10. pKR10 thus carries the *SMS1* gene with 615 bp of its upstream sequences, a unique *Not*I site preceding the *SMS1* stop codon, and 940 bp of downstream sequences from the *MAS6* gene. pKR11, which encodes Sms1p with the influenza HA epitope at its carboxyl terminus (Sms1-HA), was constructed by inserting a 114-bp *Not*I fragment containing three tandem copies of the HA epitope (Tyers *et al.*, 1992) (a gift of B. Futcher, Cold Spring Harbor Laboratory) into the *Not*I site of pKR10. The orientation of the HA piece was confirmed by DNA sequencing.

Localization of the Sms1 Protein

To determine the cellular location of the epitope-tagged Sms1 protein, we grew *sms1::TRP1* cells carrying plasmid pKR11 to an OD_{600} of 2.0 in synthetic medium containing 2% galactose as the carbon source. We added yeast extract to 1% and allowed the culture to grow until the OD_{600} was ~4. Subcellular fractionation and isolation of mitochondria were performed as described (Daum *et al.*, 1982b; Emtage and Jensen, 1993). Separation of mitochondrial outer and inner membrane vesicles was as described (Pon *et al.*, 1989; Emtage and Jensen, 1993). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970; Haid and Suissa, 1983) and transferred to Immobilon filters (Millipore, Bedford, MA) (Haid and Suissa, 1983).

To detect the epitope-tagged Sms1-HA protein, we decorated filters with a 1:25 dilution of culture supernatant from 12CA5 cells (Niman *et al.*, 1983), which produce a monoclonal antibody specific for the influenza HA epitope. As markers of cellular and mitochondrial compartments, we incubated filters with polyclonal antisera against the following proteins: Mas6p (Emtage and Jensen, 1993), the F1β protein (a gift from M. Yaffe, University of California, San Diego), OM45p (Yaffe *et al.*, 1989), CoxVp (Cumsky *et al.*, 1985), Ssc1p, and hexokinase (a gift from M. Yaffe). Immune complexes were visualized using HRP-conjugated secondary antibody (Amersham, Arlington Heights, IL) followed by chemiluminescence (ECL) (Amersham).

Import of Sms1p into Isolated Mitochondria

We first inserted the *SMS1* ORF into a vector suitable for in vitro transcription and translation as follows. We digested pGAL-SMS1 (see below) with *Sal*I and *Eco*RI, isolated the 1.1-kb insert containing *SMS1*, and ligated it into *Xho*I-*Eco*RI–digested pSP64 (Promega) to form pKR13. We produced radiolabeled Sms1p from pKR13 using 1.5 mCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham) in the SP6 TNT System (Promega Biotech) according to manufacturers' instructions. Mitochondria were isolated from wild-type strain D273-10b as described (Daum *et al.*, 1982a).

We resuspended isolated mitochondria to 1 mg/ml in import buffer (Scherer *et al.*, 1992) and used 100 μg mitochondria per import reaction. We added 5 μl of the Sms1p-containing reticulocyte lysate to each reaction and incubated at 30°C for 20 min. We terminated the reactions by transferring them to ice and adding carbonyl cyanide *m*-chlorphenylhydrazone (CCCP, Sigma Chemical, St. Louis, MO) to a final concentration of 25 μM. We digested unimported Sms1p using 100 μg/ml proteinase K (Sigma Chemical) on ice for 30 min. After all manipulations, we reisolated the mitochondrial pellet, resuspended it in Sample Buffer (125 mM tris(hydroxymethyl)aminomethane-HCl pH 6.8, 2% SDS, 20% glycerol), and analyzed the proteins by SDS-PAGE and fluorography (Bonner and Laskey, 1974).

Construction of pGAL-SMS1-HA, an Epitope-tagged Sms1p Expressed from the GAL1 Promoter

We placed the *SMS1* gene under the control of the yeast *GAL1* promoter as follows. A 1.1-kb PCR fragment was isolated from plasmid pKR7 using oligonucleotides No. 107 (5'-GCGCTCGAGCGTTATGTCAGCCG-3') and No. 99 (5'-AATACGACTCACTATAG-3'). We digested the PCR fragment with *Xho*I and *Eco*RI and inserted it downstream of the *GAL1* promoter in *Xho*I-*Eco*RI–digested plasmid pRS316GU (Sikorski and Hieter, unpublished data) to form the *URA3*-containing plasmid pGAL-SMS1. To construct pGAL-SMS1-HA, we digested pKR11 with *Bst*XI, which cuts at nucleotide 419 in the *SMS1* ORF, and with *Eco*RI, which cuts in the polylinker downstream of the ORF. We isolated a 1.1-kb fragment containing the epitope-tagged 3' end of *SMS1* and ligated it into *Bst*XI-*Eco*RI-digested pGAL-SMS1.

To construct a strain dependent on pGAL-SMS1-HA expression, we transformed a diploid heterozygous for the *sms1::TRP1* disruption (see above) with a pKR11, a *LEU2-CEN6* plasmid that carries *SMS1-HA*. We sporulated the diploid and isolated a haploid segregant

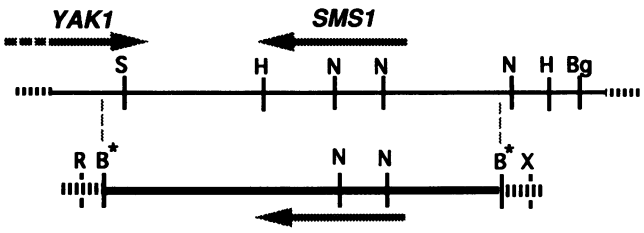

Figure 2. Restriction endonuclease map of the cloned *SMS1* gene. The top solid line indicates the relevant restriction sites in the original insert carried in plasmid pKR3. The lower solid line shows the 1750-bp partial *Sau3A* fragment in pKR7 shown to carry the *SMS1* gene. The striped lines indicate adjacent vector sequences. The shaded arrows represent the approximate locations of the *YAK1* and *SMS1* genes and their directions of transcription. Restriction endonuclease sites: Bg, *Bgl*II; H, *Hin*dIII; N, *Nco*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I; and B*, a hybrid site containing a *Bam*HI site and a *Sau3A* site.

that contained both the *sms1::TRP1* disruption and the *SMS1-LEU2* plasmid. We transformed this strain with the pGAL-SMS1-HA plasmid and transferred the transformants to medium-containing galactose. We then isolated mitotic segregants that contained only the pGAL-SMS1-HA plasmid and not the *SMS1-LEU2* plasmid.

Stability of the Altered Mas6p in mas6-1 Cells

Strains JE14-5b and JE14-5b carrying pKR7 were grown on minimal medium at 22°C to an OD_{600} of 1.0. We diluted each culture to an OD_{600} of 0.5 and shifted them to 34°C for 1 h. We then added cycloheximide to a final concentration of 100 μg/ml and continued to incubate the cultures at 34°C for 10 h. At several times during this incubation, we measured the OD_{600} of each culture and extracted total cell proteins from aliquots representing 10 OD units of cells (Yaffe and Schatz, 1984). We ran 30 μg of each protein sample on SDS-polyacrylamide gels and transferred the proteins to Immobilon filters. We visualized the Mas6 protein by immune blotting and determined the relative amount of Mas6p in each sample by densitometry (MCID, Imaging Research, Ontario, Canada).

RESULTS

Isolation of SMS1, a High-Copy Suppressor of a mas6 Mutant

To identify new proteins required for mitochondrial protein import, we isolated a high-copy suppressor of the temperature-sensitive growth defect of a *mas6* mutant. In particular, we transformed the *mas6-1 leu2* strain JE14-5b with a yeast genomic library carried in the 2μ-*LEU2* vector YEp13 (Nasmyth and Tatchell, 1980). Plasmids carrying the 2μ origin of replication are present in 10–40 copies per cell, resulting in overexpression of genes carried on these plasmids (Armstrong *et al.*, 1989). Five thousand five hundred Leu^+ transformants were isolated at 23°C and screened for the ability to grow at 34°C. We initially identified 32 colonies that grew at 34°C, but the temperature-resistant phenotype was plasmid-dependent for only four transformants. Plasmids isolated from these four transformants conferred temperature resistance when reintroduced into *mas6* cells. One of these strains is shown in Figure 1. The other temperature-resistant colonies were assumed to result from reversion of the *mas6-1* mutation and were not studied further. Restriction endonuclease digestion of the four plasmids indicated that they carried overlapping DNA inserts and thus the same gene. We showed by restriction mapping and by PCR that none of the plasmids carried wild-type *MAS6* sequences. Hence we have identified *SMS1*, a high-copy suppressor of *mas6-1*.

We localized the suppression activity from one of the plasmids to a 1750-bp partial *Sau3A* fragment (Figure 2). Initial DNA sequencing revealed that the insert contained sequences adjacent to the 3′ end of the *YAK1* gene (Garrett and Broach, 1989). Complete sequencing of this region identified a 477-bp ORF, predicted to encode a protein of 16.5 kDa (Figure 3). To confirm that this ORF contained the suppression activity, we removed an *Nco*I fragment containing nucleotides 68–262 of the *SMS1* ORF (see Figure 2) and replaced these sequences with the yeast *TRP1* gene. A 2μ-*URA3* plasmid containing this 3150-bp *sms1::TRP1* fragment no longer allowed *mas6-1* cells to grow at 34°C, indicating that overexpressed *SMS1*

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CAACGAACATTCATCGGACGCCTTTTTTACTGCTATTGTTCAACAAAGTGGGTAACCCGGCATTCTTGCGCCAAAGTTTC   80
CATTTTTATGCCAAATCCAGAGGATAAAAGCACTATTCTCATCAAAAGATGGAAAAGCTGTGAAAGAGTCAAGTTCAGAA  160
GAAAGCGTTAAGAAAAATAGAGTACACGGGAGCGTT ATG TCA GCC GAT CAT TCG AGA GAT CCA TGT CCT  229
                                     M   S   A   D   H   S   R   D   P   C   P

ATA GTC ATA CTA AAT GAT TTC GGT GGT GCT TTT GCC ATG GGT GCC ATT GGT GGT GTT GTT   298
 I   V   I   L   N   D   F   G   G   A   F   A   M   G   A   I   G   G   V   V

TGG ACT GGG ATT AAA GGT TTT AGA AAT TCG CCA TTA GGT GAG CGT GGT TCA GGA GCT ATG   367
 W   T   G   I   K   G   F   R   N   S   P   L   G   E   R   G   S   G   A   M

AGC GCC ATT AAA GCG CGT GCT CCC GTA CTG GGT GGT AAT TTT GGT GTG TGG GGT GGT TTA   436
 S   A   I   K   A   R   A   P   V   L   G   G   N   F   G   V   W   G   G   L

TTT TCG ACT TTT GAT TGC GCT GTG AAG GCC GTT AGA AAG AGA GAG GAC CCA TGG AAT GCT   505
 F   S   T   F   D   C   A   V   K   A   V   R   K   R   E   D   P   W   N   A

ATC ATT GCA GGG TTT TTC ACT GGT GGC GCT TTA GCT GTA AGA GGT GGT TGG AGG CAT ACA   574
 I   I   A   G   F   F   T   G   G   A   L   A   V   R   G   G   W   R   H   T

AGG AAC AGT TCG ATC ACG TGT GCT TGT TTG TTG GGT GTG ATT GAA GGT GTG GGA CTA ATG   643
 R   N   S   S   I   T   C   A   C   L   L   G   V   I   E   G   V   G   L   M

TTT CAA AGA TAT GCT GCT TGG CAA GCC AAA CCT ATG GCT CCT CCT TTG CCC GAA GCA CCT   712
 F   Q   R   Y   A   A   W   Q   A   K   P   M   A   P   P   L   P   E   A   P

TCC TCT CAA CCT CTG CAA GCT TAG GCATGTAGACATTATATGAGCCATTTTTTCATCGTTGTTGGAAGTACT   784
 S   S   Q   P   L   Q   A   *

TTTACTTTATTTCAGCCTTTTATTTCAAGATTACCAACCATTTCTCTCAACCATGTACATATTATATATTGAAAAAGTAC  864
CATACTTCATCTCTGAAGAGAAAATATCAACAAGCCAAAAGCTAGTGCAGAAACTTTATAATGTTACTTTATTAATTGTA  944
ATGACTATACGTATAAGGAAAATTAAGAAAAGGCAAAATTCATATTTATTTAGTATATCATTATGGCACCAAGCCGTAAT 1024
AAAATGAAAT
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Figure 3. *SMS1* encodes a 16.5-kDa protein. DNA sequence of *SMS1* and its predicted protein product. Underlined amino acids represent potential membrane-spanning domains predicted from hydropathy analysis.

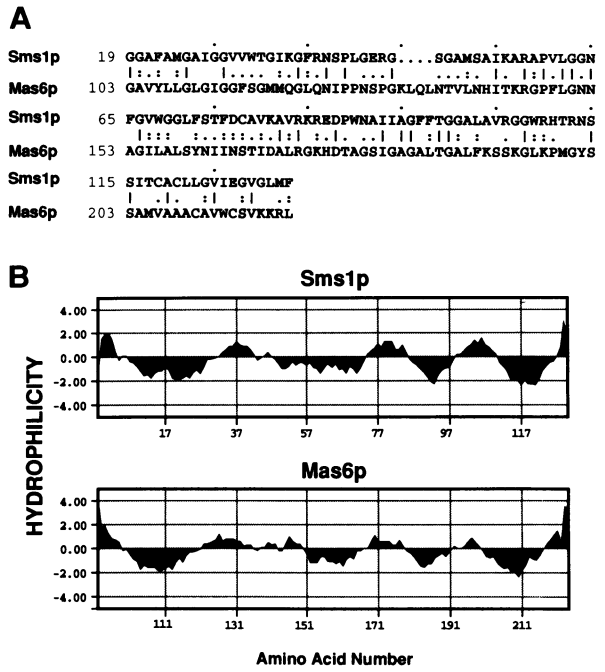

Figure 4. Sms1p is homologous to the carboxyl-terminal domain of the Mas6 protein. (A) The Sms1 protein was compared to the carboxyl-terminal domain of Mas6p (Emtage and Jensen, 1993) using the BestFit algorithm (Needleman and Wunsch, 1970; Smith and Waterman, 1981) with a gap weight of 3 and a gap length weight of 0.1. Identical amino acids in Sms1p and Mas6p are indicated by a line, and conservative amino acid substitutions are indicated by two dots. Numbers represent the positions of the amino acid residues in Sms1p or Mas6p. (B) Hydropathy plots of Sms1p and the carboxyl-terminal domain of Mas6p. Proteins were analyzed by the algorithm of Kyte and Doolittle (1982) with a window size of 12. Hydrophobic residues are indicated as shaded areas with negative values. Positions of amino acid residues in Sms1p and Mas6p are indicated.

is responsible for suppressing the *mas6-1* growth defect.

A comparison of the Sms1 protein with available databases identified Mas6p as the most significant homology. Using the BestFit alignment program (Needleman and Wunsch, 1970; Smith and Waterman, 1981), Sms1p is homologous to the carboxyl-terminal domain of Mas6p, with 46% similar (25% identical) amino acid residues (Figure 4A). Furthermore, hydropathy analysis (Kyte and Doolittle, 1982) indicates that Sms1p, like Mas6p, is a membrane protein with several potential membrane-spanning domains (Figure 4B). The hydropathy profiles of Sms1p and the carboxyl-terminal half of the Mas6 protein are strikingly similar, both in the number and the position of potential membrane-spanning domains.

SMS1 Is an Essential Gene

To determine if *SMS1* encodes an essential protein, we constructed strains carrying a chromosomal *sms1::TRP1* disruption. We transformed a linear fragment containing the *sms1::TRP1* construct (see above) into two different *trp1/trp1* diploids, strain 410 and strain YPH501. Stable Trp^+ transformants were isolated, and Southern blots of DNA from these cells confirmed that in each strain one copy of *SMS1* had been replaced by the *sms1::TRP1* disruption. When we sporulated these diploid cells and allowed the haploid progeny to grow at 23 or 30°C, only Trp^- cells were viable. *SMS1* is therefore an essential gene. Spores inferred to be *sms1::TRP1* germinated, underwent three to five divisions, and then arrested in their growth. Like cells containing a disruption of the *MAS6* gene (Emtage and Jensen, 1993), >95% of *sms1::TRP1* cells arrested as unbudded cells of differing sizes.

Whereas overexpression of *SMS1* suppresses the temperature-sensitive lethality of the *mas6-1* mutant, it does not complement a *mas6::URA3* disruption. In addition, overexpression of *MAS6* on a 2μ plasmid fails to rescue the *sms1::TRP1* disruption. These results suggest that *SMS1* and *MAS6* do not perform strictly overlapping functions in the cell.

The Sms1 Protein Is Located in the Mitochondrial Inner Membrane

We employed two approaches to determine the intracellular location of Sms1p. First, we synthesized the Sms1 protein in vitro and found that it could be imported into isolated mitochondria (Figure 5). The Sms1 protein in reticulocyte lysate is susceptible to digestion by proteinase K (lane 2), but it becomes inaccessible to protease digestion after import into mitochondria (lane 4). Sms1p seems to be synthesized without a mitochondrial presequence, as the Sms1 protein had the same apparent molecular mass before and after import (compare lanes 1 and 4). We observed a smaller protein product (*) in the reticulocyte lysate that remained in the mitochondrial pellet after the import reaction (lane 3), but this smaller protein was not protected from externally added proteinase K (lane 4). Therefore, only the full-length Sms1p is efficiently imported into isolated mitochondria.

We also directly localized the Sms1 protein in yeast cells. To follow Sms1p through the fractionation steps, we constructed a version of the Sms1 protein containing the influenza HA epitope at its carboxyl terminus. The HA epitope is recognized by the monoclonal antibody 12CA5 (Niman *et al.*, 1983). The Sms1-HA fusion protein, encoded on plasmid pKR11, rescues the lethality of *sms1::TRP1* strains. Cells expressing the Sms1-HA protein contained a single protein of 21 kDa that reacted with the 12CA5 antibodies. The size of this protein is consistent with the addition of the 4-kDa HA tag to the 16.5-kDa Sms1 protein. The 21-kDa protein was not found in wild-type cells lacking the pKR11 plasmid, and the Sms1-HA fusion was overproduced when we expressed it from the strong *GAL1* promoter (see Figure 9A). The 12CA5 antibodies are thus specific to Sms1-HA.

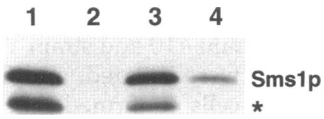

Figure 5. Sms1p is synthesized without a mitochondrial presequence and can be imported into isolated mitochondria. Mitochondria were isolated from wild-type strain D273-10b and incubated with ^{35}S-methionine-labeled Sms1p as described in MATERIALS AND METHODS. Lane 1, 40% of the Sms1 protein added to each import reaction. Lane 2, 40% of the Sms1p added to each import reaction, treated with 100 μg/ml proteinase K. Lane 3, the mitochondrial pellet after an import reaction. Lane 4, the mitochondrial pellet after an import reaction, treated with 100 μg/ml proteinase K. The full-length Sms1 protein (Sms1p) and a smaller translation product (*) are indicated.

Immune blotting of yeast cell fractions shows that Sms1p is a mitochondrial protein. When we separated a cell homogenate into a mitochondrial pellet and a crude cytosolic fraction, Sms1-HA cofractionated with the mitochondrial marker, the β-subunit of the F1-ATPase (F1β) (see Figure 6). Little or no Sms1 protein was present in the cytosolic fraction, which contained the hexokinase marker. In addition, the Sms1-HA protein behaves as an integral membrane protein. Sms1-HA, like the Mas6 protein, remained in the mitochondrial membrane pellet after treatment with 0.1 M sodium carbonate (Figure 7). In contrast, >80% of the peripheral membrane protein F1β was solubilized by carbonate treatment. Furthermore, we could not extract Sms1p from mitochondrial membranes by treating with 500 mM potassium chloride or 1.6 M urea.

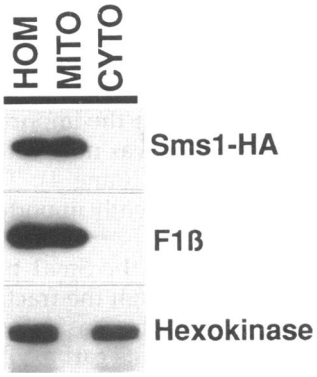

Figure 6. Sms1p is a mitochondrial protein. *sms1::TRP1* cells carrying the *LEU2-CEN6* plasmid pKR11, which express the Sms1 protein tagged with the influenza HA epitope, were grown to an OD_{600} of 2.0 in synthetic medium lacking leucine and containing 2% galactose. Yeast extract was added to 1%, and the cells were allowed to grow to an OD_{600} of 4.0. Cells were converted to spheroplasts and homogenized as described in MATERIALS AND METHODS. The homogenate (HOM) was centrifuged at 2500 × *g* for 5 min, and the supernatant was separated into a mitochondrial pellet (MITO) and a post-mitochondrial supernatant (CYTO) by centrifugation at 9600 × *g* for 10 min. Aliquots of homogenate, mitochondria, and cytosol representing equivalent numbers of cells were subjected to SDS-PAGE and analyzed by immune blotting with 12CA5 antibodies to the HA epitope tag (Sms1-HA), antiserum to hexokinase, or antiserum to the F_1-ATPase β-subunit ($F_1\beta$).

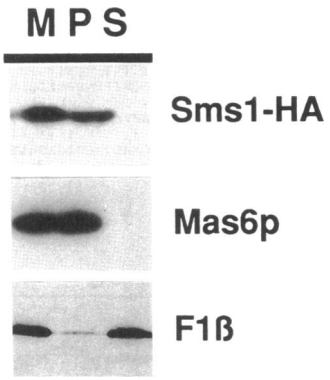

Figure 7. Sms1p is an integral membrane protein. Mitochondria (100 μg protein) were centrifuged at 12 500 × *g* for 10 min, and the mitochondrial pellet resuspended in 200 μl of 0.1 M sodium carbonate. Suspensions were centrifuged at 150 000 × *g* for 45 min in a Beckman Airfuge (Fullerton, CA). Proteins from equal aliquots of mitochondria (M), the pellet (P), and supernatant (S) after carbonate extraction were separated by SDS-PAGE and immune blotted with antibodies to the HA epitope tag (Sms1-HA), antiserum to $F_1\beta$, or antiserum to Mas6p.

Sms1p is located in the inner membrane. When we sonicated mitochondria carrying the HA-tagged Sms1 protein and separated the membrane vesicles on sucrose density gradients, Sms1-HA cofractionated with the inner membrane proteins Mas6p and F1β (Figure 8, fractions 8–10). We found little or no Sms1 protein in fractions containing the outer membrane protein, OM45p. We conclude that Sms1p is a mitochondrial inner membrane protein.

Depletion of Sms1p from Cells Causes a Defect in Mitochondrial Protein Import

SMS1 was isolated by its genetic interaction with the *mas6-1* mutation. To determine if the Sms1 protein, like

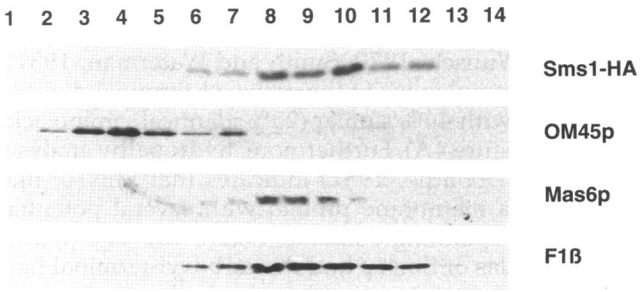

Figure 8. The Sms1 protein fractionates with the inner membrane. Mitochondria were converted to mitoplasts and sonicated as described in MATERIALS AND METHODS. Mitochondrial membrane vesicles were loaded onto 5-ml sucrose step gradients and centrifuged at 100 000 × *g* for 16 h. Fractions (0.3 ml) were collected, and an aliquot from each fraction was analyzed by immune blotting with antibodies to the HA epitope tag (Sms1-HA), antiserum to the inner membrane protein, Mas6p, antiserum to the inner membrane protein F1β, or antiserum to the outer membrane protein, OM45p. The top of the gradient is to the left.

Mas6p, is required for mitochondrial protein import, we asked whether depletion of Sms1p from cells would cause an import defect. We placed the ORF coding for the Sms1-HA fusion protein under the control of the galactose-inducible *GAL1* promoter in plasmid pGAL-SMS1-HA. We introduced the pGAL-SMS1-HA construct into cells containing a disruption of the chromosomal *SMS1* gene (*sms1::TRP1*) and examined mitochondrial protein import when production of the fusion protein was inhibited by growth on glucose (Figure 9, B and C). We also monitored the level of Sms1-HA by immune blotting (Figure 9A).

In galactose-containing medium, cells carrying pGAL-SMS1-HA overproduced Sms1p approximately fivefold compared to cells expressing SMS1-HA from the *SMS1* promoter (Figure 9A, compare the 0 h timepoint to WT). When we shifted these cells to glucose medium, which inhibits expression from *GAL1*, the level of Sms1-HA dropped. Four hours after shifting to glucose-containing medium, the amount of Sms1-HA was below the wild-type level. By 6 h, we could detect no Sms1-HA protein by immune blotting. In parallel with the loss of Sms1-HA, the precursor to the β-subunit of the F_1-ATPase accumulated (Figure 9B, F1β). This defect in mitochondrial protein import was not simply due to cell inviability, because cells containing pGAL1-SMS1-HA continued to divide for $\geq$24 h after their shift to glucose-containing medium. By 36 h, however, cells depleted of Sms1-HA stopped growing. In a similar experiment, we also observed accumulation of the precursor to the α-subunit of the F_1-ATPase 8 h after shifting the cells to glucose-containing medium.

Some mitochondrial proteins simply disappear from the cells when Sms1p is depleted, presumably because they have short half-lives and unstable precursor forms. The two isoforms of cytochrome oxidase subunit V, CoxVa and CoxVb (Cumsky *et al.*, 1985), disappeared from the cells as Sms1p was depleted (Figure 9C, CoxV). The mature form of CoxVb was present, but in reduced amounts, after 8 h in glucose, whereas by this time CoxVa was absent. We could not detect the precursor forms of these proteins, suggesting that they are rapidly degraded (Miller and Cumsky, 1993). Like the CoxV proteins, the mature form of the mitochondrial hsp70 protein, Ssc1p, also disappeared rapidly in Sms1p-depleted cells (Figure 9B, Ssc1p). We detected a very small amount of the Ssc1 precursor at 6 and 8 h. Sms1p is thus required for the import of several mitochondrial proteins.

The results above suggest that Sms1p is part of the machinery that imports proteins into the mitochondria. It is possible, however, that the role of Sms1p is to regulate the level, location, or function of Mas6p. In this case, loss of the Sms1 protein would block import indirectly, through its effect on the Mas6 protein. We found that Sms1p is not required to maintain normal levels of Mas6p in the cell. The total amount of the Mas6 protein remained unchanged in cells depleted of Sms1p (Figure 9D). Furthermore, excess Sms1p did not affect the stability of the altered Mas6 protein in *mas6-1* cells incubated at the nonpermissive temperature (Figure 10). Our results are therefore consistent with Sms1p acting directly in the mitochondrial import pathway.

DISCUSSION

We have identified a new gene, *SMS1*, as a high-copy suppressor of the temperature-sensitive growth defect

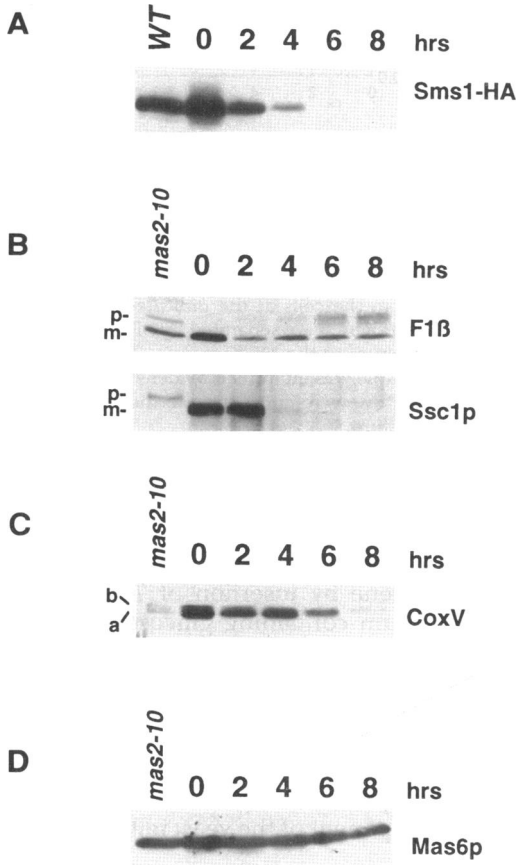

Figure 9. Cells depleted of the Sms1 protein are defective in importing several mitochondrial precursors. *sms1::TRP1* cells carrying the pGAL-SMS1-HA plasmid were grown on YP medium containing 2% galactose to an OD_{600} of 1.0. Cells were centrifuged, and pellets were resuspended in glucose medium (YPD) to an OD_{600} of 0.1. At the indicated timepoints, proteins were extracted (Yaffe and Schatz, 1984). Aliquots from different timepoints containing 80 μg protein were separated by SDS-PAGE. (A) The Sms1-HA protein was identified by immune blotting with 12CA5 antibodies to the influenza HA epitope. *sms1::TRP1* cells carrying pKR11 (WT) were grown to an OD_{600} of 1.0 in YP galactose medium, and proteins were extracted and analyzed as above. (B) The mitochondrial hsp70 protein (Ssc1p) and the β-subunit of the F_1-ATPase (F1β) were identified by immune blotting. The precursor (p) and mature (m) forms of each protein are indicated. (C) Subunit V of cytochrome oxidase was identified by immune blotting, and the CoxVa and CoxVb isoforms are indicated. (D) The Mas6 protein was identified by immune blotting. The temperature-sensitive *mas2-10* strain (Yaffe and Schatz, 1984), included to provide an example of precursor accumulation, was shifted to 37°C for 10 h in galactose-containing medium before proteins were extracted (*mas2-10*).

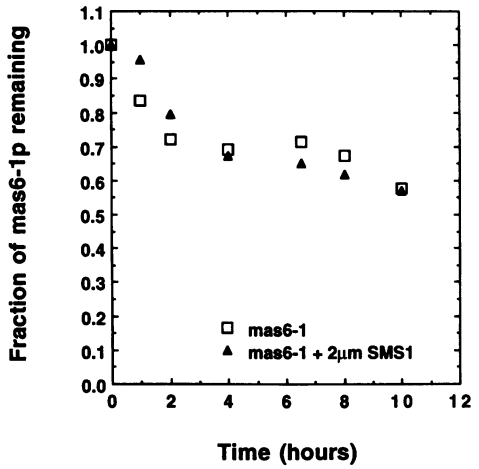

Figure 10. Overexpressing *SMS1* does not stabilize the altered Mas6 protein in *mas6-1* cells. *mas6-1* cells (strain JE14-5b) with and without pKR7, a 2μ-*SMS1* plasmid, were grown to an OD_{600} of 1.0 at room temperature, diluted to 0.5, and shifted to 34°C for 1 h. Cycloheximide was added to a final concentration of 100 μg/ml at time 0, and proteins were extracted from the cells at the indicated timepoints. Aliquots containing 30 μg protein were separated by SDS-PAGE and immune blotted with antiserum to the Mas6 protein. The relative amount of Mas6p in each sample was quantified by densitometry, and the samples from each culture were normalized to the initial Mas6p content (time 0) of that culture.

of the *mas6-1* mutant. Sms1p is located in the mitochondrial inner membrane, along with the Mas6 protein. *SMS1* also encodes an essential protein. Cells disrupted in the *SMS1* gene by insertion of *TRP1* are inviable, even on medium containing glucose. Besides Sms1p, the only other essential proteins identified in the inner membrane, Mas6p (Emtage and Jensen, 1993) and Isp45p (Maarse *et al.*, 1992; Horst *et al.*, 1993), are directly involved in import. Depletion of the Sms1 protein from yeast cells abolishes the import of several different mitochondrial proteins. Our results thus suggest that Sms1p is a component of the yeast mitochondrial import pathway.

SMS1 is predicted to encode a protein of 159 amino acids with a molecular mass of 16.5 kDa. Hydropathy analysis indicates that Sms1p has several potential membrane-spanning domains. Supporting this prediction, we find that Sms1p cannot be extracted from the mitochondrial inner membrane with high salt, urea, or carbonate. The DNA sequence of *SMS1* suggests that Sms1p is synthesized without a typical amino-terminal presequence, and there was no evidence of presequence removal when Sms1p was imported into isolated mitochondria. The import of Sms1p is thus similar to Mas6p, in that the mitochondrial targeting signal apparently resides in the mature protein.

When we compared the Sms1 protein to sequences in available databases, Mas6p was identified as the most homologous protein. Sms1p is 46% similar (25% identical) to the carboxyl-terminal half of the Mas6 protein. It is important to note, however, that most of the similarity between Mas6p and Sms1p is restricted to hydrophobic domains, which might limit amino acid variability in these regions. An alignment made after randomizing the Sms1 protein sequence with the SHUFFLE program shows no significant change in the percentage of similarity, but the identity drops from 25 to 20%. The randomized Sms1 protein no longer identifies Mas6p in database searches. We therefore argue that the similarities between Sms1p and Mas6p are significant. Supporting this conclusion, hydropathy plots suggest that the topologies of Sms1p and Mas6p in the inner membrane may be very similar.

The genetic interaction between *SMS1* and *mas6*, the inner membrane location of the two proteins, and the homology between the Sms1p and Mas6p suggest that the two proteins may play similar roles in translocating proteins across the mitochondrial inner membrane. One possibility is that Sms1p and Mas6p reside in distinct translocation complexes. In this case, overproduction of Sms1p would rescue the *mas6* mutant by increasing the activity of a different pathway into the mitochondrial matrix. We think this model unlikely because multiple copies of *SMS1* did not rescue the lethality of the *mas6* null mutant, *mas6::URA3*, and multiple copies of *MAS6* did not rescue the lethality of the *sms1::TRP1* null mutant. In addition, Sms1p and Mas6p are both required for the import of the same precursors. Yeast cells depleted of either the Sms1 protein or of Mas6p (Emtage and Jensen, 1993) are both defective in importing the F1α and F1β proteins. We favor an alternative model, in which Sms1p and Mas6p act together in a complex of proteins required for import. For example, Sms1p and Mas6p may be subunits of a protein-translocating channel in the mitochondrial inner membrane. In this case, overproduction of the Sms1 subunit of the complex compensates for the reduced activity of the Mas6 subunit in *mas6* mutants. Experiments are in progress to determine whether Sms1p physically associates with Mas6p.

To confirm that Sms1p is a component of the mitochondrial protein import machinery and to pinpoint its role in import, we are currently isolating temperature-sensitive mutations in *SMS1*, and we are determining if Sms1p can be cross-linked to precursors in transit across the mitochondrial inner membrane.

ACKNOWLEDGMENTS

We wish to thank Michael Yaffe and Jeff Schatz for antiserum to the OM45p and F1α proteins. We also thank Shawn Burgess for assistance in DNA sequencing and Jennifer Kalish for construction of strain 410. We thank Kathy Wilson, Jennifer Emtage, Shawn Burgess, Oliver Kerscher, and Elizabeth Reisinger for critical reading of the manuscript. This work was supported by grant R01-GM46803 from the United States Public Health Service and American Cancer Society Faculty Research grant JFRA-367 to R.E.J., grant R01-GM44666 from the United States Public Health Service and American Cancer Society Faculty Research grant JFRA-395 to S.G., and Medical Scientist Training Program grant GM07309 to K.R.R.

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