The ARG11 Gene of *Saccharomyces cerevisiae* Encodes a Mitochondrial Integral Membrane Protein Required for Arginine Biosynthesis*

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Prototype strain MG409 (*arg11–1*) is a severe arginine bradytroph with greatly reduced ornithine and arginine pools, although all known enzymes required for arginine biosynthesis are functional. To identify the function required for normal arginine production impaired in MG409, we have cloned, sequenced, and performed a first molecular characterization of ARG11.

We show that the ARG11 open reading frame encodes a putative 292-residue protein with a predicted molecular mass of 31.5 kDa. Sequence similarities, a tripartite organization, and six potential hydrophobic transmembrane spans suggest that Arg11p belongs to the mitochondrial integral inner membrane carrier family. We have used immuno-Western blotting and hemagglutinin epitope-tagged derivatives of Arg11p, Arg8p (a mitochondrial matrix marker), and Arg3p (a cytosolic marker) to demonstrate that Arg11p is confined to the mitochondria and behaves like an integral membrane protein.

A deletion created in ARG11 causes the same arginine-leaky behavior as the original *arg11–1* mutation, which yields a premature stop codon at residue 266. Arg11p thus appears to fulfill a partially redundant function requiring its 27 carboxyl-terminal amino acids. As a working hypothesis, we propose that Arg11p participates in the export of matrix-made ornithine into the cytosol.

Yeast strain MG409 (*arg11–1*) grows very poorly on minimal medium (generation time close to 7 h). Added arginine restores normal growth. For years this strong arginine bradytroph has been used to achieve maximal levels of expression of the arginine-biosynthetic enzymes, since severe arginine starvation results concomitantly in release from arginine-specific repression and in transactivation by Gcn4p, a general control-specific transcription factor whose translation is increased under conditions of amino acid deprivation (1). As expected, the levels of enzymes of other biosynthetic pathways subject to Gcn4p-dependent cross-pathway control are also increased in MG409; accordingly, several amino acid pools (tyrosine, phenylalanine, lysine, and others) are substantially higher than in the isogenic wild-type strain, approaching the levels observed in other amino acid-leaky mutants. Distinctive features of the *arg11–1* mutant are a 3-fold-increased glutamate pool, an expectedly reduced arginine pool (5-fold), and a drastically decreased ornithine pool (10-fold) (1). Yet all enzymes known to be required to synthesize arginine from glutamate are functional in MG409 (2, 3). This tallies with the genetic data; the *arg11–1* allele defines a new complementation class distinct from genes *ARG1* to *ARG8* (encoding the biosynthetic enzymes), and it is unlinked to these genes.1

Several hypotheses might explain the *arg11–1* mutant phenotype. (i) Expression of the arginine-catabolic enzymes at high constitutive levels causes arginine to be degraded as soon as it is produced. (ii) Highly deregulated polyamine synthesis uses up ornithine, making it unavailable for arginine synthesis. (iii) Upshifted levels of acetylornithine decaylase activity, usually low in *Saccharomyces cerevisiae* but higher in MG409 (4), might result in a shortage of acetylornithine and hence acetylglutamate, normally recycled from acetylornithine by acetylornithine-glutamate acetyltransferase (EC 2.3.1.35), leading to rate-limiting concentrations of acetylated arginine pathway intermediates. (iv) Since the first five enzyme-catalyzed steps of arginine biosynthesis involve production of ornithine in the mitochondria (5), various organelle-linked defects might explain the *arg11–1* phenotype, e.g. impaired entry of the initial substrate (glutamate) or exit of the final product (ornithine), incorrect processing of mitochondrial enzymes such as Arg5,6p (the precursor of acetyl-glutamyl kinase and acetylglutamyl-phosphate reductase) to mature enzymes (6), or, more drastically, mistargeting of some or all of the mitochondrial arginine-anabolic enzymes to the cytosol. This last hypothesis, however, would imply the existence of an unexpected arginine-specific element in the mitochondrial protein import machinery.

As a first step toward functional identification, we describe here the isolation and characterization of the ARG11 gene and demonstrate that Arg11p is a mitochondrial integral membrane protein.

MATERIALS AND METHODS

For strains and strain constructions, see Table I; for plasmids and plasmid constructions, see Table II.

Media—The basic minimal medium (M) has been described (12). Medium M.am is M supplemented with 0.02 M (NH₄)₂SO₄ as the nitrogen source. Where required, L-amino acids and uracil are added at 25 μg/ml final concentration. To prepare mitochondria, we grew cells in M.am containing 3% galactose as the carbon source, instead of the usual 3% glucose, in order to increase the organelle yield. Galactose medium

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1 M. Grenson, unpublished data.
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### Table I

| Name | Genotype | Origin/Construction |
|------|----------|---------------------|
| Σ1278b | Mate (wild type) | Gift of M. Grenson |
| MG47 | Mate (ura3<sup>−</sup>) | Gift of M. Grenson |
| MG409 | Mate (arg11<sup>−</sup>-1) | Gift of M. Grenson |
| 1c1636d | Mate (ura3<sup>−</sup>, arg11<sup>-1</sup>) | Gift of F. Messenguy |
| 11690b | Mate (ura3<sup>−</sup>, arg8<sup>-1</sup>) | Ref. 7 |
| SS1 | Mate (ura3<sup>−</sup>, arg3<sup>-AXS</sup>) | Ref. 8 |
| 10R41b | mate (ura3<sup>−</sup>, leu2<sup>−</sup>, argRII<sup>-</sup>) | This laboratory |
| RP1 | Mate (ura3<sup>−</sup>, arg11<sup>-Δ5A::URA3</sup>) | This work; obtained by gene transplacement in strain MG471 as explained in Fig. 6A. Confirmed on Southern Blot (Fig. 7). |
| RP2 | Mate (ura3<sup>−</sup>, arg11<sup>-ΔA::URA3</sup>) | This work; obtained by gene transplacement in strain RP2 as explained in Fig. 6C. Confirmed on Southern blot (see text) by demonstrating the shift of the 1.9-kb sacl fragment present in the genome of Σ1278b to two bands of 1.7 and 0.2 kb in MC157, due to the presence of the AatII restriction site in the HA-encoding sequence (data not shown). |
| MC157 | Mate (ura3<sup>−</sup>, HA-ARG11) | This laboratory |
| YM4127 | Mate (ura3<sup>-52</sup>, his3<sup>-200</sup>, ade2<sup>-101</sup>, lys2<sup>-801</sup>, trpl<sup>-903</sup>, leu2<sup>-3</sup>, 112, tyr1<sup>-501</sup>) | A gift of M. Johnston |

### Table II

| Plasmids | Type of vector | Type of insert/Mode of construction |
|----------|----------------|------------------------------------|
| pFL1-ARG11-no 1 | pFL1 = pBR322-2μ-URA3 | Bears a 7-kb Sau3A-BamHI genomic fragment from strain FL100. |
| pRP5 | pFL1 = pBR322-2μ-URA3 | Derived from pFL1-ARG11-no 1 by deleting a SacI fragment of 1.94 kb from the yeast insert (Fig. 1). |
| pRP6 | pFL1 = pBR322-2μ-URA3 | Derived from pFL1-ARG11-no 1 by deleting a BglII-BamHI fragment of 3.3 kb from the yeast insert (Fig. 1). |
| pRP10a | pFL38 = pUC19-ARS CEN-URA3 | Bears the 1.94 kb SacI fragment of pFL1-ARG11-no 1. |
| pRP10b | pFL38 = pUC19-ARS CEN-URA3 | Same as pRP10a, but insert has opposite orientation (Fig. 1). |
| pRP12 | pBR322-URA3 | Derived from pFL1-ARG11 no 1 by deletion of the 2μ EcoRI fragment. |
| pRP17 | Bluescript M13<sup>+</sup>/SK | Bears the 1.94-kb SacI fragment of pFL1-ARG11-no 1. The + strand corresponds to the ARG11 sense strand. |
| pRP18 | Bluescript M13<sup>+</sup>/SK | Bears the 1.94-kb SacI fragment of pFL1-ARG11-no 1. The + strand corresponds to the ARG11 antisense strand. |
| pRP20 | Bluescript M13<sup>+</sup>/SK | Derived from pRP17 by insertion of a URA3 1.1-kb BglII fragment (isolated from pFL38) into a BciI restriction site located 67 nt downstream of the TAA termination codon of ARG11. |
| pRP21 | Bluescript M13<sup>+</sup>/SK | Derived from pRP20 by deletion of the SfiI/Aor51HI fragment extending from −184 to +663 nt relative to the initiation codon of ARG11 (ΔSA). |
| pRP22 | Bluescript M13<sup>+</sup>/SK | Derived from pRP20 in which a deletion of about 100 nt was created by an EcoIII/S1 treatment from the Aor51HI restriction site located at +663 nt downstream of the initiation codon of ARG11 (ΔA). |
| pRP2/3/4/14 | Bluescript M13<sup>+</sup>/SK | Four independent clones bearing a 1072-nt-long BamHI-SalI fragment obtained by PCR amplification of the wild-type ARG11 gene of strain Σ1278b, from 161 nt upstream of the initiation codon to 100 nt downstream of the TAA stop codon. Primers used were pan7 (CCTCTGACTCGCCAATGATAAGTTCTCTGCTCCCTGGC-GTGGATATG) and pan8 (CCGGAGCCCAAGACGCTGGTGAGATGAGTAGTGCG-AATTTCCCATAT). |
| pPR5/8/9/10 | Bluescript M13<sup>+</sup>/SK | Four independent clones bearing a 1072-nt-long BamHI-SalI fragment obtained by PCR amplification of the arg11<sup>-I</sup> mutant gene of strain MG409, from 161 nt upstream of the initiation codon to 100 nt downstream of the TAA stop codon. Primers were as above. |
| pMC150 | Bluescript M13<sup>+</sup>/SK | Derived from pRP18; the HA epitope (YPFPDVYDA) was fused to the N-termini of Arg11p by site-directed mutagenesis using the Eckstein procedure and the MC100 oligonucleotide (GGT CGA CCT GTA ATG - TAC CCA TCA GAC GTG AGG AGT AAA AAG) as primer. The presence of an AatII restriction site allows convenient first screen of the mutagenized plasmid. pMC150 was confirmed by DNA sequencing. |
| pOS13 | pYeF2 = pBR322-2μ-URA3<sup>+</sup> | Using the plasmid pH37<sup>+</sup> (7), we amplified the ARG8 gene (from its ATG), using two primers containing BglII and NotI restriction sites, respectively, OR11 (GGCG-AGATCTCATCATGTTTAAAAGATATTTATCCAGTAC) and OR12 (GGCGGGCGGGCGGGTTCTT-GAAAATTCATATAC) and inserted it in frame upstream of the ARG11 gene (from its ATG), using two primers containing BglII and NotI restriction sites, respectively, OR11 (GGCG-AGATCTCATCATGTTTAAAAGATATTTATCCAGTAC) and OR12 (GGCGGGCGGGCGGGTTCTT-GAAAATTCATATAC) and inserted it in frame upstream of the HA epitope coding sequence present in the pYeF2 vector (9). |
| pOS16 | pYeF2 = pBR322-2μ-URA3<sup>+</sup> | Using the plasmid pMC302<sup>+</sup> (10), we amplified the ARG8 gene (from its ATG), using two primers containing BglII and NotI restriction sites, respectively, OR11 (GGCG-AGATCTCATCATGTTTAAAAGATATTTATCCAGTAC) and OR12 (GGCGGGCGGGCGGGTTCTT-GAAAATTCATATAC) and inserted it in frame upstream of the HA epitope coding sequence present in the pYeF2 vector (9). |
| pOS14 | pFLA6(L) = pUC19-2μ-LEU2 | The SacI fragment of pMC150 bearing the N-terminal HA epitope-tagged ARG11 gene was inserted in the SacI site of the vector polylinker (11). |

was also used to induce expression from the GAL1 promoter of the Arg8-HAp and Arg3-HAp fusion proteins in strains harboring plasmids pOS13 and pOS16.

Yeast Transformation and Recovery of Plasmid DNA from the Original Yeast Transformants—Yeast cells were transformed as in Ito et al. (13). Yeast plasmids were recovered as described previously (14).

DNA Sequencing Strategy—Starting with plasmids pRP17 and pRP18, we constructed families of serial deletions, using the Exonucl- ease III/nuclease S1 approach and the protocol provided with the Erase-a-base kit of Promega. Two series of 16 deletions were selected, pro-
Fig. 1. Restriction map of the yeast genomic insert complementing the arg11–1 mutation (upper lane). B, BamHI; S, SacI; StuI, BgIII, XhoI, EV, EcoRV. The three lower lanes represent the deleted versions and the subclone constructed to locate the complementing region.

Cloning of ARG11 by Functional Complementation—We first isolated yeast genes complementing the arg11–1 defect of strain 1c1636d (ura3Δ, arg11–1) by co-selecting for uracil and arginine prototrophy on minimal medium after transformation with a pFL1-based (2μ-URA3 vector; Ref. 18) yeast genomic insert fragment bearing the ARG11 gene. We used the Quiaex gel extraction kit from Quiagen to purify this fragment from agarose gels and Boehringer Mannheim’s DIG luminescent detection kit. From one end or the other. We then sequenced both DNA strands, using the dye-oxynucleotide termination procedure and the Pharmacia Biotech Inc. sequencing kit.

Southern Blotting—Standard protocols described by Sambrook et al. (15) were used for Southern blotting on Boehringer Mannheim nylon membranes. The probe used was the 1.944-kb yeast genomic SacI fragment nucleotide 1471 and is interrupted by the BglII restriction site after dephosphorylation of plasmid pKZ1 (see “Materials and Methods”). The primer was then used to generate a 1.944-kb SacI fragment that co-migrates in this karyoblot (data not shown).

Mitochondrial Preparations and Subfractionation—Mitochondria were isolated from yeast spheroplasts by differential centrifugation by the method of Daum et al. as optimized by Yaffe (16). The freely soluble matrix proteins and weakly bound membrane proteins were separated from the integral membrane proteins as by Fujiki et al. (17), except that treatment of the mitochondria with 0.1 M Na2CO3 was extended to 2 h.

RESULTS

Cloning of ARG11 by Functional Complementation—We first isolated yeast genes complementing the arg11–1 defect of strain 1c1636d (ura3Δ, arg11–1) by co-selecting for uracil and arginine prototrophy on minimal medium after transformation with a pFL1-based (2μ-URA3 vector; Ref. 18) yeast genomic library constructed by F. Lacroute. The uracil-”, arginine-”, and Proline-” character of several independent transformants was confirmed to be plasmid-linked (double auxotrophy reappearing after induction of plasmid loss by mitotic segregation on nonselective growth medium); their plasmid contents were transferred to Escherichia coli. The various plasmids identified by restriction enzyme analysis were retested for their ability to complement arg11–1 by back-transformation of strain 1c1636d; we finally identified five unrelated inserts endowed with this property, three of which fully complemented the mutation, the other two complementing it partially.

We used a genetic approach to identify inserts liable to bear the ARG11 structural gene: 2μ-deleted derivatives of the original plasmids were constructed, linearized at the level of the insert by appropriate restriction, and integrated by homologous recombination into the genome of strain 1c1636d, with selection for uracil-”, arginine-”, and Proline-” transformants. After crossing these transformants with a wild-type strain, we assessed the genetic

Evidence for linkage between the arg11–1 allele and the integrated DNA by meiotic segregation analysis. Tight coupling was observed between arg11–1 and the integrated plasmid pRP12, the 2μ version of pFL1-ARG11no1: we found only one arginine recombiant among the 14 dissected tetrads, suggesting that the 7-kb insert of this plasmid might carry the ARG11 gene. To narrow down the location of the arg11–1-complementing activity of this insert, we deleted parts of it (see Fig. 1), finally showing that a 1.944-kb SacI fragment is sufficient for complementation when subcloned in either orientation in the ARS-CEN-based monochromosomal plasmid pFL38 (see pRP10 in Fig. 1).

By hybridizing the labeled SacI fragment with the S. cerevisiae chromoblot sold by Clontech, we were able to assign it to either chromosome VII or chromosome XV, two chromosomes that co-migrate in this karyoblot (data not shown).

Nucleotide Sequencing: Arg11p Is a Member of the Mitochondrial Integral Inner Membrane Protein Family—Both strands of the SacI fragment were sequenced (see “Materials and Methods”). Only one complete open reading frame, ORF1, is present. It spans nucleotides 221–1099, thus encoding a predicted 292-residue protein with a molecular mass of 31523.5 daltons. A second, incomplete open reading frame, ORF2, begins at nucleotide 1471 and is interrupted by the SacI restriction site after 156 residues. Fig. 2 shows the nucleotide sequence of the SacI fragment and the deduced amino acid sequence for ORF1, confirmed in the next section to be ARG11.

A homology search in the SwissProt data base showed that ARG11 had not been characterized previously and revealed that Arg11p is very likely a new member of the mitochondrial integral inner membrane protein family (IM carriers). The FASTA program detected significant primary sequence similarity between the predicted Arg11p and a great many mitochondrial carriers, including the Brown fat uncoupling proteins of various organisms, several undefined "solute" carrier proteins, the human Graves disease protein, several yeast proteins, of which some are involved in RNA splicing and supposed to be solute carriers as well, a large number of ADP/ATP translocators, several oxoglutarate/malate antiporters, and a list of phosphate carrier proteins including the yeast transporter, etc.

Identity levels range from 25 to 30% for overlapping spans of 180–300 amino acid residues. Using the MOTEIFS program, we identified in Arg11p three copies of the “mitochondrial energy transfer protein signature” motif P|X|D/E|X|L/|L/V/A/T|R/K|X|L/R/H|I/L/V/M/F/Y, a feature of all IM transporter family members (19). Recently, an improved extended sequence of this signature was proposed: P|D/E|X|L/R/H|I/L/V/R/K|V/R/K/K-(20–30 amino acids)-(D/E)/G-(4 amino acids)-α-R/K/G (h representing hydrophobic and a representing aromatic amino acids) (20). Fig. 3 shows the good alignment of this consensus motif with the three found in Arg11p, starting, respectively, at amino acid residues 33, 127, and 233 (underlined in Fig. 2).
All IM carrier family members are about 300 amino acids long and characterized by a tripartite structure due to the 3-fold tandem repetition of an approximately 100-residue span; each of the three domains is assumed to consist of two hydrophobic transmembrane α-helices joined by a hydrophilic loop. For Arg11p likewise, the matrix analysis reproduced in Fig. 4 detects a 3-fold symmetry, and the hydrophobicity profile shown in Fig. 5 suggests the existence of six hydrophobic domains possibly corresponding to transmembrane regions. Secondary structure predictions (using the EMBL secondary prediction server and the Garnier program) predict the formation of α-helix stretches in each of these regions, the tendency being weaker in hydrophobic region III (data not shown). Like the other members of the IM carrier family, Arg11p is rather basic (isoelectric pH 9.2). Its N-terminal region contains several acidic amino acids (four in the first 16-residue stretch), suggesting that this region is not a matrix-targeting signal sequence.
Fig. 6; in pRP21, deletion ΔSA consists of the removal of the stretch between the SfiI and Aor51HI restriction sites, comprising the 184 nucleotides preceding the initiator codon plus those encoding the 220 N-terminal amino acids of the ORF1 product; in pRP22, the short deletion ΔA, estimated to consist of the removal of about 100 nucleotides on the basis of restriction band shifts, was obtained by limited ExoIII/SI digestion initiated at the Aor51HI restriction site that overlaps codons 221 and 222 of ORF1. Liberating the shortened SacI fragment from either pRP21 or pRP22, we selected uracil transformants in strain MG471(ura3Δ2) on a medium containing arginine. Gene transplacement is expected to occur by homologous recombination and requires formation of a stable duplex. In the case of the pRP21 insert, only 37 nucleotides are available upstream from the deletion, so the homologous DNA pairing depicted in Fig. 6A cannot be very stable. It is thus not surprising that among the 200 uracil transformants screened, only four exhibited the arginine deficiency expected of an arg11 deletion mutant. One such transformant, named strain RP1, was investigated further by Southern blot analysis. When the SacI fragment of pRP22 was used to transform MG471 to uracil prototrophy, we obtained a majority of arginine-deficient transformants (75%), as expected in this situation more favorable to transplacement (see Fig. 6B). One strain, RP2, was analyzed further by Southern blot analysis. The Southern blots confirmed the occurrence of the correct gene transplacements in both the RP1 and RP2 genomes: probing with the wild-type SacI fragment revealed (Fig. 7) the expected size shifts among the SacI and HindIII restriction fragments. Deletions in ORF1 thus result in a requirement for arginine. The deletions, furthermore, were not complemented in diploids made by crossing strain c1636d(Mata, ura3Δ2, arg11–1) with either RP1 or RP2. We conclude that ORF1 and ARG11 are one and same gene.

Strains RP1 and RP2 both exhibit severe arginine bradytrophe like the in vivo selected mutant alleles arg11–1 to arg11–4. By carefully comparing growth in streaks on minimal medium, we determined that RP2 and especially RP1 are slightly more bradytrophic than the arg11–1 strain. The null mutant does retain some ability to grow in the absence of arginine. Remarkable is the appearance of some big mutant colonies in the poorly growing streaks of even the null mutant, suggesting the existence of either other cryptic iso-functional proteins or of external suppressors, in keeping with the isolation of several complementing genes, distinct from ARG11, in our initial genetic screen. In the arg11–null allele, we detected no phenotype other than arginine bradytrophe, in particular no respiratory defect in cells grown on glycerol as the carbon source (YE medium), even at 37°C, and no thermosensitivity of growth on glucose-rich medium.

Cloning and Sequencing of the arg11–1 Mutant Allele: the 27 Carboxyl-terminal Amino Acids Are Functionally Necessary—The Southern blot data presented in Fig. 7 reveal no size differences between the ARG11-bearing SacI restriction fragments from the genomes of strains FL100 (from which the pFL1-Yeast library and thus our original ARG11 clone are derived), Σ278b (the wild-type strain used in our laboratory), and MG409 (arg11–1 mutant derived from Σ278b) (compare lanes 1 and 2 and lanes 3 and 6). Any modifications must thus
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be fairly minor. Acting on the hypothesis that the arg11-1 allele might be a point mutation in an essential domain of the protein, we amplified by PCR, cloned, and sequenced the arg11-1 allele present in MG409 and its wild-type counterpart present in the isogenic parental strain \( \Sigma 1278b \) (see pPR plasmids description in Table II). Not a single nucleotide was found to differ in the sequence from \( \Sigma 1278b \) and FL100. In the MG409 sequence, a premature TGA stop codon was found instead of the CGA triplet encoding arginine 266. Since the arg11-1 mutation and the major SA deletion cause almost equally severe bradytrophic growth, we conclude that the 27 C-terminal residues of Arg11p are essential to its function.

Arg11p Is Located in the Mitochondria—We next sought to determine the subcellular location of Arg11p. Having no functional assay, we used epitope tagging to monitor the protein. Given the functional importance of the C-terminal domain and because the N-terminal amino acid composition suggests there is no mitochondrial targeting presequence, we chose to fuse the tag to the amino terminus. We used oligonucleotide-mediated site-directed mutagenesis to add the HA epitope coding sequence to ARG11 (see pMC150, Table II).

The HA-Arg11p fusion protein proved functional, since strain RP2 (arg11-ΔA::URA3) could be transformed back to arginine prototrophy by gene transplacement in the presence of the SacI fragment bearing the tagged gene. Transformants were obtained by selecting simultaneously for the arg+ phenotype and for 5-fluoro-orotic acid resistance conferred by loss of the URA3 gene integrated downstream from ARG11 in the engineered strain RP2. Southern blotting revealed that half of the arginine+, uracil+ transformants bore the HA-ARG11 fusion in their genomes, as demonstrated by the characteristic 1.72- and 0.22-kb subfragments obtained by cutting the 1.944-kb HA-ARG11-bearing SacI fragment at the AatII restriction site present in the HA coding sequence (data not shown). Since the tagged protein remained active, we inferred that it was properly localized.

In cell fractionation experiments undertaken to determine the subcellular location of overexpressed HA-Arg11p, we additionally monitored the distribution of two enzymes whose location is known: the cytosolic enzyme ornithine transcarbamoylase (5), product of ARG3, and the mitochondrial enzyme acetylornithine transaminase (bidem), product of ARG8. The HA-ARG11-bearing SacI fragment was subcloned in pFL46L(11), a 2μ-LEU2 plasmid (resulting plasmid: pOS14). We carboxytagged the two control enzymes by PCR-amplifying the ARG3 and ARG8 genes (using primers with suitable restriction sites at their 5' ends) and subcloning them in plasmid pVeP2 (9). In the resulting plasmids (pOS16 and pOS13, respectively), ARG3 or ARG8 is placed under the control of the GAL1 promoter and inserted in frame upstream from the HA coding sequence. Plasmid pOS16 was introduced into strain SS1, an arg3 deletion mutant, and pOS13 was introduced into strain 11S60b, an arg8 mutant. In both cases, the criterion for transformant selection was the presence of the vector's URA3 marker. Both carboxytagged enzymes proved functional, complementing the arginine auxotrophy of galactose-grown SS1 or 11S60b.

We then transformed strain 10R41b with pOS14, pOS16, and pOS13 separately, selecting for uracil or leucine prototrophy. Total protein extracts of the transformants and of the untransformed 10R41b control were analyzed by immunoblotting. The bands corresponding to HA-Arg11p, Arg3-HAp, and Arg8-HAp were unambiguously identified (data not shown), each tagged protein giving rise to a single, intense band of the expected apparent molecular mass (32.5, 39, and 46.6 kDa, respectively). The HA-Arg11p band was reproducibly somewhat fainter than the other two; probably the natural ARG11 promoter is weaker than the GAL1 promoter. In all extracts, including the negative control, we also observed a faint, unidentified band of about 46 kDa.

We proceeded similarly with mitochondrial and cytosolic fractions of galactose-grown 10R41b transformed with pOS14 + pOS16 or pOS14 + pOS13; Fig. 5A shows clearly that both HA-Arg11p and Arg8-HAp are confined strictly to the mitochondria, whereas Arg3-HAp accumulates exclusively in the cytosol. The faint unidentified 46-kDa band is cytosolic.

Mitochondrial Subfractionation: Arg11p Behaves Like an Integral Membrane Protein—To determine the submitochondrial location of HA-Arg11p, we treated mitochondria isolated from strain 10R41b (pOS13 + pOS14) with 100 mM Na2CO3, a treatment known to separate membranes of subcellular organelles and their attached integral membrane proteins from the soluble and peripheral membrane proteins (17). After treatment (Fig. 5B), HA-Arg11p remains totally associated with the pelleted membrane fraction, whereas matrix protein Arg8-HAp is fully released from the organelle into the supernatant. We conclude that Arg11p is a mitochondrial integral membrane protein.

DISCUSSION

We have identified a new mitochondrial integral membrane protein, Arg11p, required for normal arginine biosynthesis. We further show that in addition to sharing, over its entire length, significant primary sequence identity with many IM carrier family members, Arg11p displays all of the distinctive features
of this protein family: (i) a tripartite structure (3-fold tandem repetition of a stretch of some 100 residues); (ii) the presence of two hydrophobic regions in each of the three domains; and (iii) 3-fold repetition of a conserved “family signature” motif. According to the topological model proposed for mitochondrial transporters (21, 23), each of the three domains consists of two transmembrane α-helices linked by a long polar segment (loops A, B, and C between helices I and II, III and IV, and V and VI, respectively); shorter stretches of hydrophilic amino acids (loops a’ and b’) should connect the three tandem repeats. This implies that the N and C termini of the polypeptide chain are on the same side of the membrane, together with segments a’ and b’, while hydrophilic regions A, B, and C should be on the other side. Many recent topological investigations, reviewed by Palmieri (20), confirm this proposed model and concur in locating A, B, and C on the matrix side. From Palmieri’s review and a report by Walker (22), it appears that the conserved motifs at the extremities of the extended IM transporter signature can be considered markers of the termini of helices I, III, and V and of the beginnings of helices II, IV, and VI, respectively: the required specific amino acids and their absolutely conserved spacing probably contribute to a common structural framework. In contrast, the hydrophilic loops, exposed domains varying considerably in length and in sequence, are believed to contribute to substrate and functional specificities. Using the BLASTP program, we sought best alignments of the known IM carriers that should highlight these regions, hoping to gain insight into the function of Arg11p. We found a possibly significant relationship (five or six identities and about seven similarities (24) in 22-residue stretch) between the putative b’ region of Arg11p and the equivalent loops of Neurospora crassa gene arg-13 (reported in the data base as an amino acid transporter) and S. cerevisiae genes YMC1 (25) and YMC2 (SWISS-PROT, accession number P38087) of unknown function (see below).

Another interesting question is how IM carriers are targeted to the inner membrane. Most are synthesized without a matrix-targeting presequence (e.g. several ADP/ATP translocators, uncoupling proteins, oxoglutarate carriers, the yeast phosphate carrier), but others carry cleavable leader sequences (e.g. several mammalian phosphate and citrate carriers) (26–28). No presequence seems present in Arg11p, given the amino acid composition of its N-terminal sequence (four acidic amino acids among the 16 first residues), the predicted length of the full-sized protein (292 residues, corresponding to the expected three-fold tandem repetition of a 100-residue domain), and the fact that the amino-tagged HA-Arg11p accumulates as such in the mitochondria. This is suggestive of a “nonconservative” pathway of import into the mitochondria, obeying rules less well known than those governing the more frequent ATP- and membrane-potential-dependent import via the matrix.

What is the function of Arg11p? Our null allele has no detectable phenotype other than severe arginine bradytropy. From the normal growth observed both at 30 and 37 °C on YPG medium, the mutant appears unaffected in oxidative phosphorylation. Hence, while we cannot formally exclude at this stage the possibility that Arg11p fulfills some “general” function, we deem more probable that it is directly and specifically involved in arginine, or more precisely ornithine biosynthesis, given the amino acid pools measured in MG409 (arg11–1). Our demonstration that Arg11p is a mitochondrial membrane protein and its structural resemblance to the IM-carrier proteins strongly suggest a transporter role. In S. cerevisiae, arginine biosynthesis involves ornithine production in the mitochondria from glutamate, imported into the organelle, and acetyl-CoA, produced essentially within it; ornithine is then exported toward the cytosol, where it is further processed to arginine. The arg11 mutant phenotype might thus most simply be explained by a role of Arg11p in either glutamate import or ornithine export. The former hypothesis can reasonably be excluded; such a defect should have pleiotropic effects, since glutamate is required for protein synthesis in the matrix. Furthermore, MG409 retains its requirement for arginine when proline is the nitrogen source and glutamate is produced directly in the mitochondria where proline is degraded (data not shown). Involvement of Arg11p in ornithine export seems more plausible. It would certainly explain the mutant’s 5-fold-reduced arginine pool; it is not incompatible with the observed 10-fold-reduced ornithine pool, taking into account that in a wild-type strain more than 90% of the ornithine accumulates in the vacuole (29). How the remaining ornithine distributes between mitochondria and cytosol in the wild type is unknown, but it might accumulate exclusively in the mitochondria in arg11 mutants.

R. L. Weiss has called our attention to a N. crassa mutation producing a phenotype similar to the arg11–1 phenotype. The affected gene, arg-13, was characterized by Q. Liu and J. C. Dunlap, and its sequence was deposited in the EMBL data bank under accession number L36378. On the basis of its structure, arg-13 is obviously also an IM carrier family member; it was registered in the databank as an “amino acid transporter,” but no published data are yet available. Importantly, however, in N. crassa ornithine is carbamoylated in the mitochondria, so citrulline, not ornithine, is the intermediate exported to the cytosol. One might alternatively formulate a quite different hypothesis: the mutant phenotype might be due to a defect in one or several enzymatic steps of ornithine synthesis; since all five activities involved are very high in the arg11–1 mutant (2, 3), we must consider the possibility of enzyme mistargeting. Interestingly, one member of the IM carrier family, the yeast phosphate carrier, is proposed to be bifunctional and to work not only as an ion transporter but also as an import receptor on the outer membrane (30, 31). In this light, it is worth considering the hypothesis that Arg11p might play a dual role, transporting some metabolite and importing ornithine-biosynthetic enzymes into the matrix.

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Addendum—The ORF O3299, sequenced recently as part of the European Union yeast genome project (32), encodes Arg11p; alignment of both nucleotide sequences reveals a single difference resulting in a serine in position 105 in our protein (codon TCC) instead of a phenylalanine in the protein encoded by strain S288C (codon TTC). This difference is not in the inner membrane conserved signature.

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