A Novel Small-Subunit Ribosomal Protein of Yeast Mitochondria That Interacts Functionally with an mRNA-Specific Translational Activator

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Mitochondrial translation of the mRNA encoding cytochrome *c* oxidase subunit III (coxIII) specifically requires the action of three position activator proteins encoded in the nucleus of *Saccharomyces cerevisiae*. Some mutations affecting one of these activators, PET122, can be suppressed by mutations in an unlinked nuclear gene termed PET123. PET123 function was previously demonstrated to be required for translation of all mitochondrial gene products. We have now generated an antibody against the PET123 protein and have used it to demonstrate that PET123 is a mitochondrial ribosomal protein of the small subunit. PET123 appears to be present at levels comparable to those of other mitochondrial ribosomal proteins, and its accumulation is dependent on the presence of the 15S rRNA gene in mitochondria. Taken together with the previous genetic data, these results strongly support a model in which the mRNA-specific translational activator PET122 works by directly interacting with the small ribosomal subunit to promote translation initiation on the coxIII mRNA.

Protein synthesis in mitochondria of *Saccharomyces cerevisiae* depends on nuclear genes that encode components of the general translation machinery such as mitochondrial ribosomal proteins, translation factors, and tRNA synthetases (1, 21; M. C. Costanzo and T. D. Fox, Annu. Rev. Genet., in press). In addition to these general components, other proteins are required specifically to promote the translation of particular mitochondrial mRNA species (1, 18, 21; Costanzo and Fox, in press). For example, the proteins coded by the nuclear genes PET122, PET54, and PET494 have been shown to be required specifically for mitochondrial translation of cytochrome *c* oxidase subunit III (coxIII) (2, 5, 8, 10, 26, 27, 31). All three of these coxIII-specific translational activators have been shown to accumulate in mitochondria (5, 9, 35; unpublished results), and their site (or sites) of action has been mapped genetically to the 5'-untranslated leader of the coxIII mRNA (6). A possible explanation for these findings is that one or more of the three translational activator proteins interact directly both with the coxIII 5' leader and with some component of the general mitochondrial translation apparatus to activate coxIII translation.

To understand better the mechanism of action of the coxIII-specific translational activators, we have been isolating and analyzing allele-specific nuclear suppressors of mutations in PET122, PET54, and PET494. Such allele-specific suppressors can frequently identify genes encoding proteins that directly interact (23, 25). Previously, we genetically characterized a suppressor mutation in the newly identified gene PET123 which restores coxIII translation in certain *pet122* mutants (22a). PET123 function is required generally for the translation of mitochondrial mRNAs, since heat-sensitive *pet123* mutations dramatically reduce the synthesis of all mitochondrially coded proteins at nonpermissive temperatures (22a). Furthermore, like null mutations in other known components of the translation machinery (16, 32, 33), a *pet123* deletion destabilizes [*rho*⁺] mitochondrial DNA

(22a). However, since the predicted sequence of PET123 exhibited no strong sequence similarity to any known proteins (22a), its role in translation remained to be determined.

Here we report the initial characterization of the PET123 protein. We find that PET123 specifically accumulates in mitochondria and is a component of the small subunit of mitochondrial ribosomes. On the basis of the characteristics of the PET123 protein and the phenotype of *pet123* mutants, our data strongly indicate that PET123 is a mitochondrial ribosomal protein that interacts with the PET122 protein. Thus, mitochondrial translation of coxIII mRNA appears to require an interaction between at least one mRNA-specific factor and the small ribosomal subunit.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The wild-type *S. cerevisiae* strains used in this study were DAU2 (*MATa ura3 ade2 [rho*⁺*]*) (6) and DBY947 (*MATa ura3-52 ade2-101 [rho*⁺*]*) (34). The *pet123* null mutant strain PTH78 (*MATa pet123::URA3 ura3-52 ade2-101 [rho*⁺*]*) is described elsewhere (22a). The [*rho*[−]] strains used in the experiment illustrated in Fig. 5 were ROS-31, which carries the complete 15S rRNA gene (19); 18D, which carries the complete *cox3* gene (C. Sengstag and T. D. Fox, unpublished data); 8-6, which carries a portion of the *cox2* gene (17); dD22, which carries the complete *cob* gene (38); and a [*rho*[−]] strain carrying the complete 21S rRNA gene and isogenic to strain COP U7 (obtained from T. L. Mason). The [*rho*[−]] strains were checked for retention of their mitochondrial genetic markers. The percentage of retention was at least 90% for all cultures used. [*rho*⁰] strains were generated as described elsewhere (20). To confirm the complete loss of mitochondrial DNA, total DNA from these cells (37) was analyzed by Southern blotting with ³²P-labeled wild-type mitochondrial DNA as a probe.

YPD and SD media and routine genetic manipulations were as described by Sherman et al. (39). YPEG and YPGal media were the same as YPD medium except that 3% (vol/vol) ethanol and 3% (vol/vol) glycerol were substituted for glucose in YPEG medium and 2% (wt/vol) galactose was

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substituted for glucose in YPGal medium. SGal medium was the same as SD medium except that 2% (wt/vol) galactose was substituted for glucose. Yeast transformation was performed as described previously (7).

Plasmids. The multicopy plasmid pPHY44, which carries *PET123*, was created by ligating the *Bam*HI-to-*Hind*III fragment from pPHB5 (22a) into the 2- μ m vector pCEG137 (7) cut with the same restriction enzymes. A plasmid expressing a *trpE*-*PET123* fusion protein was constructed as follows. An approximately 590-base-pair *Dra*I-*Eco*RI fragment of pPHB5 containing most of the amino-terminal part of *PET123* was inserted into the vector pUC18 (43), generating pPHB8. From pPHB8, an approximately 450-base-pair *Hind*III-*Xba*I fragment was excised and ligated, together with a 1.5-kilobase-pair *Pvu*II-*Hind*III fragment from pATH3 (14), between the *Hinc*II and *Xba*I sites of an *Nde*I deletion derivative of pPHB5. The resulting plasmid, pPHB11, contains almost the entire open reading frame of *PET123* (interrupted by some polylinker sequences) fused at codon 11 to the carboxy terminus of the *Escherichia coli* *trpE* gene.

Generation of anti-*PET123* antiserum. The *trpE*-*PET123* fusion plasmid pPHB11 (see above) was transformed into *E. coli* DH5 α , and those cells were induced with 3 β -indoleacrylic acid (14) to produce large amounts of the *trpE*-*PET123* fusion protein. The insoluble fraction enriched in the fusion protein was isolated as described previously (14) and was electrophoresed into sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were lightly stained with Coomassie brilliant blue R, and the band corresponding to the fusion protein was cut from the gels. The fusion protein was electroeluted from the gel slices, dialyzed against 10 mM Tris-100 mM NaCl (pH 7.6), and concentrated. An initial injection of 100 μ g of the fusion protein and a booster of 50 μ g were prepared and injected into rabbits intradermally as previously described (5). Crude serum was prepared as described previously (3). To remove cross-reacting material from the crude serum, mitochondrial proteins from the yeast strain PTY78 were bound to Affi-gel 10 (Bio-Rad Laboratories) according to the instructions of the manufacturer. A 1:50 dilution of the crude antiserum was incubated with the matrix overnight at 4°C and then removed from the matrix and stored at -20°C.

Polyacrylamide gel electrophoresis and immunoblotting. The separation of proteins by one-dimensional gel electrophoresis was as described elsewhere (28). Mitochondrial ribosomal proteins were extracted with acetic acid as described elsewhere (29) and analyzed by two-dimensional gel electrophoresis as described elsewhere (30) except that the first-dimension gels were run for 12 h at 1.5 mA per gel and the second dimension was a 15% polyacrylamide-SDS gel. Proteins were transferred from the gel to Immobilon-P membranes (Millipore Corp.) and processed for immune detection as described in the instructions of the manufacturer. The anti-*PET123* and anti-cytochrome *c* oxidase subunit II (anti-coxII) antisera were used at a dilution of 1:1,000, and ¹²⁵I-protein A was used to detect the antibody-antigen complexes. The polyclonal anti-coxII antiserum was a generous gift of G. Schatz. The monoclonal anti-MRP7 and anti-MRP13 antisera, generous gifts from T. Mason, were used at a dilution of 1:7, and detection of these antibody-antigen complexes was accomplished by using ¹²⁵I-anti-mouse immunoglobulin G. Total yeast protein samples were prepared as follows. Equal numbers of cells from each culture were incubated in a solution containing 1.2 M sorbitol and 0.25 mg of Zymolyase 20T (ICN Immunobiologicals,

Inc.) per ml for 15 min at 23°C and then washed twice with 1.2 M sorbitol. The cells were disrupted in 10 mM Tris (pH 7.5)-1 mM phenylmethylsulfonyl fluoride by vortexing vigorously in the presence of an equal volume of glass beads (diameter, 0.45 mm) for 30 s. A portion of each sample was added to an equal volume of 2 $\times$ SDS sample buffer (28), heated to 100°C for 4 min, and stored frozen at -20°C. Protein concentrations were determined for samples of the extracts before sample buffer addition by the Bio-Rad protein assay according to the instructions of the manufacturer.

Isolation of mitochondria and mitochondrial ribosomes. Mitochondria were prepared from cells grown to late exponential phase (approximately 2 $\times$ 10⁸ cells per ml) by the procedure of Daum et al. (13), except that spheroplasts were disrupted with a Parr-Bomb (Parr Instrument Co., Moline, Ill.) pressurized to 300 lb/in² for 10 min as described previously (4, 11). Mitochondria were purified by equilibrium density gradient centrifugation by suspending the crude mitochondrial pellet in buffer (pH 7.5) containing 0.6 M sorbitol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 1 mM EDTA and centrifuging this suspension into a 20 to 60% sucrose gradient (pH 7.5) containing 100 mM NaCl, 20 mM HEPES, and 1 mM EDTA at 100,000 $\times$ *g* for 16 h at 3°C. The procedure of Singh et al. (40) (method 1) was used to isolate mitochondrial ribosomes and to separate the ribosomal subunits by sucrose density centrifugation. Appropriate fractions containing either small or large subunits of mitochondrial ribosomes were pooled, diluted by half with buffer (pH 7.5) containing 50 mM NH₄Cl, 10 mM Tris, and 10 mM MgCl₂, and centrifuged at 200,000 $\times$ *g* for 16 h. The pellets were stored frozen at -70°C. The procedure of Partaledis and Mason (36) was used to analyze mitochondrial ribosomes by sucrose gradient centrifugation directly from disrupted mitochondria.

RESULTS

Detection and mitochondrial localization of *PET123*. As a means of identifying and studying the *PET123* protein biochemically, we prepared an anti-*PET123* antiserum by immunizing a rabbit with a *trpE*-*PET123* fusion protein (see Materials and Methods). To test for an immune response, total yeast proteins, separated by SDS-gel electrophoresis and transferred to a membrane, were incubated with either the immune serum or preimmune serum. The resulting immunoblot revealed an immune-specific reaction to a 39-kilodalton (kDa) protein from wild-type yeast (Fig. 1, lane 2). The identification of this 39-kDa protein as the *PET123* product was confirmed by two additional observations. First, a yeast strain harboring multiple copies of the *PET123* gene exhibited increased amounts of the 39-kDa protein (Fig. 1, lane 4). Second, the 39-kDa protein was undetectable in proteins from a strain, PTH78, in which the *PET123*-coding sequence was disrupted by insertion of *URA3* (Fig. 1, lane 3). The small discrepancy between the size of the protein predicted from the DNA sequence, 41.6 kDa (22a) and the size measured by gel electrophoresis may reflect a proteolytic processing event common to many mitochondrially imported proteins (15, 24).

Our previous experiments (22a) indicated that *PET123* is required for translation of all mitochondrial mRNAs. The *PET123* protein might play a direct role in mitochondrial translation, in which case its cellular location would be within mitochondria. This possibility was first addressed by fractionating disrupted cells into a crude mitochondrial pellet and a postmitochondrial supernatant. The *PET123* protein

FIG. 1. Anti-PET123 antiserum recognizes a 39-kDa protein in yeast. Total cell proteins (100 μ g) were separated by SDS-gel electrophoresis, blotted to Immobilon-P membranes, and incubated with either preimmune serum (lane 1) or immune serum (lanes 2 to 4) as described in Materials and Methods. Proteins were prepared from the following strains: lanes 1 and 2, wild-type DBY947; lane 3, the *pet123::URA3* disruption strain PTH78; and lane 4, DBY947 transformed with the multicopy *PET123* plasmid pPHY44.

was quantitatively recovered in the crude mitochondrial pellet, while essentially no PET123 protein was detected in the postmitochondrial supernatant, as judged by immunoblotting of equivalent amounts of each fraction (Fig. 2A). To determine whether PET123 was specifically associated with mitochondria, the material in the crude mitochondrial pellet was subjected to equilibrium density gradient centrifugation (see Materials and Methods). Each fraction of the gradient was analyzed by immunoblotting of the mitochondrial inner membrane protein *coxII* as a marker for mitochondria (Fig. 2B). The PET123 protein from the crude mitochondrial fraction was found in the gradient at the same position as *coxII*, indicating that the PET123 protein was completely and specifically associated with mitochondria.

PET123 is a small-subunit ribosomal protein. To investigate the possibility that the PET123 protein is involved directly in mitochondrial translation, purified mitochondria were solubilized with detergent and the soluble contents were sedimented into a sucrose gradient (see Materials and Methods). The positions in the gradient of the mitochondrial ribosomal subunits were determined first by measuring the A_{260} of each gradient fraction and then by immunoblotting proteins from each fraction and probing those blots with antibodies to either the known large-subunit protein MRP7 (16) or the known small-subunit protein MRP13 (36). The positions of each of the known ribosomal proteins coincided with one of the two A_{260} peaks, as expected (Fig. 3). An identical blot was treated with the anti-PET123 antiserum, revealing that the PET123 protein was present only in those fractions containing the small subunit of the mitochondrial ribosome. When mitochondrial ribosomal subunits obtained by pelleting from the initial mitochondrial lysate through a high-salt sucrose cushion (see Materials and Methods) were analyzed by sucrose gradient centrifugation, results identical to those shown in Fig. 3 were obtained (data not shown). The observation that the PET123 protein remained associated with the small subunit of the ribosome despite the high salt

FIG. 2. PET123 is specifically associated with mitochondria. Spheroplasts of the wild-type strain DAU2 were disrupted and fractionated into a postmitochondrial supernatant and a mitochondrial pellet as described in Materials and Methods. The crude mitochondrial pellet was suspended in isotonic buffer to a volume equivalent to that of the postmitochondrial supernatant. (A) To compare the relative amounts of PET123 in each fraction, equal proportions (volumes) of the subcellular fraction were electrophoresed into an SDS gel and immunoblotted with the anti-PET123 antiserum. The samples were as follows: lane 1, total yeast extract; lane 2, postmitochondrial supernatant; and lane 3, crude mitochondrial pellet. (B) Crude mitochondria were suspended in isotonic buffer and centrifuged to their equilibrium density in a 20 to 60% sucrose gradient as described in Materials and Methods. Equal volumes of each of the fractions of the gradient were electrophoresed into an SDS gel and immunoblotted with either the anti-PET123 antiserum or an antiserum directed against *coxII*.

concentration (0.5 M NH_4Cl) used in the suspension buffer and the sucrose gradient indicates that PET123 is a tightly associated component of the mitochondrial small ribosomal subunit.

To compare the amount of PET123 present in ribosomes relative to the amounts of other ribosomal proteins, proteins of gradient-purified small subunits were separated by one-dimensional SDS-gel electrophoresis and either stained (Fig. 4A, lane 1) or transferred to a membrane and reacted with the anti-PET123 antiserum (Fig. 4A, lane 2). Immunologically detected PET123 corresponded to a protein band with a staining intensity stronger than those of the surrounding protein bands, suggesting that either PET123 is present in above-stoichiometric amounts or that the stained band containing PET123 represents more than one protein. To investigate this further, proteins of purified ribosomal small subunits were separated by two-dimensional gel electrophoresis (Materials and Methods) and stained (Fig. 4B). The same gel was then blotted to a membrane for immunoblot analysis (Fig. 4C), revealing that the position of the PET123 immune reaction was identical to that of a prominent stained protein spot (Fig. 4B). In this analysis, the staining intensity of the PET123 protein was similar to those of other small subunit proteins, indicating that PET123 is a stoichiometric ribosomal protein. (Note that there is no generally accepted

FIG. 3. PET123 is associated with the small subunit of the mitochondrial ribosome. Purified mitochondria were disrupted with deoxycholate, and their contents were sedimented by centrifugation into a 15 to 30% sucrose gradient containing 500 mM NH_4Cl as described in Materials and Methods. The absorbance (A_{260}) profile of the gradient is shown in the top panel. Equal volumes of each of the fractions of the gradient were immunoblotted with a monoclonal antibody against the large-subunit ribosomal protein MRP7 (16), a monoclonal antibody against the small-subunit protein MRP13 (36), and antiserum against PET123, as indicated.

two-dimensional map of yeast mitochondrial ribosomal proteins.)

The accumulation of at least one other yeast mitochondrial small-subunit ribosomal protein is strongly reduced in cells lacking mitochondrial DNA ($[\text{rho}^0]$) (36). The PET123 protein behaved similarly: its level was drastically reduced, although it was still detectable, in $[\text{rho}^0]$ cells (Fig. 5, lanes 1 and 2). Since PET123 is strongly associated with the small subunit of the ribosome, its accumulation might be dependent upon the presence of the 15S rRNA. To investigate this, we asked whether various $[\text{rho}^-]$ strains containing different regions of the mitochondrial genome showed any differences in accumulation of the PET123 protein. A $[\text{rho}^-]$ strain, ROS-31, that retained the 15S rRNA gene showed normal accumulation of the PET123 protein (Fig. 5, lane 3). However, a $[\text{rho}^0]$ strain derived from ROS-31 (Fig. 5, lane 4) contained very low levels of PET123, similar to those of other $[\text{rho}^0]$ strains and $[\text{rho}^-]$ strains retaining different parts of the mitochondrial genome (Fig. 5, lanes 6 to 10). The fact that PET123 accumulation is dependent on the presence of the 15S rRNA gene suggests that the protein interacts with the 15S rRNA directly or indirectly and that this interaction promotes protein stability.

To investigate the submitochondrial location of the PET123 protein in the $[\text{rho}^-]$ strain containing the 15S rRNA

FIG. 4. PET123 is a major component of the small subunit. Mitochondrial ribosomal subunits were purified by gradient centrifugation. Appropriate fractions were pooled and concentrated as described in Materials and Methods. (A) Small ribosomal subunit proteins were dissolved in SDS sample buffer, electrophoresed into an SDS-15% polyacrylamide gel, and either stained with Coomassie brilliant blue R (lane 1) or immunoblotted with the anti-PET123 antiserum (lane 2). (B) Small ribosomal subunit proteins were separated by two-dimensional gel electrophoresis as described in Materials and Methods, stained with Coomassie brilliant blue R, and photographed. The spot corresponding to PET123 is indicated by the arrow (see below). (C) The gel shown in panel B was soaked in 2% SDS for 15 min and then transferred to a membrane, which was then treated with the anti-PET123 antiserum. The staining pattern was visible on the membrane, facilitating the identification of the stained protein spot indicated by the arrow in panel B as the spot corresponding to the immunologically detected PET123 protein.

gene, mitochondria from this strain were disrupted and the contents were centrifuged into a sucrose gradient. Immunoblot analysis of each fraction of the gradient revealed that the PET123 protein sedimented into the gradient in a manner similar to that of mitochondrial ribosomal small subunits from wild-type yeast (not shown). While both MRP13 and 15S rRNA accumulated in this strain and were present in the same gradient fractions as PET123, most of the MRP13 and

FIG. 5. Accumulation of PET123 is dependent upon the presence of the 15S rRNA gene in mitochondria. Equal amounts (100 μg) of total cell protein from various yeast strains were electrophoresed on an SDS gel and immunoblotted with the anti-PET123 antiserum. Proteins were prepared from the following strains: lane 1, the $[\text{rho}^+]$ strain DAU2; lane 2, an isogenic strain lacking mitochondrial DNA, DAU2 $[\text{rho}^0]$; lane 3, a $[\text{rho}^-]$ strain, ROS-31, carrying the 15S rRNA gene; lane 4, a $[\text{rho}^0]$ strain derived from ROS-31; lane 5, the $[\text{rho}^+]$ strain COP U7; lane 6, a $[\text{rho}^-]$ strain carrying the 21S rRNA gene and isogenic to COP U7; lane 7, a $[\text{rho}^0]$ strain isogenic to COP U7; lane 8, a $[\text{rho}^-]$ strain, 18D, carrying the *cox3* gene; lane 9, a $[\text{rho}^-]$ strain, 8-6, carrying part of the *cox2* gene; and lane 10, a $[\text{rho}^-]$ strain, dD22, carrying the *cob* gene.

15S rRNA trailed behind the rapidly sedimenting complex (data not shown). Since the $[rho^-]$ strain lacks the mitochondrially coded small subunit protein var1 (22, 41, 42), the rapidly sedimenting complex must represent incomplete small subunits.

DISCUSSION

Mitochondrial translation of coxIII requires the specific positive activators PET122, PET494, and PET54 (8, 10, 27, 31), which activate translation of the coxIII mRNA through a site(s) in 5'-untranslated leader (6). The mechanism by which these nuclearly encoded but mitochondrially located proteins (5, 9, 35; unpublished results) function is not understood. One attractive hypothesis is that they could mediate an interaction between the coxIII mRNA and some component of the general translation machinery. We have sought to identify general translation components that might interact with the coxIII mRNA-specific activators by isolating allele-specific suppressors of *pet122*, *pet494*, and *pet54* mutations. Allele-specific suppression can be used to reveal likely protein-protein interactions (23, 25).

PET123 is one gene in which allele-specific suppressors of certain *pet122* mutations arise (22a). The suppressible *pet122* mutations cause carboxy-terminal truncations of the PET122 protein. The suppressor mutations that alter PET123 probably restore an interaction with PET122 that was weakened by the carboxy-terminal deletions, although more indirect mechanisms cannot be ruled out.

Our previous analysis of the phenotypes of *pet123* mutants indicated that the PET123 protein is a component of the general mitochondrial translation system (22a). However, since PET123 did not show any strong sequence similarity to other known proteins (22a), its role in mitochondrial translation was unclear.

Four lines of evidence indicate that the PET123 protein is a bona fide mitochondrial small-subunit ribosomal protein. First, the PET123 protein was specifically and quantitatively associated with mitochondria and thus cannot be a component of cytosolic ribosomes. Second, PET123 remained specifically associated with the small subunit of mitochondrial ribosomes even in the presence of high salt concentrations, showing that it is tightly associated with the small subunit. Third, PET123 was present in amounts roughly equivalent to those of other small-subunit proteins, suggesting that it is a stoichiometric component of all small subunits. Finally, the accumulation of PET123 appears to be dependent upon the presence of the 15S rRNA, since a $[rho^-]$ strain that retains the mitochondrial 15S rRNA gene exhibited wild-type levels of the PET123 protein, while other $[rho^-]$ strains and $[rho^0]$ strains lacking 15S rRNA showed a drastic reduction in the accumulation of PET123.

In view of the fact that accumulation of the PET123 protein was very low in $[rho^-]$ cells lacking 15S rRNA, it is perhaps surprising that respiring cells containing extra copies of the *PET123* gene accumulated higher than normal levels of the PET123 protein (Fig. 1). In such strains, the excess PET123 was quantitatively recovered in mitochondria, although it did not appear to be associated with small ribosomal subunits (data not shown).

Taken together with our previous work, the data presented here strongly support a model in which the coxIII-specific translational activator PET122 functions to mediate an interaction between the coxIII mRNA and the small ribosomal subunit. This model is further supported by identification of a second gene which can mutate to suppress the

same *pet122* mutations (P. Haffter, T. W. McMullin, and T. D. Fox, submitted for publication). This second suppressor gene encodes the previously described mitochondrial ribosomal protein MRP1 (32), which is also a component of the small subunit (12).

ACKNOWLEDGMENTS

We thank T. L. Mason for the gift of strains and antibodies to known mitochondrial ribosomal proteins and G. Schatz for the gift of the coxII antiserum.

T.W.M. was a postdoctoral associate of the Cornell Biotechnology Program during the early phases of this investigation. P.H. was a recipient of a European Molecular Biology Organization long-term fellowship and a fellowship from the Swiss National Science Foundation. This work was supported by a Public Health Service grant GM29362 from the National Institutes of Health.

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