DRS1 to *DRS7*, Novel Genes Required for Ribosome Assembly and Function in *Saccharomyces cerevisiae*

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To identify *Saccharomyces cerevisiae* mutants defective in assembly or function of ribosomes, a collection of cold-sensitive strains generated by treatment with ethyl methanesulfonate was screened by sucrose gradient analysis for altered ratios of free 40S to 60S ribosomal subunits or qualitative changes in polyribosome profiles. Mutations defining seven complementation groups deficient in ribosomal subunits, *drs1* to *drs7*, were identified. We have previously shown that *DRS1* encodes a putative ATP-dependent RNA helicase necessary for assembly of 60S ribosomal subunits (T. L. Ripmaster, G. P. Vaughn, and J. L. Woolford, Jr., Proc. Natl. Acad. Sci. USA 89:11131–11135, 1992). Strains bearing the *drs2* mutation process the 20S precursor of the mature 18S rRNA slowly and are deficient in 40S ribosomal subunits. Cloning and sequencing of the *DRS2* gene revealed that it encodes a protein similar to membrane-spanning Ca^{2+} ATPases. The predicted amino acid sequence encoded by *DRS2* contains seven transmembrane domains, a phosphate-binding loop found in ATP- or GTP-binding proteins, and a seven-amino-acid sequence detected in all classes of P-type ATPases. The cold-sensitive phenotype of *drs2* is suppressed by extra copies of the *TEF3* gene, which encodes a yeast homolog of eukaryotic translation elongation factor EF-1 γ . Identification of gene products affecting ribosome assembly and function among the DNAs complementing the *drs* mutations validates the feasibility of this approach.

The eukaryotic ribosome is a complex organelle which in most species contains more than 75 different proteins and four different RNA molecules. The primary sequences of eukaryotic rRNAs and many eukaryotic ribosomal proteins (r-proteins) (reviewed in references 65 and 66) have been determined or inferred from sequences of the respective genes. Regulation of expression of these genes has been studied in some detail (65, 66). Despite the large volume of data concerning the structure and expression of individual components of eukaryotic ribosomes, the pathway of assembly of ribosomal subunits and the functional roles that r-proteins and rRNAs play during the translation process are not well understood.

Ribosome biogenesis occurs in the nucleolus of eukaryotic cells, where rRNA is transcribed and processed, and associated with r-proteins imported from the cytoplasm (reviewed in references 19, 65, and 66). Preribosomal particles containing rRNA precursors, r-proteins, and non-r-proteins have been detected within the nucleolus (59, 63). Most of the r-proteins assemble into subunits while in the nucleolus, although some final steps of maturation occur after transport of subunits to the cytoplasm (61). The ribosomal subunits are then competent to catalyze protein synthesis.

Reconstitution and genetic studies have been applied successfully to study the ribosome assembly pathway in prokaryotic cells. Functional 30S and 50S ribosomal subunits of *Escherichia coli* can be reconstituted from purified rRNA and r-proteins in vitro (38, 60). This technique facilitated a detailed understanding of prokaryotic ribosome assembly, first with the elucidation of assembly maps (32) and more recently by elegant studies detecting changes in rRNA secondary structure during 30S subunit assembly (55). Many

cold-sensitive strains of *E. coli* and *Salmonella typhimurium* that have defects in ribosomal subunit assembly have been identified (18, 57). Although a few of these mutants were shown to contain defective r-proteins, the molecular bases of most of the assembly defects have not been determined.

Genetic approaches have been taken with *Saccharomyces cerevisiae* to explore the nature of eukaryotic ribosome biogenesis. *S. cerevisiae* mutants blocked in rRNA processing or unable to synthesize rRNAs or r-proteins have been obtained. Inactivation or depletion of individual r-proteins or rRNAs blocks proper assembly of the subunit of which that rRNA or protein is a part and leads to rapid degradation of the other rRNAs and proteins of that subunit (1, 33, 34, 37, 64). Heat- and cold-sensitive mutants that have ribosomal subunit deficits have been identified by monitoring changes in the steady-state levels of ribosomal subunits, although in most cases the corresponding genes have not been cloned (3, 7, 17, 54, 56). Two temperature-sensitive mutants of *S. cerevisiae* that fail to process rRNA have been isolated, although steady-state levels of ribosomal subunits have not been examined (30, 52).

Mutations in several yeast genes encoding non-r-proteins that affect levels of ribosomal subunits have been isolated. Depletion of nucleolar protein Nop1p or Gar1p or small nucleolar RNA U3 or U14 affects early steps in 35S rRNA processing. Dramatic reductions of mature 18S rRNA in all four cases cause deficiencies of 40S subunits (15, 20, 29, 58). Nucleolar protein Nop3p is necessary for production of 18S and 25S rRNAs (44). Striking changes in the levels of 60S subunits have been observed in strains having a mutation in either the *SPB4* or *RRP1* gene, whose products are involved in processing of the 27S precursor to the mature 25S rRNA (1, 45). Strains bearing a null allele of *NSR1* were shown to have defects in processing of the 20S rRNA precursor to 18S rRNA as well as changes in the steady-state level or distribution of 40S ribosomal subunits (28). Mutations in two genes encoding translation factors, *GCD2* (14), and *SUI1*

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(67), affect steady-state levels of 80S monoribosomes and polyribosomes.

To more fully characterize the assembly and function of the eukaryotic ribosome, we sought cold-sensitive yeast mutants with decreased amounts of 40S or 60S ribosomal subunits or striking changes in the monoribosome or polyribosome profiles. By monitoring changes in the ratio of free 40S to 60S ribosomal subunits, we expected to recover mutations residing in at least three classes of genes: those encoding (i) r-proteins, (ii) proteins involved in ribosome assembly and rRNA processing, and (iii) proteins required for certain aspects of protein synthesis. Cloning the genes defined by these mutations should identify gene products important for assembly and function of the eukaryotic ribosome.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* DS94 (*MAT α ura3-52 trp1-1 leu2-3,112 his3-11,15 lys2-801*), obtained from Elizabeth Craig (University of Wisconsin, Madison), was the wild-type parent from which cold-sensitive mutants were derived by ethyl methanesulfonate mutagenesis. Yeast cells were mutagenized to a survival rate of 20 to 50% as described by Drain and Schimmel (13). Single colonies were patched onto yeast extract-peptone-dextrose (YEPD), replica plated to YEPD, and scored for growth at 13°C. These cold-sensitive strains were isolated by Janine Maddock in our laboratory. *drs* mutants were outcrossed to *S. cerevisiae* DBY1034 (*MAT α ura3-52 his4-51 lys2-801*), obtained from David Botstein (Stanford Medical School, Stanford, Calif.). GRF123 (*MAT α rrp1-1 ade1 trp1 lys1 gal1*) containing the *rrp1-1* mutation was obtained from Anita Hopper (Pennsylvania State University, Hershey). JWY749 (*MAT α /MAT α ura3-52/ura3-52 trp1-101/trp1-101 lys2-801/lys2-801 leu2-1/leu2-1 his3- Δ 200/his3- Δ 200*) was the parent diploid in which the *drs2::TRP1* disruption was constructed. Standard yeast genetic methods were employed (35, 51). Yeast cells were grown either in YEPD or in defined synthetic medium supplemented with 2% glucose as a carbon source (51). Yeast cells were transformed by the lithium acetate method (21).

Materials. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). *E. coli* DNA polymerase holoenzyme was a gift of William E. Brown, Carnegie Mellon University. [α -³²P]dATP, α -³⁵S-dATP, and L-[methyl-³H]methionine were obtained from New England Nuclear (Boston, Mass.).

Fractionation and characterization of polyribosomes. Yeast strains were grown to a density of 4.0×10^7 to 6.0×10^7 cells per ml in 200 ml of YEPD or defined synthetic medium at 30°C then shifted to 13°C for 2 h. Polyribosomes were extracted and analyzed on 35-ml 7 to 47% sucrose gradients by using an ISCO density gradient fractionator (model 640) as described by Baim et al. (2).

DNA manipulations, Southern analysis, and RNA blotting. Construction and analysis of recombinant DNAs and electrophoresis, blotting, and hybridization of DNA were performed as described by Sambrook et al. (46). Gel electrophoresis, blotting, and hybridization of RNA were performed as described by Larkin and Woolford (27). Nytran membrane (Schleicher and Schuell, Keene, N.H.) was used for both RNA blots and DNA blots. Poly(A)⁺ RNA was isolated from *S. cerevisiae* as described by Couto et al. (9). Subclones designed to be used in both *E. coli* and *S. cerevisiae* were cloned into the pUN series of vectors (53).

The bacteriophage λ and cosmid libraries of overlapping yeast genomic fragments were probed as described by Bender and Pringle (5).

DNA sequence determination and analysis. To sequence *DRS2* and *TEF3* by the dideoxy chain termination method (47), restriction fragments from plasmids pDRS2A and pDRS2C were subcloned into Bluescript pKS⁺ and pSK⁺ (Stratagene, La Jolla, Calif.). Synthetic oligonucleotides synthesized by Operon Technologies (Alameda, Calif.) were used as primers to complete sequencing when necessary. Sequence analysis and data base searches for sequence similarities were performed by using computer programs in the Genetics Computer Group's Sequence Analysis Software Package developed at the University of Wisconsin Biotechnology Center (12). We accessed this software through the courtesy of the Pittsburgh Supercomputing Center.

Construction of the *drs2::TRP1* null allele. A deletion-disruption allele of *DRS2* was constructed by cloning the 3.6-kb *Bam*HI fragment containing *DRS2* into pKS⁺ and then replacing the 1.4-kb *Nco*I-*Bgl*II fragment in the *DRS2* coding region with a 0.8-kb fragment containing the yeast *TRP1* gene. The *Bam*HI fragment bearing the *drs2::TRP1* disruption allele was used to transform homozygous diploid strain JWY749 to tryptophan prototrophy.

Pulse-chase analysis of rRNA. Labeling, extraction, and electrophoresis of rRNAs were performed exactly as described by Moritz et al. (34).

Nucleotide sequence accession numbers. The GenBank accession number for *DRS2* is L01795. The GenBank accession number for *TEF3* is L01879.

RESULTS

Identification of seven unique complementation groups deficient for ribosomal subunits. We initiated a screen of cold-sensitive mutants of *S. cerevisiae* to identify genes important for ribosome assembly and function. The rationale for screening cold-sensitive mutant strains is based on the thermodynamic argument that an assembly process, such as ribosome assembly and function, would be unfavorable at low temperatures because of the hydrophobic interactions driving protein-protein interactions (6). A bank of 194 conditional cold-sensitive yeast strains was generated by ethyl methanesulfonate mutagenesis and screened by sucrose gradient centrifugation for changes in relative amounts of free 40S and 60S ribosomal subunits or altered profiles of 80S monoribosomes and polyribosomes. Several reconstruction experiments were first conducted to test the feasibility of detecting changes in the ratio of ribosomal subunits when more than one mutant extract was loaded on each gradient. Mixing experiments were conducted with the yeast *rrp1* strain, a temperature-sensitive mutant that is blocked in processing of 27S to 25S rRNA and thus is deficient in 60S ribosomal subunits (1). Extracts were prepared from the *rrp1-1* mutant strain grown at the permissive temperature of 23°C and from the same strain after a 30-min shift to the nonpermissive temperature of 37°C. The deficit of 60S ribosomal subunits could still be detected when the extract prepared from cells at the permissive temperature and the extract made from cells shifted to the restrictive temperature were mixed at a 6-to-1 ratio and loaded onto a sucrose gradient (data not shown). On the basis of this result, cell extracts were prepared from the bank of cold-sensitive mutant strains and screened in groups of four to allow recovery of mutants with defects more subtle than that of the

FIG. 1. Cold-sensitive mutants carrying *drs1* to *drs7* have diminished 40S or 60S ribosomal subunits or altered polyribosome profiles at 13°C. Mutant cells grown at 30°C to 4.0×10^7 cells per ml were shifted to 13°C for 2 h. Cell lysates containing ribosomal subunits and polyribosomes were separated by centrifugation on 7 to 47% sucrose velocity gradients. Peaks representing free 40S and 60S subunits and 80S monoribosomes are labeled. Fractions containing halfmer polyribosomes are indicated by vertical arrows.

rrp1-1 mutant strain. Four cultures grown at the permissive temperature of 30°C to equal cell concentrations were pooled and shifted to the nonpermissive temperature of 13°C for 2 h. Extracts were prepared, loaded onto 7 to 47% sucrose gradients, subjected to centrifugation, and fractionated. Strains from any pool of extracts that showed a change in ribosomal subunit ratios or altered monoribosome or polyribosome levels were then assayed individually.

A total of 18 mutant strains with altered ratios of ribosomal subunits or striking changes in monoribosomes or polyribosomes were identified in this manner. To determine whether the mutant phenotype is due to a mutation in a single nuclear gene and whether it is dominant or recessive, the mutant strains were mated to wild-type strain DBY1034 and sporulated, and tetrads were dissected. Spores were scored for cold sensitivity, altered ribosomal subunit ratios or polyribosome profiles, and altered sensitivity to a variety of drugs that affect protein synthesis. Seven strains were identified in which the ribosomal subunit deficit segregated 2+:2– and cosegregated either with the cold-sensitive phenotype or with altered sensitivity to translation inhibitors. To define complementation groups, cold-sensitive or drug-sensitive haploid spores from the outcross that had ribosomal subunit defects were then crossed to each other in pairwise combinations. The cold-sensitive and drug-sensitive phenotypes of the resulting diploids were then tested to determine whether the mutations resided in the same or different genes. Seven different complementation groups of recessive mutations were identified in this manner and were designated *drs1* to *drs7* (deficient for ribosomal subunits). The ribosomal subunit and polyribosome profiles for the wild type and the *drs1* to *drs7* mutants grown at the permissive temperature of 30°C and shifted to the nonpermissive tem-

perature of 13°C for 2 h are shown in Fig. 1. Because the 60S ribosomal subunit contains approximately twice as much rRNA as the 40S subunit, the A_{260} ratio between peaks of 40S and 60S ribosomal subunits is 1:2 in wild-type yeast cells. A deficit of ribosomal subunits in mutant strains is reflected by altered 40S/60S subunit ratios (Fig. 1).

The cold sensitivity and the subunit deficit each segregated 2+:2– and cosegregated in five mutant strains, the *drs1* to *drs4* mutants and the *drs6* mutant, indicating that each mutation conferring a cold-sensitive phenotype resides in a single nuclear gene and is the cause of the subunit deficiency. The *drs1* mutant has a deficit of 60S ribosomal subunits, has reduced amounts of monoribosomes and polyribosomes, and contains halfmer polyribosomes (Fig. 1B). The *drs2* mutant has a deficit of 40S ribosomal subunits and a reduced 80S monoribosome peak (Fig. 1C). The cold-sensitive spores bearing the *drs2* mutation are also sensitive to 10 mM hygromycin B, an aminoglycoside that acts on the 40S ribosomal subunit to inhibit polypeptide chain elongation (16). The *drs3* and *drs4* mutants both have a deficit of 40S ribosomal subunits (Fig. 1D and E). The *drs3* mutation confers both a cold-sensitive and a heat-sensitive phenotype as well as sensitivity to 10 mM hygromycin B. The *drs6* mutant strain has a deficit of 40S subunits and is sensitive to neomycin (Fig. 1G).

Although the subunit deficits of the *drs5* and *drs7* mutants did not cosegregate with cold sensitivity, they did cosegregate with altered drug sensitivities. The *drs5* mutant has a deficit of 40S ribosomal subunits and decreased monoribosomes and polyribosomes (Fig. 1F). Cells bearing this mutation are hypersensitive to the glutarimide antibiotic cycloheximide at concentrations as low as 100 μ M, to which most wild-type strains are resistant. The *drs7* mutant has a 60S

TABLE 1. Phenotypes cosegregating with ribosomal subunit deficiencies

Mutation	Subunit deficit	Phenotype				
		Cs ^{-a}	Ts ^{-b}	Hyg ^{sc}	Neo ^{sd}	Cyh ^{sse}
<i>drs1</i>	60S	+	-	-	-	-
<i>drs2</i>	40S	+	-	+	-	-
<i>drs3</i>	40S	+	+	+	-	-
<i>drs4</i>	40S	+	-	-	-	-
<i>drs5</i>	40S	-	-	-	-	+
<i>drs6</i>	40S	+	-	-	+	-
<i>drs7</i>	60S	-	-	-	+	+

^a +, unable to grow at 13°C.^b +, unable to grow at 37°C.^c +, sensitive to 10 mM hygromycin B.^d +, sensitive to 10 mM neomycin.^e +, hypersensitive to 100 μM cycloheximide.

ribosomal subunit deficit (Fig. 1H), and is sensitive to neomycin and hypersensitive to cycloheximide. A summary of the subunit deficiencies and the drug sensitivity phenotypes of the *drs* mutants is shown in Table 1.

DRS2 encodes a putative membrane-associated ATPase. Previously we cloned *DRS1* and demonstrated that it encodes a putative ATP-dependent RNA helicase required for rRNA processing (41). To further characterize the mutations causing the ribosomal subunit and polyribosome defects in the *drs2* mutant strain, the wild-type *DRS2* gene was cloned by transformation with yeast genomic libraries cloned in the low-copy-number centromere plasmid YCp50 (42), with screening for complementation of the cold-sensitive phenotype. Ura⁺ transformants were grown at the permissive temperature on YEPD plates, replica plated to YEPD, and assayed for growth at 13°C. Plasmids that allowed the *drs2* mutant strain to grow at 13°C were identified by shuttling the plasmids into *E. coli* and back into the *drs2* mutant.

Two plasmids containing identical inserts of genomic DNA, pDRS2A and pDRS2B, that allowed growth of the *drs2* mutant at 13°C were recovered. To determine whether DNA derived from the pDRS2A or pDRS2B plasmid actually contained the *DRS2* gene, a 3.6-kb *Bam*HI fragment was cloned into plasmid YIp5, containing *URA3*, and integrated into the genome of a *drs2* strain to create yeast strain JWY2067. Integration of one copy of this DNA at the homologous locus was confirmed by genomic Southern blotting (data not shown). JWY2067 was tested and found to remain cold sensitive. JWY2067 was crossed to wild-type yeast strain DBY1034 in order to analyze the segregation pattern of both the cold-sensitive mutation and the linked *URA3* marker. Cosegregation of Ura⁺ and cold sensitivity in the 24 tetrads analyzed indicated that the DNA integrated at or near the *drs2* locus and therefore contains the *DRS2* gene.

Initial subcloning indicated that the 5.8-kb *Eco*RI fragment of pDRS2A was able to complement the cold-sensitive phenotype of the *drs2* mutant (Fig. 2A). The 3.6-kb *Bam*HI fragment and either of the *Xho*I-*Eco*RI fragments subcloned from the 5.8-kb *Eco*RI fragment failed to complement the cold-sensitive phenotype of the *drs2* mutant, indicating that the *DRS2* open reading frame (ORF) spans one or both of the *Bam*HI sites and the *Xho*I site. The 2.2-kb *Eco*RI-*Bgl*II fragment hybridized to both a 1.1- and a 4.3-kb poly(A)⁺ mRNA (Fig. 2B), while the 0.4-kb *Bam*HI-*Xho*I fragment hybridized to only the 4.3-kb mRNA (data not shown), indicating that the 4.3-kb mRNA is transcribed from *DRS2*. Sequencing of the DNA from the 5.8-kb *Eco*RI fragment of pDRS2A revealed a 4,065-bp ORF that spans both *Bam*HI sites and the *Xho*I site. Subsequently, a 2.2-kb *Eco*RI-*Bgl*II fragment of pDRS2A, containing the first 528 codons of the ORF, was shown to complement the cold sensitivity of the *drs2* mutant.

The complete nucleotide sequence of the *DRS2* gene plus flanking DNA is shown in Fig. 3. *DRS2* contains a 4,065-

FIG. 2. Restriction map of yeast genomic DNA containing the *DRS2* gene and Northern blot analysis of the *DRS2* mRNA. (A) Yeast genomic DNA insert from the original complementing plasmid, pDRS2A. The abilities of various subclones to complement the cold sensitivity of the *drs2* mutant were tested by transformation into the *drs2* mutant strain. The ORF determined by sequencing of *DRS2* is indicated by the horizontal arrow. (B) The 2.2-kb *Eco*RI-*Bgl*II fragment was radioactively labeled with ³²P and hybridized to a blot of poly(A)⁺ RNA. mRNAs of 4.3 and 1.1 kb were detected. Further analysis indicated that the 4.3-kb RNA is transcribed from *DRS2*.

nucleotide ORF encoding a 1,355-amino-acid protein with a predicted molecular weight of 153,843. The size of the *DRS2* ORF is consistent with the length of the poly(A)⁺ mRNA that hybridizes to *DRS2* DNA (Fig. 2B). Comparison of the amino acid sequence inferred from *DRS2* with other known protein sequences indicated that *DRS2* encodes a putative membrane-spanning protein involved in transporting cations at the expense of ATP hydrolysis. The sequence of amino acids 894 to 901 is similar to the phosphate-binding loop (P-loop) or "A" consensus site found in numerous proteins that bind ATP or GTP, including ATP-binding proteins involved in active transport, several kinases, and many elongation factors (24, 48). There is additional conservation of residues in the predicted amino acid sequence of Drs2p with those in the 10 domains found in all P-type ATPases (43). An alignment of 5 of the 10 domains common to P-type ATPases (domains b, f, g, h, and i) found in the predicted amino acid sequence of Drs2p with those from several calcium-transporting ATPases from *S. cerevisiae* is shown in Fig. 4A. There is additional, although weak, conservation of residues in domains a, c, d, e, and j (data not shown). The sequence DKTGTLT, from amino acid residues 560 to 566, is present in all classes of P-type ATPases identified to date (43). P-type ATPases transport a variety of cations. The conserved aspartate residue found in the DKTGTLT consensus sequence forms an aspartyl phosphate intermediate in the course of ATP hydrolysis (62). Secondary structural analysis predicts the presence of 7 to 10 transmembrane helices within Drs2p (26). The hydrophobicity profile of Drs2p is shown in Fig. 4B. Also diagrammed are the regions of Drs2p containing the seven most prominent predicted membrane-spanning or associated segments.

***DRS2* maps to chromosome I.** The *DRS2* gene was localized to the left arm of chromosome I, centromere distal to *MAK16*, by hybridizing *DRS2* DNA first to yeast chromosomes separated by contour-clamped homogeneous field electrophoresis and then to a set of bacteriophage λ and cosmid clones that contain overlapping fragments from the yeast genome (5). The nucleotide sequence from pDRS2A, 3' to the *DRS2* coding region, is 100% identical to the sequence of the 3' end of *MAK16*, placing the *DRS2* gene exactly centromere distal to *MAK16* on chromosome I.

***DRS2* is required for mitotic growth at 23°C or below.** To test whether the *DRS2* gene is required for mitotic growth, a null allele of *DRS2* was created. The 1.4-kb *Bgl*II-*Nco*I fragment of *DRS2*, coding for amino acids 528 to 1,000, which includes the ATP-binding and ATPase domains, was replaced with the yeast *TRP1* gene. One of the wild-type alleles of *DRS2* from diploid strain JWY749 was replaced with *drs2::TRP1* by transforming JWY749 to tryptophan prototrophy with the cloned *drs2::TRP1* fragment. The expected transplacement was verified by genomic Southern analysis (data not shown). Upon sporulation of the transformed diploid, 45 four-spore tetrads that showed 2+:2- segregation for Trp were recovered at 30°C. Therefore, the *DRS2* gene is dispensable for mitotic growth at 30°C. When tetrads were dissected and the spores were allowed to germinate at 23°C, two viable Trp⁻ spores and no viable Trp⁺ spores were recovered for each tetrad. The viable Trp⁺ spores recovered at 30°C were unable to grow at 23 or 13°C. These results indicate that the product of the *DRS2* gene is required for mitotic growth at temperatures of 23°C and below.

Processing of the 20S rRNA precursor to 18S rRNA is slowed in *drs2* cells at the nonpermissive temperature. To assay the synthesis and stability of rRNAs in the *drs2*

mutant, pulse-chase analysis was performed. Cells bearing the *drs2* mutation were grown to a density of 4.0×10^7 cells per ml; one half of the culture was maintained at 30°C, while the other half was shifted to 13°C for 2 h. Cells were pulse-labeled with L-[methyl-³H]methionine for 5 min and then chased with excess cold methionine (1 mg/ml) for 15, 30, and 120 min. Total RNA was extracted at each time point and subjected to electrophoresis on a 1.0% formaldehyde gel to resolve 18S and 25S rRNAs as well as any intermediates that might accumulate. At 30°C, processing of both the 18S and 25S rRNAs in the *drs2* mutant is comparable to that in the wild-type strain (Fig. 5A). In both the *drs2* mutant and the wild-type *DRS2* strain at 13°C, mature 25S rRNA accumulates by the 30-min chase (Fig. 5B). In contrast to wild-type cells, processing of the 20S rRNA to 18S rRNA is considerably slowed in the *drs2* mutant at 13°C. The 20S rRNA precursor was detectable in the 15- and 30-min chase samples of *drs2* but not *DRS2* cells (Fig. 5B). The accumulation of the 20S rRNA precursor suggests that a late step of assembly of 40S subunits is slowed in the *drs2* mutant strain at 13°C and may be the cause of the steady-state deficit of 40S ribosomal subunits.

A yeast homolog of translation elongation factor EF-1 γ suppresses the mutation in *drs2*. During cloning of *drs2*, two other plasmids able to complement the cold sensitivity of *drs2* were recovered and shown to be different from each other and from pDRS2A and pDRS2B by cross-hybridization, Southern blotting, and restriction mapping (data not shown). One of these plasmids, pDRS2C, promotes weak growth of the *drs2* mutant at the nonpermissive temperature of 13°C. The amino acid sequence inferred from the ORF contained within pDRS2C is very similar to that of translation elongation factor EF-1 γ from *Artemia salina* (31); hence, the gene was named *TEF3*. A restriction map of the portion of pDRS2C containing the *TEF3* gene, the nucleotide sequence of *TEF3*, and the inferred amino acid sequence of Tef3p are shown in Fig. 6. The amino acid sequence of the complete ORF encoded within the 3.0-kb *Pst*I-*Sal*I genomic fragment of pDRS2C is 35.6% identical to that of *A. salina* translation elongation factor EF-1 γ . When conservative substitutions are included, the similarity of the yeast Tef3 protein to *A. salina* EF-1 γ is 57%. The *Xenopus laevis* homolog of EF-1 γ has recently been cloned by using antibodies generated against the EF-1 α , β , γ complex isolated from oocytes (8). The identity between the *Xenopus* and *Artemia* proteins is 60%, which increases to 75% similarity with conservative substitutions. A human cDNA encoding EF-1 γ has also been identified (25). A comparison of the predicted amino acid sequences of the human (25), *Xenopus* (8), and *Artemia* (31) EF-1 γ proteins with that of the *TEF3* gene product is shown in Fig. 7. From the degree of similarity between the *TEF3* gene product and EF-1 γ from other species, Tef3p appears to be a homolog of EF-1 γ in *S. cerevisiae*. We note that the carboxyl-terminal portion of EF-1 γ is more highly conserved among all four species than is the amino terminus.

Although the low-copy-number YCp50 plasmid containing the entire *TEF3* gene weakly suppresses the cold-sensitive phenotype of the *drs2* mutant, a high-copy-number plasmid containing the 2.4-kb *Sal*I fragment of *TEF3* (YEp351-*Sal*I), encoding the carboxy-terminal 276 amino acids of Tef3p, allows wild-type growth of the *drs2* mutant at 13°C (Fig. 6). To investigate effects on the translational apparatus, extracts prepared from the *drs2* mutant harboring YEp351-*Sal*I or the vector YEp351 were analyzed by sucrose gradient centrifugation (Fig. 8). Although the cold-sensitive phenotype of the

q.

Pmr1p	172	**	GDLVHFRIGDRIPADIRI	Pmr1p	346	*	MAKRKAIVRRLLPSVETLGSVNVICSDKTGTLT	Pmr1p	500	***	VKGAF	
Pmr2p	149	** *	GDICLVKVGDTIPADLRL	Pmr2p	344	* *	MVSRNVI VRKLDSLEALGAVNDICS DKTGTLT	Pmr2p	560		GKGAF	
Pmalp	199		GDILQLEDGT VIPTDGRI	Pmalp	353	*****	LAKKQAIVQKLSAIESLAGVEILC SDKTGTLT	Pmalp	473		VKGAP	
Drs2p	305		GDIIRVKSEEP IADTII	Drs2p	535		KDTP TVVRTSSLVEELGQIEYIFSDKTGTLT	Drs2p	720		CKGA.	
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Pmr1p	596	*	GGVHIIMI TGDSSENTAV	Pmr1p	656	*	ATPEHKLNIVRAL.RKRGDVVAMTGDGVNDAPSLKKADTGIAV					
Pmr2p	665	***	AGINVHMLTGDFVGTA K	Pmr2p	733	*	CSPQTKVRMIEAL.HRRKFCTMTGDGVNDSPSLKMANVGIAM					
Pmalp	550	*	LGLRVKMLTGDAVGIAK	Pmalp	610	* *	VFPNHKYRVVEIL.QNRGYLVAMTGDGVNDAPSLKKADTGIAV					
Drs2p	827		AGIKIWVL TGDROETA V	Drs2p	929	* * *	VSPLQKALVVKMVKRKSSSLLLAIASGANDVSMLOAAHVGVGI					

Drs2D

FIG. 4. Drs2p contains domains common to P-type ATPases and seven prominent membrane-spanning or associated domains. (A) Alignment of 5 of the 10 domains common to P-type ATPases found within Drs2p with those from *PMR1* (43), *PMR2* (43), and *PM41* (50). (B) Hydrophilicity profile (26) of the entire *DRS2* coding region. Also shown are the profiles of the seven most prominent predicted membrane-spanning domains (shaded).

Genomic Southern analysis indicated that *TEF3* DNA hybridized to at least two genomic fragments, indicating a possible duplication of this gene (data not shown). Many of the genes coding for components of the translational apparatus are duplicated in *S. cerevisiae*, including *TEF1* and *TEF2*, the genes encoding elongation factor 1 α (EF-1 α) (36, 49), and *EFT1* and *EFT2*, the genes encoding EF-2 (40). The *TEF3* gene was localized to the left arm of chromosome XVI, centromere distal to the *RAD1* gene, by hybridization of *TEF3* DNA to yeast chromosomes separated by gel electrophoresis and to the library of overlapping yeast genomic DNA fragments. *TEF3* DNA weakly cross-hybridized to a DNA that maps to chromosome XI. The 3' end of

an additional ORF that is similar in sequence to *TEF3* has been identified on chromosome XI adjacent to a gene encoding subunit C of yeast vacuolar ATPase (4). Our subsequent analysis has shown that this ORF represents a second unique gene encoding EF-1 γ , which we designate *TEF4* (23a).

DISCUSSION

To identify mutants defective in ribosome biogenesis or function, a bank of cold-sensitive yeast mutants was screened by sucrose gradient centrifugation for strains with altered ratios of 40S to 60S ribosomal subunits or altered polyribosome profiles. Seven complementation groups, *drs1* to *drs7*, were identified, each of which is defined by a recessive mutation in a single nuclear gene. Each mutation causes a cold-sensitive phenotype or altered drug sensitivity that cosegregates with the ribosomal subunit deficit. The *DRS2* gene was cloned by transformation and complementation of the cold-sensitive phenotype of the *drs2* mutant. In

FIG. 5. The 20S precursor to mature 18S rRNA is processed slowly in the *drs2* mutant. RNAs were extracted from the wild-type *DRS2* strain and the *drs2* mutant strain, which were labeled with L-[methyl-³H]methionine for 5 min and chased for 15, 30, and 120 min with excess cold methionine at the permissive temperature of 30°C and after a 2-h shift to the nonpermissive temperature of 13°C. RNA was resolved by denaturing gel electrophoresis, blotted to Nylon membranes, sprayed with En³Hance, and exposed to X-ray film at -70°C. The positions of the 27S precursor of 25S rRNA, the 20S precursor of 18S rRNA, and mature 25S and 18S rRNAs are indicated.

addition, *TEF3*, encoding translation elongation factor EF-1 γ , was identified as a gene dosage suppressor of *drs2*.

A significant fraction of cold-sensitive mutants of *E. coli* and *S. typhimurium* have defects in ribosome assembly.

Many of these mutants also have altered sensitivities to translational inhibitors (18, 57). Likewise, approximately 10% of the cold-sensitive yeast strains screened in this study have altered ratios of 40S to 60S ribosomal subunits or dramatic reductions in 80S monoribosomes or polyribosomes. A majority of these mutants also have altered drug sensitivities. The enrichment among cold-sensitive yeast strains for those containing altered ratios of ribosomal subunits, plus the ability to screen more than one mutant extract per sucrose gradient, makes this a feasible approach to identify genes involved in ribosome assembly or function.

In our screen for *drs* mutants, we expected to recover mutations in genes encoding non-r-proteins involved in ribosomal assembly or rRNA processing, genes encoding proteins required for certain aspects of protein synthesis, and r-protein genes. We previously reported that the product of the *DRS1* gene is a putative ATP-dependent RNA helicase involved in rRNA processing (41). Cloning and sequencing of *DRS2* suggests that Drs2p is a membrane-associated ATPase. Although the role that Drs2p plays in ribosome assembly or function is unknown, the *drs2* mutation is suppressed by extra copies of *TEF3*, the gene encoding an *S. cerevisiae* homolog of translation elongation factor EF-1 γ . The successful isolation of genes involved in rRNA processing and translational elongation fulfills the expectations of the screen.

The apparent deficit of free 40S ribosomal subunits in the *drs2* mutant may be due to impaired processing of the 20S rRNA precursor to 18S rRNA, as shown by pulse-chase analysis. Processing of 20S pre-rRNA to mature 18S rRNA occurs during the late stages of 40S ribosomal subunit assembly in the cytoplasm (61). Perhaps the transport of 40S ribosomal subunits from the nucleus to the cytoplasm is slowed in the *drs2* mutant strain. Alternatively, the deficit of 40S subunits in the *drs2* mutant may result from an inability to detect certain populations of ribosomes in the cell lysate, for example, those inappropriately associated with membrane fractions. Our method for centrifugation of cell lysates prior to loading extracts on sucrose gradients clears the extract of membranous components and most nuclei (unpublished observation). Using this method of preparation, we are unlikely to detect 40S ribosomal subunits trapped in the nucleus or 40S subunits that are bound to membranes.

We do not understand why the 2.2-kb *EcoRI-BglII* fragment of *DRS2*, containing the coding region for just the first 528 amino acids of Drs2p, complements the cold-sensitive phenotype of the *drs2* mutant. Perhaps the cold-sensitive mutation in *drs2* could lie within the first 528 codons, such that expression of the truncated wild-type portion of Drs2p would complement the *drs2* mutation by interacting productively with the wild-type portion of Drs2p expressed in the *drs2* strain. The 2.2-kb *EcoRI-BglII* fragment of *DRS2* does not relieve the cold sensitivity of a haploid strain containing the *drs2::TRP1* null allele, nor does it rescue the inability of a *drs2::TRP1* spore to germinate at 23°C when introduced into the heterozygous *DRS2*-disrupted diploid. These results support the data that the 2.2-kb *EcoRI-BglII* fragment does not contain the entire *DRS2* ORF (Fig. 2A).

The *TEF3* gene, encoding a homolog of EF-1 γ , suppresses the cold-sensitive phenotype of the *drs2* mutant. *A. salina* translation elongation factor EF-1 consists of three subunits, EF-1 α , -1 β , and -1 γ . EF-1 is required for binding of aminoacyl-tRNA to the ribosome, a step requiring GTP hydrolysis. The EF-1 α subunit binds aminoacyl-tRNA in a GTP-dependent manner. The EF-1 β and -1 γ subunits are tightly associated with each other and facilitate exchange of EF-

FIG. 6. Restriction map and nucleotide sequence of *TEF3* and inferred amino acid sequence of Tef3p, an extragenic suppressor of the *drs2* mutation. (A) Restriction map of the *TEF3* gene. Yeast genomic DNA is indicated by the heavy line. The dashed line indicates YCp50 DNA. (B) Nucleotide sequence of *TEF3* and inferred amino acid sequence of Tef3p. Nucleotides are numbered above the sequence. Amino acids are numbered to the left of each line.

1 α · GDP to EF-1 α · GTP. EF-1 β alone has nucleotide exchange activity, although in *A. salina*, this activity is stimulated by EF-1 γ (22). The *TEF3* gene is identical to the *CAM1* gene, recently cloned by Kambouris et al. (23). Tef3p (Cam1p) was found to be tightly associated with a polypeptide of 34 kDa, which is identical to the published molecular mass for *S. cerevisiae* elongation factor EF-1 β (11). Therefore, we speculate that the EF-1 β , γ complex functions as a nucleotide exchange factor in *S. cerevisiae*.

The product of the *CAM1* (*TEF3*) gene was identified by

Creutz et al. during a screen for yeast proteins that bind microsomal membranes in a calcium-dependent manner (10). Additionally, Cam1p was found to be highly insoluble as an isolated protein. Purified EF-1 γ from *A. salina* has also been shown to have properties of a membrane-associated protein; it is insoluble in aqueous buffer and tends to aggregate in the absence of detergent (22). Additionally, in vitro association of EF-1 γ with tubulin and lipids has led to speculation that EF-1 γ facilitates anchorage of the EF-1 β , γ complex to membrane structures and to the cytoskeleton (22). A mech-

1	Xenopus	MAQDTLYT Y POKWAKPL IAAQSGPFI KVASBAPEQ POKWRTPEP LKQPLGKVP
	Human	MAQDTLYT Y POKWAKPL IAAQSGPFI KVASBAPEQ POKWRTPEP LKQPLGKVP
	Artemia	MAQDTLYT Y POKWAKPL IAAQSGPFI KVASBAPEQ POKWRTPEP LKQPLGKVP
	Yeast	MAQDTLYT Y POKWAKPL IAAQSGPFI KVASBAPEQ POKWRTPEP LKQPLGKVP
51	Xenopus	AFBKGKQFCL FBSAIAHYV ... GNDILR ... OTTP LQAQVQVYV SFBDHIVVP
	Human	AFBKGKQFCL FBSAIAHYV ... GNDILR ... OTTP LQAQVQVYV SFBDHIVVP
	Artemia	AFBKGKQFCL FBSAIAHYV ... GNDILR ... OTTP LQAQVQVYV SFBDHIVVP
	Yeast	AFBKGKQFCL FBSAIAHYV ... GNDILR ... OTTP LQAQVQVYV SFBDHIVVP
111	Xenopus	ASAMVFFPLQ IMHAKQATE NAKSEPRVIL QLDVATLPR FTVGBRITVL ADIVVCTLL
	Human	ASAMVFFPLQ IMHAKQATE NAKSEPRVIL QLDVATLPR FTVGBRITVL ADIVVCTLL
	Artemia	ASAMVFFPLQ IMHAKQATE NAKSEPRVIL QLDVATLPR FTVGBRITVL ADIVVCTLL
	Yeast	ASAMVFFPLQ IMHAKQATE NAKSEPRVIL QLDVATLPR FTVGBRITVL ADIVVCTLL
171	Xenopus	MLYKQVLEPS PROPFGNIVR HPTVCVNOPE FRAVLGEVIL CDRMAQFAR KPAHQPKKE
	Human	MLYKQVLEPS PROPFGNIVR HPTVCVNOPE FRAVLGEVIL CDRMAQFAR KPAHQPKKE
	Artemia	MLYKQVLEPS PROPFGNIVR HPTVCVNOPE FRAVLGEVIL CDRMAQFAR KPAHQPKKE
	Yeast	MLYKQVLEPS PROPFGNIVR HPTVCVNOPE FRAVLGEVIL CDRMAQFAR KPAHQPKKE
218	Xenopus	TPKKEKPAKE PK...KEKEK KKAATPAPA PEEDESEK ALAAEPKRO PPAHLKPSF
	Human	TPKKEKPAKE PK...KEKEK KKAATPAPA PEEDESEK ALAAEPKRO PPAHLKPSF
	Artemia	TPKKEKPAKE PK...KEKEK KKAATPAPA PEEDESEK ALAAEPKRO PPAHLKPSF
	Yeast	TPKKEKPAKE PK...KEKEK KKAATPAPA PEEDESEK ALAAEPKRO PPAHLKPSF
258	Xenopus	INDSEFKYS NEDTLVALP YVWDFKQKQ MSIMVAYEP PEELOQFMS CHLTHQFOR
	Human	INDSEFKYS NEDTLVALP YVWDFKQKQ MSIMVAYEP PEELOQFMS CHLTHQFOR
	Artemia	INDSEFKYS NEDTLVALP YVWDFKQKQ MSIMVAYEP PEELOQFMS CHLTHQFOR
	Yeast	INDSEFKYS NEDTLVALP YVWDFKQKQ MSIMVAYEP PEELOQFMS CHLTHQFOR
328	Xenopus	LKLRKQYGA SVLLQTNIN SRSIGVWYR QDEL...APFL SEDQVDESS YNRKLDGSS
	Human	LKLRKQYGA SVLLQTNIN SRSIGVWYR QDEL...APFL SEDQVDESS YNRKLDGSS
	Artemia	LKLRKQYGA SVLLQTNIN SRSIGVWYR QDEL...APFL SEDQVDESS YNRKLDGSS
	Yeast	LKLRKQYGA SVLLQTNIN SRSIGVWYR QDEL...APFL SEDQVDESS YNRKLDGSS
383	Xenopus	SECKTLVKEV PAMEGPKV G...KPHQOK IFK
	Human	SECKTLVKEV PAMEGPKV G...KPHQOK IFK
	Artemia	SECKTLVKEV PAMEGPKV G...KPHQOK IFK
	Yeast	SECKTLVKEV PAMEGPKV G...KPHQOK IFK

FIG. 7. Alignment of the predicted amino acid sequences of yeast Tef3p and *Xenopus*, human, and *Artemia* translation elongation factor EF-1 γ . Shaded areas indicate the high degree of amino acid identity and conservative substitutions among these four proteins. The numbering above each row of sequences corresponds to the amino acid sequence of Tef3p.

anism to direct components of the translational apparatus toward membranous components of the cell has been postulated since the discovery that many secretory proteins are cotranslationally translocated across the endoplasmic reticulum (39). It is interesting to speculate that EF-1 γ encoded by *TEF3* is somehow involved in the process of membrane-associated translational elongation.

The finding that the form of EF-1 γ encoded by the *TEF3* gene may be a membrane-associated protein is also intriguing with respect to the ability of a low-copy-number plasmid bearing the full-length *TEF3* gene or a high-copy-number plasmid bearing a portion of the *TEF3* gene to suppress a defect in a putative membrane-associated cation-transporting ATPase Drs2p. Perhaps Drs2p is responsible for changes in the local concentration of calcium, which could facilitate binding of EF-1 γ to membranous components of the cell, thereby sequestering the translational apparatus at the site at

FIG. 8. Extra copies of the *SalI* fragment of pDRS2C suppress the cold sensitivity but not the 40S ribosomal subunit deficit of the *drs2* mutant. Polyribosome profiles of the *drs2* strain transformed with YEp351 vector or YEp351 containing the 2.4-kb *SalI* fragment of pDRS2C (YEp-SalI) are shown. Plasmid-containing *drs2* cells were grown to a density of 4.0×10^7 cells per ml at 30°C and then shifted to the nonpermissive temperature of 13°C for 2 h. The free 40S ribosomal subunits, free 60S ribosomal subunits, and 80S monoribosome peaks are labeled.

which it is needed. Determination of the subcellular location of Drs2p is currently in progress to aid in further understanding the role it may be playing in these processes.

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