Mitochondria have specialized ribosomes that have diverged from their bacterial and cytoplasmic counterparts. We have solved the structure of the yeast mitosomal large subunit using single-particle cryo–electron microscopy. The resolution of 3.2 angstroms enabled a nearly complete atomic model to be built de novo and refined, including 39 proteins, 13 of which are unique to mitochondria, as well as expansion segments of mitoribosomal RNA. The structure reveals a new exit tunnel path and architecture, unique elements of the E site, and a putative membrane docking site.

Mitochondria are organelles in eukaryotic cells that play a major role in metabolism, especially the synthesis of adenosine triphosphate (ATP). During evolution, mitochondria have lost or transferred most of their genes to the nucleus, substantially reducing the size of their genome (1). In yeast, all but one of the few remaining protein-coding genes encode subunits of respiratory chain complexes, whose synthesis involves insertion into the inner mitochondrial membrane along with incorporation of prosthetic groups (2). For the translation of these genes, mitochondria maintain their own ribosomes (mitoribosomes) and translation system. The mitoribosomal RNA (rRNA) and several transfer RNAs (tRNAs) are encoded by the mitochondrial genome, whereas all but one of its ribosomal proteins are nuclear-encoded and imported from the cytoplasm. Mitoribosomes have diverged greatly from their counterparts in the cytosol of bacterial and eukaryotic cells and also exhibit high variability depending on species (table S1) (3). Several genetic diseases map to mitoribosomes (4). In addition, the toxicity of many ribosomal antibiotics, in particular aminoglycosides, is thought to be due to their interaction with the mitoribosome (5). The 74S yeast mitoribosome has an overall molecular weight of 3 MD, some 30% greater than that of its bacterial counterpart. It consists of a 54S large subunit (1.9 MD) and a 37S small subunit (1.1 MD).

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Supplementary Materials
www.sciencemag.org/content/343/6178/1478/suppl/DC1
Materials and Methods
Supplementary Text
Tables S1 to S26
References (S2–112)
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High-resolution crystal structures have been solved for bacterial (8, 9) and eukaryotic ribosomes (10–12), as well as for an archaeal large subunit (13). In comparison, the only structures to date for mito-
riboosomes come from cryo-electron microscopy (cryo-EM) reconstructions at 12 to 14 Å (14–16).

The very low yields of mitoribosomes from most tissues have impeded their crystallization. However, the advent of high-speed direct electron detectors (which allow accounting for particle movement during imaging) (17, 18) and improved algorithms for the classification and alignment of particles (19) allowed us to determine the structure of the large subunit of the yeast mitoribosome (hereafter referred to as 54S) to an overall resolution of 3.2 Å, resulting in a nearly complete, stereochemically refined atomic model.

Structure Determination
Ribosomes purified from yeast mitochondria were contaminated with cytoplasmic ribosomes and free 54S (fig. S1A). The use of maximum likelihood image classification techniques (20) enabled sorting of these classes of particles into separate groups (fig. S1B). A preliminary re-
construction of the 74S mitochondrial ribosome yielded a density map with overall resolution of 3.6 Å. However, the small subunit was found to adopt a wide range of orientations with respect to the large subunit, resulting in markedly worse resolution for this region (~5 Å). Attempts to further classify the mitoribosomes into separate groups failed to yield maps that were suitable for de novo model building of the small subunit. We focused on subsequent efforts on the large subunit only. Density for this region was improved further by reconstructions that combined the large subunit regions of particles consisting of the 74S mitoribosome with those of the free 54S subunit, in a refinement in which the small subunit was masked away. The resulting total of 47,124 particles yielded an overall resolution of 3.2 Å by the “gold standard” Fourier shell correlation (FSC) = 0.143 criterion (fig. S2A) (21).

The local resolution (22) is better than 3 Å for most of the interior of the 54S subunit (Fig. 1 and fig. S2B) but decreases toward the periphery, especially for elements such as the L1 and L7/L12 stalks, which are often disordered even in crystal structures of the ribosome. Even at the surface, the resolution mostly appears to be better than 4 Å (fig. S2C).

The high resolution for much of the molecule allowed us to construct a nearly complete model for the large subunit, including one-third of the molecule for which no previous structural homology was known. In some regions, the quality of the map allowed identification of amino acids directly from side-chain densities without any additional information, leading to an unambiguous assignment of specific mitoribosomal proteins to their corresponding densities. For other regions, the identification of specific proteins required a combination of secondary structure prediction, homology modeling, and molecular replacement (23). X-ray crystallographic tools such as Coot (24) and REFMAC (25) were modified for model building, refinement, and cross-validation (23). The final model (Fig. 2A) has good stereochemistry and agreement with the density (table S2).

The model does not include surface exposed regions for which only partial density is visible, such as the L1 and L7/L12 stalks. In addition, some of the mitochondria-specific rRNA expansions are highly mobile and thus could not be completely resolved. Therefore, we included only the parts for which clear density is apparent in appropriately sharpened maps. The relative orientation of the two subunits in the major class is shown in fig. S3.

Overall Structure
Both protein and RNA moieties are substantially larger than their bacterial counterparts. From the total mass of 1.9 MD, 0.4 MD are from mitochondria-
specific proteins and extensions of known bacte-
rial homologs, 0.2 MD are from rRNA expansion segments (ESs), and 1.3 MD have structural equivalents in bacteria. Most of these additional elements occur on the surface of the conserved core (Fig. 2B), giving the 54S a markedly different overall shape from the large subunits of cytoplasmic ribosomes.

In particular, there is an expansion of the central protuberance (CP) and a new protuberance formed at the membrane-facing surface. In both cases, the rRNA expansion serves as a scaffold for the addition of new proteins. Such progressive expansion shares principles with the development of modern ribosomes themselves from primordial ancestors (26). In addition to these changes at the surface, there is a substantial remodeling of the tunnel through which the nascent protein chain proceeds as it emerges from the ribosome.

rRNA Expansion Segments
The 21S RNA of the 54S subunit contains 3296 nucleotides, of which 2714 have been modeled in our structure. A secondary structure diagram was constructed using base-pairing information directly from the structure, supplemented by predictions for the unmodeled regions (Fig. 3 and figs. S4 and S5). Conserved rRNA helices are numbered corresponding to those in Escherichia coli (27), with ESs denoted by the preceding con-
served helix. Despite the lower sedimentation coefficient relative to E. coli 23S, there has been a considerable expansion of rRNA, resulting in 392 additional nucleotides spread over 11 ESs. Only a few insertions correlate with those of the eukaryotic ribosome. However, as in the eukaryotic case, many of the insertions are flexible expansion segments that project out from the core and are not well resolved in the map. It is not clear whether this similarity is a coincidental growth of complexity by neutral means (28) or whether both evolved independently under selective pressure for functional reasons.

Mitochondria-Specific Protein Elements
Of the 39 proteins built into the 54S, 26 share homology with bacterial ribosomes, 8 are present in both yeast and mammalian mitochondria, and 5 are unique to yeast mitochondria (table S3 and fig. S6). Following a recent proposal by the ribosome community, proteins universal to all domains of life have a “u” prefix and bacterial numbering, those with only a bacterial homolog have a “b” prefix and bacterial numbering; and proteins unique to mitochondria have an “m” prefix. Bacterial ribosomal proteins L18, L20, and L25 are not present in the yeast mitoribosome. However, a homolog of L35 that was previously thought not to be part of the mitoribosome (29) was clearly identified in the maps. Mitochondrial homologous recombination protein 1 (MHRI) was also found to be a component of the mitoribosome (29). Density is absent for mL53, mL54, and mL61, which were present in our sample as judged by

Fig. 1. Examples of the density in cryo-EM maps of the 54S subunit. (A) RNA showing a double helix (top) and a base pair (bottom). (B) An α helix and a β turn in proteins showing side chains. (C) Mg ion that coordinates a tight turn in RNA.
mass spectrometry, indicating that they are disordered or dissociated during grid preparation.

Most of the homologous proteins are considerably larger, with an average mass of ~25 kD compared to ~13.5 kD for their bacterial counterparts (table S3 and fig. S6). Similar to eukaryotic ribosomes, many of the extensions to bacterial proteins consist of long tails and loops extending from globular domains. For example, uL24 has approximately three times the number of residues and extends 115 Å across the surface of the mitoribosome (fig. S7A). As in the eukaryotic ribosome (11, 12), many of these extensions contact other extensions and unique proteins and rRNA (Fig. 2), suggesting a role in the assembly of mitoribosome-specific components. Some of the flexible extensions are highly basic and probably assist rRNA folding by neutralizing negative charge, as suggested for bacterial and archaeal ribosomes (13, 30). Only the extensions to uL17 and bL27 form compact domains. uL17 is characterized by a four-helix bundle fold, whereas bL27 forms a domain with no homology to known structures (fig. S7B); both domains interact with rRNA.

As a result of protein extensions, the number of protein-protein contacts in the 54S is not only larger than in bacteria, but also exceeds that observed in the eukaryotic large subunit (11). Each protein in the mitoribosome has on average 4.5 neighbors (fig. S8), in comparison with only 1.5 in bacteria. Despite many more proteins overall, the number that contact only rRNA is halved relative to bacteria. Some of the new interactions include bridging different sites of the subunit (naturally performed by 55 rRNA in cytoplasmic ribosomes), whereas others are involved in the architecture of the exit tunnel.

All of the 13 mitochondria-specific proteins interact with rRNA, including those that have domains not previously shown to have a role in binding nucleic acids. Of these, all except mL43 and mL49 are bound to rRNA ESs. Analyses of the unique proteins show that three of them have similarity with RNA binding proteins (mL44, mL49, and mL57), two with DNA binding proteins (mL58 and mL60), two with nucleotide-binding proteins (mL58 and mL46), and two with lipid-binding proteins (mL38 and mL50; table S3). These proteins have lost their catalytic or ligand-binding residues and perform other roles in the mitoribosome.

The Central Protuberance (CP)

One site of major expansion is the CP, which because of its location is thought to facilitate communication between various functional sites of the ribosome in a manner that is not fully understood (31, 32). In bacterial, archaeal, and eukaryotic ribosomes, the CP consists of a separate 55 rRNA core interacting with several ribosomal proteins and 23S rRNA.

Yeast 54S lacks 55 rRNA and the 55 rRNA-binding proteins L18 and L25. It is extensively remodeled (Fig. 4A) and consists of mitochondria-specific proteins (mL38, mL40, and mL46), rRNA expansion clusters (82-ES and 84-ES1), and the extensions of the five homologous proteins (uL5, uL16, bL27, bL31, and bL33). These elements do not structurally mimic 55 rRNA but occupy its location in bacterial ribosomes (Fig. 4B). The bottom “toe” of 55 rRNA is replaced by 44-ES1, which interacts with uL16 and mL60. The large extension of bL27 binds the 82-ES1-3 to form part of the CP, illustrating how expansions in both proteins and rRNA have been used to reform the CP (fig. S7B). Despite the absence of 55 rRNA, the CP is triple the volume of the bacterial version. There is a larger network of interactions with the rest of the large subunit, as well as potential new contacts with the head of the small subunit (fig. S3).

A Membrane-Facing Protuberance

A second site enriched with mitochondria-specific proteins and rRNA is located adjacent to the mouth of the exit tunnel and thus must be close to the point of attachment of the ribosome to the translocon in the membrane (fig. S9). This protuberance consists of mitochondria-specific mL43, 44, 50, 57, and 58 (Fig. 2A) and rRNA expansion segments 0-ES1, 0-ES2, and 44-ES3. Two inactive members of the ribonuclease III family, mL44 and mL57, form a heterodimer and bend the 0-ES by an angle of 90°, forming an “L-shaped” helix (Fig. 5, B and C). The rest of the protuberance involves a second rRNA ES (44-ES3), the C-terminal part of mL50, N- and C-terminal extensions of uL22 and mL43, which binds predominantly to helix 46.

Yeast mitoribosomes have been shown to be permanently associated with the inner mitochondrial membrane in both translationally active and inactive states (33, 34). Moreover, mitoribosomes remain docked to the membrane even in the absence of the protein insertion machinery associated with the tunnel exit, indicating that additional and as yet uncharacterized anchors exist (35). The location of the membrane-facing protuberance makes it a candidate to be involved in the tethering of mitoribosomes to the membrane, independent of nascent peptide insertion.

Fig. 2. Overview of the yeast 54S ribosomal subunit. (A) Cytoplasmic and side views of final model with 21S rRNA colored in gray. (B) Cytoplasmic and interface views colored showing regions conserved with bacteria (blue), found in both yeast and mammalian mitochondria (red) and present only in yeast mitochondria (yellow).
E-Site tRNA

The yeast mitoribosomes copurified with a tRNA bound in the exit (E) site. The density for the complete tRNA is relatively weak, presumably because of a combination of partial occupancy and mixed species of tRNA (fig. S10A). Nonetheless, we were able to model the acceptor stem including the conserved 3′-CCA. The terminal adenine intercalates between bases 2688 (E. coli: 2421) and 2689 (2422) in a manner identical to bacteria and eukaryotes (fig. S10B). The tRNA appears to be stabilized by an additional contact not seen in bacteria. A C-terminal extension in bL33 creates a highly electropositive patch that stabilizes the phosphate backbone of the tRNA acceptor stem (fig. S10C). The extension of bL33 is stabilized by a change in the rRNA path prior to helix 82-ES1, which is itself partially stabilized by the unique protein mL59. Thus, the E site of ribosomes from archaea (36), bacteria, (9) and mitochondria (this work) all bind the terminal A76 in an essentially identical manner by intercalating between two purines of rRNA, while at the same time the rest of the E site has diverged among these classes of ribosomes.

The Exit Tunnel

During translation, the growing nascent chain proceeds from the peptidyl transferase center (PTC) through a tunnel to the opposite end of the large subunit where it emerges ~100 Å away. Given its universality, the tunnel is highly conserved among previously studied ribosomes. Despite this conservation, a superposition with bacterial ribosomes showed that the path of the bacterial tunnel is blocked in the 54S by a section of a long mitochondria-specific extension to uL23 that is well anchored in the 54S on either side and thus unlikely to be displaced (Fig. 6A). Instead, a new tunnel path is seen that deviates from the bacterial one ~60 Å from the PTC (Fig. 6B). This is the only channel in the yeast mitoribosome that extends from the PTC to the exterior of the ribosome. The boundaries of the new tunnel are defined by extensions of uL22, uL23, and uL24, and by rRNA 3-ES. The tunnel leads to a new exit site located roughly 35 Å away from where the bacterial tunnel would have emerged (Fig. 6, B and C, and fig. S9) and is surrounded by two additional unique proteins, mL44 and mL50, which are also involved in forming the membrane-facing protuberance (Fig. 6B). This suggests that the attachment to a translocon-like entity for insertion of the nascent peptide into the mitochondrial membrane must be very different in the 54S than in bacterial ribosomes. In support of this idea, uL24 was chemically found to form cross-links with uL23, mL44, and mL50 (37). The exit of the tunnel is also wider than in bacteria, possibly to allow cotranslational assembly of oxidative phosphorylation complexes (35, 38).

The entrance to the tunnel just beyond the PTC is noticeably narrower than in all previously determined structures of the large subunit (Fig. 6D). In part this is due to a base pair between U2877 and A1958 that appears unique to yeast mitochondria and could only be identified on the basis of the structure. This pair brings opposing strands of 21S rRNA closer together by about 8 Å. This location is the binding site of macrolide antibiotics, which, unlike many aminoglycosides, do not bind to mitochondrial ribosomes (39) and thus do not have the same toxicity. A superposition of the macrolide erythromycin in its binding site shows that it would clash with the narrower tunnel entrance in mitochondria, so in yeast mitoribosomes there is an additional mechanism for resistance to these antibiotics beyond sequence variation of the binding pocket. Beyond the entrance, the tunnel is lined by conserved elements (Fig. 6C), including the constriction site formed by uL22 and uL4. In addition, near the point where the path diverges from the path of the bacterial tunnel, two aromatic residues of uL22 and uL24 narrow the tunnel to a diameter of ~9 Å (Fig. 6C).

This work shows that recent advances in cryo-EM can be used to determine structures of comparable quality to x-ray crystallography but from much smaller amounts of more heterogeneous material. It also reveals that this highly divergent ribosomal subunit differs substantially from those of bacteria, eukaryotes, and archaea, with several unique features including an altered path of the exit tunnel.
Fig. 5. A membrane-facing protuberance. (A) Site of expansion of the 54S relative to bacteria. (B) ESO of 21S rRNA serves as an anchor for the protuberance. (C) Two views of the protuberance showing how mitochondria-specific proteins bind to the expansion segment.

Fig. 6. The putative exit tunnel in the 54S subunit. (A) The bacterial tunnel (top, path in orange) is blocked by a mitochondria-specific extension to uL23 (detail in bottom panel). (B) The 54S tunnel (blue mesh) has been extensively remodeled by mitochondria-specific protein elements (colored) (top), resulting in a tunnel exit (bottom) that is substantially different from that in bacteria. In (A) and (B) the lower panels are 90° rotations of the upper panels. (C) Conservation of elements that make up the entrance and top part of the tunnel. The constriction at the top is narrower, and two aromatic amino acids that line the tunnel make a close approach. The path of the bacterial tunnel (orange) would exit 35 Å away. (D) Narrowing of the 54S tunnel entrance (yellow) due to an AU base pair not found in bacteria (white) that would cause a steric clash with macrolides such as erythromycin (pink).