A Mitochondrial Protein from *Neurospora crassa* Detected Both on Ribosomes and in Membrane Fractions

ANALYSIS OF THE GENE, THE MESSAGE, AND THE PROTEIN*

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We have isolated clones representing at least three nuclear genes for mitochondrial ribosomal proteins from *Neurospora crassa* by screening a *agt11* cDNA library with an antiserum against a mixture of these proteins. The cDNA and genomic DNA sequence for one of these genes, *mrp-3*, was determined. The MRP3 protein was purified by immune-affinity chromatography, using a monoclonal antibody probe, and subjected to amino acid sequence analysis to identify the mature amino terminus and a prospective mitochondrial-targeting presequence. MRP3 was identified as the largest, least basic protein detected from the small subunit of ribosomes which had been salt-washed and fractionated on sucrose gradients. However, the mRNA and protein products of *mrp-3* were found to be present in excess over those of other *N. crassa* mito-ribosomal protein genes. Using a solution hybridization/S1 nuclease assay, we found three-fold more mRNA for *mrp-3* than for another mito-ribosomal protein gene. In addition, a 30- to 50-fold excess of non-ribosomal MRP3 protein was discovered. The additional protein was localized in mitochondrial membrane fractions; none was detected in matrix fractions after removal of the ribosomes. An immunologically related protein was detected in ribosome and membrane fractions of mitochondria from *Saccharomyces cerevisiae*. The functional significance of this dual localization remains an enigma.

Mitochondria are attractive subjects for the study of both evolution and gene regulation. As the descendants of an endosymbiotic prokaryote, mitochondria contain their own genome and protein synthesizing system, the latter of which shares a number of characteristics with those of modern bacteria (reviewed by Borst and Grivell, 1971). During evolution, however, most of the genetic information has been transferred from the mitochondrion to the nucleus. As a consequence, mitochondria encode and synthesize only a fraction of the components required for their function. Mitochondrial tRNAs and rRNAs, a few protein subunits of the respiratory enzymes and ATP synthase, and in fungi, one ribosomal protein are products of the organellar system. However, most mitochondrial proteins are encoded in the nucleus, synthesized on cytoplasmic ribosomes, and imported into the organelle for assembly with the mitochondrial products. Thus, several complexes, such as the ribosomes, the F,P,-ATPase, and most of the respiratory chain enzymes are hybrids composed of products from the nucleo-cytoplasmic system plus products from the organellar system (reviewed in Schatz and Mason, 1974; Tzagoloff et al., 1979; Hay et al., 1984; and Hurt and van Loon, 1986). Since the components of these hybrid complexes are functionally related, they provide useful models for the study of coordinate gene regulation, mitochondrial import, and complex assembly.

We have chosen to study the nuclear genes for proteins of the mitochondrial ribosome from *Neurospora crassa* as a model system. These ribosomes are composed of two rRNA molecules and over 55 proteins. Both RNAs and one protein of the small subunit (S5) are products of the organelle, whereas the remaining proteins are products of the nucleo-cytoplasmic system (Lambowitz et al., 1976). Thus, two separate systems must interact to produce mito-ribosomal components as needed and in the proper proportions. In addition, all but one of the mito-ribosomal proteins must be transported across both mitochondrial membranes to assemble with the three mitochondrial products. Presently, little is known about the structure of the genes encoding the imported ribosomal proteins or about the signals that control their expression or that specify the import of their protein products. A comparative structural analysis of these genes will provide insights into their evolution and regulation, the structure of the encoded proteins, and the nature of protein import signals.

We report the isolation of cDNA clones representing at least three nuclear genes for mitochondrial ribosomal proteins from *N. crassa*. The nucleotide sequence for one of these genes, *mrp-3*, is presented from both cDNA and genomic DNA clones. It encodes a protein of 458 amino acids, which shows some similarities to *Escherichia coli* ribosomal protein S1. We note an abundance of cDNA clones and a more highly biased pattern of codon usage for *mrp-3* when compared with other mito-ribosomal protein genes, suggesting that *mrp-3* is expressed at an elevated level. Indeed, we detect 3-fold more mRNA for *mrp-3* than for another mito-ribosomal protein gene. Using a monoclonal antibody probe, we demonstrate that the MRP3 protein is strongly associated with the small ribosomal subunit. However, to our surprise, we find a 30- to 50-fold excess of MRP3, which appears to be associated with one or both mitochondrial membranes. In addition, we detect a presumably analogous protein in ribosome and membrane...
fractions of mitochondria from Saccharomyces cerevisiae by immune cross-reaction.

MATERIALS AND METHODS

RESULTS

Isolation and Classification of cDNA Clones for N. crassa Mitochondrial Ribosomal Protein Genes—A polyclonal antiserum was raised against a mixture of N. crassa mito-ribosomal proteins for use as a probe to screen an N. crassa genomic library in λgt11. As a preliminary test, polyclayamide gel patterns of mitochondrial and cytoplasmic ribosomal proteins were probed with the antisem. Several proteins from both large and small subunits of mito-ribosomes reacted; however, no cross-reactio

The polyclonal antisem was treated to remove contaminating reactivity against E. coli proteins, and used to screen a library of N. crassa cDNA in λgt11. Four clones were isolated from 7 × 10⁵ to 7 × 10⁶ plaques of an amplified library (RPm1–RPm4), and an additional nine clones were isolated from approximately 10⁶ plaques of the original, unamplified library (RPM5–RPm16; RPM9, -10, and -14 failed to reproduce an antigenic response upon enrichment).

To classify the 13 clones and to determine the number of genes represented, purified phage DNAs were labeled by nick translation and hybridized with HindIII patterns of N. crassa genomic DNA (data not shown). The clones were classified into four groups, presumably representing four distinct genes, based upon their hybridization patterns: RPM1–6, -8, -11, -12; RPM7, -15; RPM16; and RPM13.

To facilitate restriction map and nucleotide sequence determinations, the N. crassa cDNA inserts were removed from the phage vectors and recloned in pUC8 to generate pRP1–16. Preliminary nucleotide sequence data and identification of the encoded proteins have confirmed that the first three groups represent genes for three distinct mito-ribosomal proteins. Analysis of the pRP3 family of cDNA clones, its genomic counterpart, and gene products are presented below. Findings on the pRP15 family will be reported elsewhere. Studies with pRP16 and 13 are in progress.

Isolation of a Genomic Clone for mrp-3—Preliminary nucleotide sequence data indicated that none of the members of the pRP3 family of clones contained a full-length cDNA for the mitochondrial ribosomal protein gene, mrp-3. To complete the 5'-coding and upstream sequences, a clone of genomic DNA was isolated. The insert from the longest cDNA clone, pRP3, was 5'-end-labeled and used as a hybridization probe to screen an N. crassa genomic library in λJ1 (Orbach et al., 1986). A single clone was isolated, and hybridizing fragments were subcloned for sequence analysis.

Nucleotide and Amino Acid Sequence Analyses—The nucleotide sequence of mrp-3 was determined from four overlapping clones, three of cDNA and one of genomic DNA (Fig. 1). Both coding and noncoding strands were analyzed, except for nucleotides 1331–1442 and 1615–1623, which were determined (repeatedly) solely from one strand. This sequence, shown in Fig. 2, includes 300 nucleotides of the 5'-untranslated region; 1374 nucleotides of coding sequence, interrupted by an intron of 305 nucleotides; and 594 nucleotides of the 3'-flanking region.

Several lines of evidence indicate that the reading frame and translational start site shown for mrp-3 are correct. The reading frame presented is in frame with the coding sequence for β-galactosidase in the λgt11 clone RPM3, and thus, would be expressed as the carboxyl terminus of the fusion protein that is recognized by the antisem. In addition, the pattern of codon usage for mrp-3 in this reading frame matches that reported for other N. crassa protein genes more closely than do the two other reading frames (discussed below). Preliminary S1 nuclease mapping indicated that the transcriptional start site lies in a region centered at nucleotide -36 (data not shown). The ATG at position +1 would be the first from the start of transcription, and there are termination codons in all three reading frames between positions -35 and +1. In addition, as discussed below, the mature MRP3 protein begins with Ala-29. The ATG at position +1 is the only Met codon between the amino terminus of the mature protein and the upstream termination codons.

The nucleotide composition immediately 5' to the starting methionine is quite different from the coding region; the 5'-untranslated region is very A+T-rich for about 90 nucleotides before the start of translation. The start of transcription is centered at position -69. In contrast, the entire coding region is quite rich in guanine. A similar pattern has been observed for other N. crassa protein genes (Schechtman and Yanofsky, 1983; Legerton and Yanofsky, 1985). The sequence CTCACC, found immediately before the proposed translational start site, is in good agreement with the consensus for N. crassa (ATACA/C; Legerton and Yanofsky, 1985).

The encoded protein, MRP3, is composed of 458 amino acids and has a calculated molecular weight of 48,564. It contains 54 basic and 56 acidic residues for an overall charge of -2.

Since most matrix proteins which have been studied are directed into the mitochondrion by a cleavable amino-terminal extension, we were interested in determining what portion of the protein encoded by mrp-3 is present inside the organelle, and thereby, to identify its prospective matrix-targeting presequence. To this end, the MRP3 protein was purified from detergent-solubilized mitochondria by immune-affinity chromatography and subjected to amino acid sequence analysis. The first 12 amino acids of the mature protein are Ala-Ser-Tyr-Pro-Pro-X-Thr-Val-Val-Lys-Met-Pro, indicating that MRP3 is synthesized as a precursor protein with a 28 amino acid extension (Fig. 2). Thus, the mature protein is composed of 430 residues, its calculated molecular weight is 45,406, and it has an overall charge of -6. Presumably, the amino-terminal extension serves as a mitochondrial targeting sequence. Indeed, it contains 4 Arg, 3 Ser, 1 Thr, 13 hydrophobic, and no acidic residues, a composition similar to known targeting signals (von Heijne, 1986). In addition, these residues are spaced such that they could form an amphipathic helix, as demonstrated for several known import sequences (Roise et al., 1986, 1988).

A computer search of the Protein Identification Resource data base using FASTP (Sidman et al., 1988) was conducted to identify proteins having an amino acid sequence similar to MRP3. No extensive homologies were discovered; however, the search did identify regions of MRP3 that are similar to known proteins. The amino-terminal portion of MRP3 is 20% identical to E. coli ribosomal protein S1 over a region of 96 amino acids (Fig. 3). No similarity was found between MRP3 and the carboxyl-terminal domain of S1. Other sequence comparisons received higher "homology scores" than did the MRP3/S1 alignment; however, their significance is less ob

1 The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 W. M. Weaver and J. E. Heckman, manuscript in preparation.

3 P. Andrews, personal communication.
For example, an interesting stretch of MRP3, from amino acids 148 to 160, has a proline at every second position (Ala-Pro-Thr-Pro-Ala-Pro-Thr-Pro-Ala-Pro-Glu-Pro). Similar motifs are found at the amino termini of L1 myosin light chains from chicken and rat (Matsuda et al., 1981), and near the middle of ompA outer membrane proteins from several bacteria (Chen et al., 1999; Braun and Cole, 1982; Freudl and Cole, 1983). However, the extent of these structures is unknown.

The pattern of codon usage for mrp-3 shows a pronounced bias, similar to that seen for highly expressed genes in N. crassa. In general, codons ending in a pyrimidine, especially cytosine, are used preferentially, whereas codons ending in purines, especially adenine, are avoided (Kinnaird and Fincham, 1983). Presumably, these preferred codons are read most efficiently by the cytoplasmic population of tRNAs. In Fig. 4, the codon usage for mrp-3 is compared with another of the mito-ribosomal protein genes from N. crassa, mrp-15. Mrp-3 adheres more strictly to the rules for codon preference in N. crassa than does mrp-15. For example, only 3 of the 33 Leu codons in mrp-3 end in a purine, whereas 15 of the 33 Leu codons in mrp-15 end in G. The degree of codon bias seen for mrp-3 is similar to that for genes which are expressed at fairly high levels, such as those for β-tubulin (Orbach et al., 1986), glutamate dehydrogenase (Kinnaird and Fincham, 1983), and a cytoplasmic ribosomal protein (Kreader and Heckman, 1987). On the other hand, the less stringent bias found for mrp-15 is similar to that for genes expressed at more moderate levels, such as those for amino acid biosynthetic enzymes his-3 (Legerton and Yanofsky, 1985) and trp-1 (Schechtman and Yanofsky, 1983). Other nuclear genes encoding mito-ribosomal proteins, cyt-21+ from N. crassa (Kuiper, 1987) and MRPl and 2 from Saccharomyces cerevisiae (Meyers et al., 1987), are similar to mrp-15 and have a codon usage bias resembling that for moderately expressed genes in their respective organisms.

Relative Abundance of mRNA for mrp-3—Although the antisera used to screen the lact1/cDNA library recognized 10 to 13 different mito-ribosomal proteins (data not shown), most of the cDNA clones isolated (9 out of 13) represent mrp-3. Several of the mrp-3 clones produced stable fusion proteins whose immune response was much more intense than those from any other group, making these mrp-3 clones easier to detect and isolate. In addition, however, we suspected that mrp-3 might have a more abundant message than the other mito-ribosomal protein genes. To investigate this possibility, the relative levels of message for mrp-3, mrp-15, and crp-1 (a cyto-ribosomal protein gene) were compared using a solution hybridization/S1 nuclease assay (Quarless and Heinrich, 1986). Uniformly labeled cRNA probes were hybridized with total N. crassa RNA, carrier tRNA, or a combination of the two. The hybridization mixtures were treated with the single strand-specific nuclease, S1, to eliminate unhybridized probe, and the products were analyzed by electrophoresis on non-denaturing gels. Product bands were localized by autoradiography and excised from gels, and the amount of radioactivity was measured in a scintillation counter.

In a preliminary experiment, the amount of hybrid produced was analyzed using a fixed amount of crp-1 probe and increasing amounts of total N. crassa RNA. It was found that the amount of product formed was strictly proportional to the amount of N. crassa RNA used, indicating that more than enough probe was available for all mRNA present and that sufficient nuclease was used to degrade essentially all unhybridized probe (data not shown).

In Fig. 5, the relative amounts of mRNA for crp-1, mrp-3, and mrp-15 are compared. A illustrates the positions where probes (solid arrows) hybridize to their respective messages. These probes are roughly the same length (400 nucleotides) and contain nearly the same number of C residues (100). Identical conditions were used to prepare these probes so that their specific activities would be similar. Equal numbers of counts for each probe (the same amount as used in the preliminary experiment) were hybridized with 100 µg of either carrier tRNA (−mRNA lanes) or total N. crassa RNA (+mRNA lanes). As seen in the autoradiogram (Fig. 5B), the amount of mRNA for mrp-3 is intermediate between that for crp-1 and mrp-15. Quantitation of the radiolabeled hybrids indicated that the relative levels of mRNA are 10:3:1.

The experiment was repeated using freshly prepared probes and a different preparation of N. crassa RNA (data not shown). Again, the ratio of mrp-3:mrp-15 mRNA was 3:1.
FIG. 2. Nucleotide sequence of mrp-3 and deduced amino acid sequence of the encoded protein.

Probable region for the start of transcription is underlined. The amino terminus of the mature protein begins after the slash.

However, there was far less mRNA for the mito-ribosomal proteins relative to the mRNA for crp-1 (crp-l:mrp-3 mRNA was 13:1 as opposed to 3:1). These differences in relative mRNA abundance may relate to growth phase of the culture, a possibility we plan to investigate. Nonetheless, mRNA for mrp-3 was consistently more abundant than that for at least one other mito-ribosomal protein gene.

We suspect that the presence of multiple message-dependent bands of similar intensity, as seen for mrp-3 and -15 are a consequence of the fact that these probes hybridize fully (mrp-3) or partially (-15) to 3'-untranslated regions of the message. The population of mRNAs from several N. crassa genes vary in the length of their 3'-untranslated region (Legrerton and Yanofsky, 1983; Kuiper, 1987; and Footnote 4), and multiple S1 nuclease products result when hybridization probes that span these alternate 3' ends are used. Unfortunately, probes complementary to coding regions of mrp-3 and -15 (dashed arrows in Fig. 5A) produced unsatisfactory results. These probes were quite resistant to S1 nuclease in the absence of hybridizing mRNA, presumably due to the formation of stable secondary structures. As a result, it was impossible to detect message-dependent bands among the background of message-independent bands (data not shown).

Localization of the MRP3 Protein on the Small Mitro-


Fig. 3. Comparison of the amino acid sequences for MRP3 and E. coli ribosomal protein S1. The deduced amino acid sequence of MRP3 is aligned with that for the amino-terminal domain of S1 (Kimura et al., 1982; Schnier et al., 1982; Subramanian, 1983). Identical residues are indicated with double dots; conservative substitutions are indicated with single dots. The amino terminus of mature MRP3 begins after the sequence of S1 (Kimura et al., 1982; Schnier et al., 1982; Subramanian, 1983).

Fig. 4. Comparison of codon usage for mrp-3 and mrp-15.

Fig. 5. Comparison of mRNA levels for crp-1, mrp-3, and mrp-15. A, maps of mRNAs and positions of complementary RNA probes. Heavy bars represent coding regions; thin bars represent flanking regions. Vertical lines indicate the known ends of transcripts. Arrows below the transcripts mark regions complementary to hybridizing probes. Solid arrows are probes used in B; dashed arrows are probes used in a separate experiment (described in the text). Numbers below each indicate the lengths of probes in nucleotides and the number of C residues (in parentheses). B, autoradiogram of solution hybridization/S1 nuclease products fractionated on a 5% polyacrylamide, nondenaturing gel. Probes indicated by solid lines in A were hybridized with 100 μg of tRNA (−) or total N. crassa RNA (+).

Mito-ribosomal protein—As shown above, mrp-3 has a more abundant message than expected for a mito-ribosomal protein gene. In addition, the degree of codon bias suggested that mrp-3 mRNA could be translated efficiently. Therefore, it was imperative to identify the encoded protein and ensure that it is a mito-ribosomal protein.

To study the protein product of the mrp-3 gene, monoclonal antibodies were generated against the β-galactosidase/MRP3 fusion protein encoded in the λgt11 clone, RPM12. RPM12, which encodes amino acids 86-297 (Fig. 3), was the most antigenically responsive of the 13 clones isolated using the mixed polyclonal antiserum. As expected, its fusion protein induced a strong immune response in a mouse, and scores of monoclonal antibody-producing hybridoma cell lines all reacted with a single band of protein from different cell lines. Two of these antibodies were used in subsequent investigations.

In preliminary experiments, MRP3 was detected in post-ribosomal supernatants from detergent-lysed mitochondria. During the usual preparation, mitochondria were isolated in 5 The abbreviations used are: SDS, sodium dodecyl sulfate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride.
buffers containing EDTA to dissociate cytoplasmic ribosomes and then purified on 44/55% sucrose step gradients. Purified mitochondria were lysed with Nonidet P-40, and mito-ribosomes were pelleted through 60% sucrose in a high salt solution (0.5 M KCl) to remove loosely associated proteins (Lambowitz, 1979). It seemed possible that incompletely solubilized membrane or some other particle could co-purify with these mito-ribosomes. To determine whether MRP3 is a true mito-ribosomal protein or merely a highly antigenic contaminant in the ribosome preparations, mito-ribosomes were purified further on sucrose gradients using conditions which did or did not result in subunit separation. Gradient profiles are shown in Fig. 6. In A, ribosomes remained intact. In B, the salt concentration was higher, and ribosomes were pretreated with puromycin to dissociate subunits. Equal portions of even-numbered fractions from both gradients were run on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with one of the MRP3-specific monoclonal antibodies. Peak immune response coincided with the absorbance peak for intact ribosomes (Fig. 6A, fractions 40 and 42) or for small ribosomal subunits (Fig. 6B, fraction 32). Therefore, MRP3 appears to be strongly associated with the small subunit of N. crassa mito-ribosomes, rather than a contaminant in the preparation.

To determine which small subunit protein is encoded by mrp-3, a two-dimensional protein pattern was probed with this same monoclonal antibody (Fig. 7). The first dimension separation was based mostly upon charge, whereas the second dimension separation was based upon molecular weight. A comparison of the immune blot (Fig. 7B) with the Coomassie-stained gel pattern (Fig. 7C) demonstrates that MRP3 is the largest, least basic small subunit protein detected on this gel system. It was noted that the intensity of the Coomassie-stained protein is similar to the intensities of other proteins, indicating that there is about the same amount of MRP3 as the other MRPs on the small subunit. To obtain an estimate of molecular weight, ribosomal proteins were run next to molecular weight markers in one dimension using the same gel conditions as the second dimensions in B and C (Fig. 7A). The apparent molecular mass of MRP3 is 65 kDa on this gel system, whereas it migrates at around 50–55 kDa on the Laemmli (1970) gel system (see below). A second, much weaker response to the monoclonal antibody is seen for a smaller protein (27 kDa; Fig. 7B). Although the latter may be a second mito-ribosomal protein having an antigenic determinant in common with MRP3, we suspect that it is a degradation product of MRP3. It is frequently detected in mito-membrane fractions with MRP3, whereas the other MRPs are not detected in these fractions (see below).

Localization of the MRP3 Protein with Mitochondrial Membranes—In preliminary experiments, MRP3 was detected in the post-ribosomal fraction of detergent-lysed mitochondria as well as in the ribosomal fraction. To determine what proportion of MRP3 is associated with ribosomes versus the total mitochondrial content, half of a mitochondrial preparation was treated with detergent, and the ribosomes were collected as described above, whereas total mitochondrial protein was extracted from the other half. As a control, equal proportions of the two preparations were fractionated and probed with the mixed anti-MRP serum (Fig. 8B). Much of the antibody reactivity against MRP3 was removed before performing the blot (as described in the figure legend) because the immune response from MRP3 and its degradation products using such a large amount of total mitochondrial protein was so intense that it masked the signals from the other mito-ribosomal proteins. As shown in Fig. 8B, there are equal amounts of these other proteins in ribosomal and total mito-protein lanes (for example, a 37- and a 28-kDa protein), indicating that the ribosomes were recovered very efficiently from these mitochondria. A similar pattern, containing one-tenth the amount of protein, was probed with an MRP3-specific monoclonal antibody. As shown in Fig. 8A, there is 30- to 50-fold more MRP3 in mitochondria than the amount detected on ribosomes.

The post-ribosomal supernatant of detergent-lysed mito-
Fig. 7. Identification of the MRP3 protein. A, immune blot of a one-dimensional gel pattern. Aliquots of peak fractions for small subunit (S), large subunit (L), and total ribosomes (T) from gradients illustrated in Fig. 6 were fractionated on a 7.5–15% linear polyacrylamide gradient-SDS gel (Chua and Bennoun, 1976), transferred to nitrocellulose, and probed with an MRP3-specific monoclonal antibody. Molecular masses are in kilodaltons. B, immune blot of a two-dimensional gel pattern. Small subunit fractions from gradient B in Fig. 6 and three similar gradients were pooled and their proteins extracted. One-fourth of this preparation was fractionated in two dimensions as described by Lambowitz (1979). First dimension gels contain 4% polyacrylamide and 8 M urea at pH 5.0 (Mets and Bogarad, 1974). Second dimension gel conditions and immune blots were as in A, C, Coomassie-stained two-dimensional gel pattern. Gel as in B, but containing twice as much protein. Arrows indicate immunoreactive proteins.

N. crassa Mitochondrial Ribosomal/Membrane Protein

Cytoplasm, cytoplasmic ribosomes, and the mitochondrial fractions were tested for the presence of citrate synthase, a matrix marker. SDS-polyacrylamide gel patterns were probed with an antisera against citrate synthase of S. cerevisiae (Fig. 9A, lanes 1 and 2). Citrate synthase was detected only in the matrix fraction, indicating that these mitochondria had been thoroughly broken to release the soluble matrix. Therefore, the membrane fraction does not contain significant amounts of intact mitochondria or mitoplasts with trapped matrix.

Since all but one of the products from protein synthesis on the mito-ribosomes are membrane proteins, it may be that these ribosomes are anchored to the membrane for efficient coupling of translation and membrane insertion. To determine whether MRP3 associates with the mitochondrial membranes alone or as a component of intact ribosomes, a pattern similar to that in Fig. 9B was probed with antibodies against a second mito-ribosomal protein, MRP15.5 MRP15 was detected only in the mito-ribosome fraction (Fig. 9B, lane 4). No trace was found in the membrane fraction, even when 2- and 4-fold more membrane proteins were analyzed (lanes 6 and 7). Preliminary experiments indicated that the other mito-ribosomal proteins recognized by the mixed anti-MRP serum are absent from membrane fractions as well (data not shown). Therefore, although the mito-ribosomes may or may not associate with the inner membrane in vivo, the entire ribosome was not isolated in association with the membranes.

Identification of an MRP3 Analogue from S. cerevisiae—It was discovered that a polyclonal serum raised against a mixture of small ribosomal subunit proteins from mitochondria of S. cerevisiae cross-reacted with a single mito-ribosomal protein from N. crassa.6 Additional experiments identified this protein as MRP3 and demonstrated that neither the polyclonal antisera against N. crassa mito-ribosomal proteins nor the MRP3-specific monoclonal antibody cross-react with any mito-ribosomal proteins of S. cerevisiae (data not shown).

To identify the yeast protein responsible for this immune reaction, the N. crassa protein was used as an affinity probe to isolate MRP3-specific antibodies from the mixture of anti-yeast small subunit antibodies (see "Materials and Methods"). The selected antibodies were used to probe patterns of yeast proteins. Yeast mitochondria were fractionated as described above for N. crassa mitochondria. Most citrate synthase was detected in the matrix fraction (Fig. 9A, lane 3 versus 4) and 97% of the cytochrome oxidase activity was present in the membrane fraction (data not shown). Therefore, matrix and membrane fractions were separated fairly cleanly. The yeast homologue of MRP3 was detected in both mito-ribosome and membrane fractions (Fig. 9D, lanes 4 and 5), as for the N. crassa protein. Interestingly, the yeast protein appears to be somewhat larger than the N. crassa protein (lanes 5 versus 6; 60 kDa versus 50 kDa).

DISCUSSION

We have begun a study of the nuclear genes for mitochondrial ribosomal proteins from N. crassa, with the ultimate goal of investigating the coordinate control of their expression.

6 B. D. Fornier and W. M. Weaver, unpublished results.
and the import of their protein products into mitochondria. The first gene to be analyzed, mrp-3, has presented unexpected, although intriguing results.

The highly biased pattern of codon usage found for mrp-3 indicated the potential for greater expression than expected for a mito-ribosomal protein gene. Indeed, 3-fold more mRNA was detected (Fig. 5) for mrp-3 than for mrp-15, a second gene under investigation.² In addition, mrp-3 has a more biased pattern of codon usage than other mito-ribosomal protein genes which have been analyzed (Meyers et al., 1987; Kuiper, 1987; and Footnote 2), suggesting that its mRNA may be translated more efficiently. Indeed, although the amount of MRP3 protein found associated with ribosomes appears to be similar to that of other MRPs (Fig. 7), there is a 30- to 50-fold excess of non-ribosomal MRP3 (Fig. 8).

An analysis of mito-ribosomes purified on sucrose gradients demonstrated that MRP3 co-fractionates specifically with the small subunit under conditions where the subunits dissociate, or lower on the gradient, with intact ribosomes under conditions where the subunits do not dissociate (Fig. 6). Therefore, MRP3 appears to be a true ribosomal protein and not merely a contaminant in the ribosome preparations.

The bulk of MRP3, however, appears to be associated with one or both of the mitochondrial membranes (Fig. 9B). Its amino acid sequence reveals no extensive hydrophobic stretches, and thus, it may associate with the membrane via protein-protein interactions. Although it is possible that MRP3 associates with membranes under the isolation conditions used and is normally soluble in the matrix under physiological conditions, not a trace was detected in the matrix fraction after removal of the ribosomes. In contrast, citrate synthase, a soluble matrix enzyme, was found exclusively in

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**Fig. 8.** Proportion of MRP3 on ribosomes versus total mitochondrial content. Protein preparation is described in the text. Ribosomal (R) or total mitochondrial (T) proteins from equal proportions of these preparations were fractionated on a 10% polyacrylamide-SDS gel (Laemmli, 1970). B contains 10-fold more protein than A. Protein patterns were transferred to nitrocellulose and probed with an MRP3-specific monoclonal antibody (A) or with the anti-N. crassa MRP serum (B). In B, the primary antiserum was preincubated with the RPM12 fusion protein immobilized on nitrocellulose (Johnson et al., 1985) to remove most of the antibodies against MRP3. Molecular masses are in kilodaltons.

**Fig. 9.** Submitochondrial localization of MRP3. Cells and mitochondria were fractionated as described under "Materials and Methods." Except where noted, 25-µg aliquots of protein from these fractions were separated on 15% polyacrylamide-SDS gels (Laemmli, 1970), transferred to nitrocellulose, and probed with antibodies. Molecular masses are in kilodaltons. A, immune blot using anti-yeast citrate synthase. Lanes 1 and 2, N. crassa mito-matrix and membranes; lanes 3 and 4, S. cerevisiae mito-matrix and membranes. B, immune blot of N. crassa fractions with an MRP3-specific monoclonal antibody. Lane 1, cytoplasm; lane 2, cytoplasmic ribosomes; lane 3, mito-matrix; lane 4, mito-ribosomes; lane 5, mito-membranes. C, immune blot of N. crassa fractions with anti-MRP15. Lanes 1–5, as in B; lane 6, 50 µg of mito-membrane; lane 7, 100 µg of mito-membrane. D, immune blot of S. cerevisiae fractions with anti-MRP3, affinity-purified from antiserum against S. cerevisiae mito-ribosomal small subunit proteins. Lanes 1–5, as in B, except that here they are fractions of yeast cells; lane 6, N. crassa mito-membranes.
this same matrix fraction (Fig. 9A). The other mito-ribosomal proteins recognized by a mixed polyclonal antiserum were conspicuously absent from this mito-membrane fraction. Therefore, MRP3 seems to associate with mito-membranes alone and not as a component of intact ribosomes.

This dual localization suggests that MRP3 has more than one function in the mitochondrion. Multi-functional proteins are not unknown in N. crassa. For example, his-3 (Legerton and Yanofsky, 1985) and trp-1 (Schechtman and Yanofsky, 1983) encode enzymes which function in two and three steps along these amino acid biosynthetic pathways. In addition, a more bizarre combination has been reported for a nuclear-encoded mito-protein, that of the mitochondrial tyrosyl-tRNA synthetase which has a second role in mRNA splicing (Akins and Lambowitz, 1987). The latter may have interesting implications concerning the evolution of mitochondria in particular and of mRNA splicing in general.

A low level amino acid sequence homology was found between the amino termini of MRP3 and E. coli ribosomal protein S1. In addition, MRP3 and E. coli S1 demonstrate very similar electrophoretic mobilities on the Mets and Bogorad (1974) two-dimensional gel system (Fig. 7 in the present study and Fig. 1 in Subramanian, 1974) such that the least, basic protein detected from the small subunit of its ribosome. Since mitochondrial and E. coli ribosomes presumptively evolved from a common ancestor, one might expect to find structural similarities. Indeed, the mito-ribosomal proteins encoded by mrp-15° and cyt-21° (Kuiper, 1987), from N. crassa, and MRP2 of S. cerevisiae (Meyers et al., 1987) all have regions of amino acid sequence that are somewhat similar to regions of E. coli ribosomal proteins. The amino-terminal domain of S1 is involved in ribosome association via protein-protein interactions (Bonì et al., 1982). The similarity between S1 and MRP3 in this region suggests an analogous role for the amino-terminal portion of MRP3. However, S1 is known to dissociate from the E.coli ribosome in the presence of 1 m NH4Cl, a condition we have not yet tested for MRP3. The carboxy-terminal portions of these two proteins bear no obvious resemblance, and furthermore, no one has reported detecting a huge excess of S1 associated with the plasma membrane of E. coli. Therefore, we suspect that MRP3 has a non-ribosomal function in addition to whatever role it plays on the ribosome.

It was of great interest to identify a protein from S. cerevisiae immunologically related to MRP3 and similarly localized in mito-ribosome and membrane fractions (Fig. 9D). This discovery indicates that the existence of the MRP3 protein and its function(s) are not confined to N. crassa. In addition, yeast are more amenable to genetic manipulation via gene replacement than N. crassa. We hope to utilize the yeast system to investigate the function of MRP3.

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N. crassa Mitochondrial Ribosomal/Membrane Protein


Supplementary Material to
A mitochondrial protein from Neurospora crassa detected both on ribosomes and in membrane fractions: analysis of the gene, the message, and the protein
Carol A. Kreider, Cornelia S. Laenger, and Joyce F. Harvey

Production of antibodies against cytochrome oxidase as a nuclear gene product in N. crassa, by recombinant DNA techniques (G. Robert, personal communication). This work is protected by copyright and is subject to a license agreement.

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N. crassa Mitochondrial Ribosomal/Membrane Protein

Preparation and analysis of proteins. The fungus N. crassa was cultivated as described above for RNA preparation. Subcellular fractionation strain 2923-106 was cultivated, and spheroplasts were prepared and washed as described by Dau et al. (1982). Spheroplasts (both wild and JAW) were broken and their mitochondria were isolated and purified on step gradients, essentially as described by Lamberts (1979), except that PSF was mixed into 1 ml during the initial homogenization of cells. For most purposes, ribosomes were prepared from detergent (Nonidet P-40) lysed mitochondria according to Lamberts (1979). These ribosomes were purified further on 15-30% sucrose gradients, using conditions for submitochondrial particles described by Lamberts (1979) or Van den Berg and de Vries (1979). Ribosomal subunits were collected and their proteins extracted as described by Lamberts (1979).

Mitochondria used for subfractionation and WPS localization were recovered from sucrose step gradients, divided into 0.2 volumes of 10 mM Tris-HCl, pH 7.5, and pelleted at 15,000 g for 30 min. These mitochondria were resuspended in 10 mM Tris-HCl, pH 7.5, and incubated on ice for 1 h with agitation. 20 min. Supernatant volume of moving buffer (Dau et al., 1982) was added to the washed mitochondria. After 1 h in 1 ml mitochondria (pre-incubation) were broken by two 30 s bursts of ultratrans (80,000 rpm, with a 30 s pause between treatments. Particles fractions were sequentially recovered from the resulting suspension: mitochondrial membranes (inner membrane) were pelleted at 150,000 rpm for 2 h in a Beckman Ti50 rotor at 4°C, then ribosomes were recovered from the post-mitochondrial supernatant by pelleting through 40% sucrose in high salt buffer (Lamberts, 1979). Cytosolic ribosomes were recovered from post-mitochondrial supernatant by pelleting as for mitochondrial ribosomes. Proteins were recovered from both post-mitochondrial supernatant solutions (Nonidet P-40 and Nonidet) by adding 0.2 volumes of laemur solution and precipitating overnight at 4°C. Precipitated proteins were pelleted at 15,000 g for 30 min at 4°C, dried under vacuum, and resuspended in 0.1 M NaOH by heating at 25°C for 1 h. Ribosomal pellets were resuspended in 10 mM Tris-HCl, pH 7.5. Mitochondrial membrane pellets were isolated in 10 mM Tris-HCl, 0.40 g Trichloroacetic acid (TCA), 200 mM sucrose, 1 mM EDTA (in Brazil, 1977).

Cytochrome oxidase activity was determined using conditions described by Mason et al. (1972). A 1% solution of cytochrome c (Sigma, horse heart type II) in assay buffer was reduced by adding 0.2 pH adjusted to 6.5 medium containing 1 mg/ml of cytochrome c, and the absorbance at 550 nm had increased 2 to 2.5 fold. Aliquots of isolated membrane were diluted 10 or 100-fold with 0.5 triton X-100, 2% glycerol, 130 mM Tris-HCl, pH 7.5. Triton X-100 and glycerol were added to portions of post-mitochondrial supernatants (before removal of ribosomes), to 20 and 20% of the final volumes. Particles were pelleted at 12,000 g for 10 min (Brinck, 1977). Aliquots of the resulting superantibodies were tested for citochrome oxidase activity in 0.1 M KPi, pH 6.0, 0.01 M sucrose, 10 µM reduced cytochrome c (Mason et al., 1973) by monitoring the rate of absorbance decrease at 550 nm.

Protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Standards and blanks contained the same buffer but were not treated for interference from chromic substances. 0.18% SDS was included in all assay mixtures.

Proteins were fractionated in one dimension by polyacrylamide gel electrophoresis according to Laemmli (1970) or Oua and Baren (1978), and in two dimensions according to Weber and Dugard (1974) with modifications described by Lamberts (1979) for the second dimension. Proteins were visualized by staining with Commassie Brilliant Blue (CBB, gel fixed in 20% methanol, 7.5% acetic acid, stained in 0.25% CBB, destained in 7.5% acetic acid, 0.25% CBB, destained in fixing solution), or by gel electrophoresis at 4°C (Schleicher and Schuell, 1946, 0.4 g) according to Towbin et al. (1976) and stained with amido black (0.1% in fixing solution, 0.1% methanol, 0.1% acetic acid, Sigma). Immune assays were as described above for screening the U20K bank, except that BSA and horse serum were replaced with 1% casein dry milk for all washes (Johnson et al., 1985), and 30% antisera (not treated to remove cross-reactivity to E. coli protein) were used.

WPS was purified from the post-mitochondrial supernatant of detergent lysed mitochondria by immune-affinity chromatography, essentially as described by Sibley and Brooks (1983). The protein was concentrated by sodium phosphate precipitation, washed from residual contaminants by SDS-polyacrylamide gel electrophoresis, and transferred to Semethyl PPIV membranes onto polyvinylidene difluoride (Millipore) as described by Matsudaira (1980). The amino acid sequence for the anchor-domain of WPS was determined by F. Anderson ( Purdue University, W. Lafayette, IN) using an automated protein sequencer. (Applied Biosystems).