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Qsr1p, a 60S Ribosomal Subunit Protein, Is Required for Joining of 40S and 60S Subunits

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***QSR1* is a recently discovered, essential *Saccharomyces cerevisiae* gene, which encodes a 60S ribosomal subunit protein. Thirty-one unique temperature-sensitive alleles of *QSR1* were generated by regional codon randomization within a conserved 20-amino-acid sequence of the *QSR1*-encoded protein. The temperature-sensitive mutants arrest as viable, large, unbudded cells 24 to 48 h after a shift to 37°C. Polysome and ribosomal subunit analysis by velocity gradient centrifugation of lysates from temperature-sensitive *qsr1* mutants and from cells in which Qsr1p was depleted by down regulation of an inducible promoter revealed the presence of half-mer polysomes and a large pool of free 60S subunits that lack Qsr1p. In vitro subunit-joining assays and analysis of a mutant conditional for the synthesis of Qsr1p demonstrate that 60S subunits devoid of Qsr1p are unable to join with 40S subunits whereas 60S subunits that contain either wild-type or mutant forms of the protein are capable of subunit joining. The defective 60S subunits result from a reduced association of mutant Qsr1p with 60S subunits. These results indicate that Qsr1p is required for ribosomal subunit joining.**

The eukaryotic ribosome is a complex organelle that consists of 4 RNA molecules and 80 proteins (50, 51). The goal of determining the primary amino acid sequence of all the proteins of the mammalian 40S and 60S ribosomal subunits is near completion. A recent three-dimensional reconstruction of the 80S eukaryotic ribosome at a resolution of 38 Å (47), like the 25-Å description of the *Escherichia coli* 70S ribosome (17), is an important step in our understanding of these complex structures but is not informative about the location of the ribosomal proteins, rRNA, tRNA, mRNA, and active site. A greater understanding of the function of individual subunit proteins, including the complex interplay of elongation and initiation factors that regulates protein synthesis, is needed to elucidate the biochemistry of protein synthesis.

Many ribosomal proteins are highly conserved, with sequence homology extending between mammals and bacteria (50). Although many similarities exist between the bacterial and eukaryotic ribosomes, the *E. coli* ribosome is smaller and has fewer protein subunits than the eukaryotic ribosome (47). The study of proteins unique to the eukaryotic ribosome may highlight the differences between eukaryotic and prokaryotic protein synthesis and shed light on eukaryotic ribosomal structure and function. *Saccharomyces cerevisiae* has been useful in providing insight into protein synthesis and has potential use in studies to further unravel the mechanisms of ribosome function such as translational control (see reference 21 for a review). The conservation of yeast and mammalian ribosomal proteins is high, with an average identity of 60%; only 13 yeast counterparts to rat ribosomes have not yet been identified (50), and there are examples of functional substitution of essential yeast ribosomal protein genes with mammalian homologs (15).

The recently discovered 25.4-kDa yeast 60S ribosomal protein Qsr1p is highly conserved (13), and *QSR1* can be functionally substituted for by the corn and human homologs (8). cDNAs containing homologs of *QSR1* were isolated from various eukaryotes by differential or subtractive hybridization methods on the basis of the mRNA being down-regulated in differentiating or slower-growing cells and tissues (9, 10, 19, 29, 39), which is not surprising since genes for ribosomal proteins are highly expressed in growing cells (51).

QSR1 is an essential, single-copy gene in yeast and was discovered in a synthetic-lethal screen in which a single chromosomal copy of *QCR6*, the gene which encodes a nonessential subunit of the mitochondrial cytochrome bc_1 complex, can rescue an otherwise lethal point mutation in *qsr1-1* (46). *QCR6* cannot substitute for *QSR1*, and rescue of the *qsr1-1* mutation is independent of the function of Qcr6p in mitochondrial respiration. How a nucleus-encoded respiratory protein can cover the *qsr1-1* mutant is not presently understood.

There are few examples of temperature-sensitive alleles of eukaryotic ribosomal proteins. Temperature-sensitive mutants of the yeast ribosomal proteins L1 and L16 are defective in the assembly of 60S subunits (6, 32). A cold-sensitive mutant of the ribosomal protein gene *RPL46* suppresses a null allele of *pab1*, the gene encoding the poly(A) binding protein of yeast, which demonstrates an important link between the 60S subunit and the poly(A) binding protein requirement for translation initiation (41). To learn more about the protein encoded by *QSR1* and its role in 60S subunits, we isolated temperature-sensitive alleles of *QSR1* and characterized the mutant phenotypes. The mutants are defective in subunit joining. Depletion of *QSR1* and in vitro subunit-joining assays confirm that 60S subunits without Qsr1p cannot join with a 40S subunit.

MATERIALS AND METHODS

Plasmids and *S. cerevisiae* strains. Plasmids were transformed into *E. coli* DH5α, and DNA was sequenced with an Applied Biosystems no. 373 DNA sequencer. Plasmid pDEQ2 was constructed by ligating a 1.9-kb *Sca*I-*Bam*HI *QSR1* fragment that harbors 1.0 and 0.3 kb of 5′- and 3′-flanking sequence, respectively, into pRS315 (44) cut with *Pst*I and *Bam*HI such that the *Pst*I and *Sca*I sites are destroyed. The galactose-inducible *QSR1* construct pDEGQ2 was made as follows. A 1.2-kb *Cla*I-*Spe*I *QSR1* fragment was made blunt ended with T4 polymerase and cloned into *Hin*dIII-blunt-ended pRS316 (44) to generate pDEGQ1, such that the 5′ end of *QSR1* is on the *Xho*I side of the polylinker. A

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365-bp *Sau*3A-*Dde*I fragment harboring the upstream activating sequence from the GAL1-10 promoter was excised from pBM258, made blunt ended, and cloned into the *Xho*I site of pDEGQ1 to make pDEGQ2 (42). Plasmid pHFF22 has been described previously (46).

Yeast cells were grown in yeast extract-peptone-dextrose (YPD), synthetic complete (SC), or synthetic dropout (SD) medium in which relevant nutritional supplements were omitted (1). The yeast cells were transformed with lithium acetate, and tetrads were dissected after sporulation of diploids (1). Yeast strains were derived from W303 (*MAT***a**/*MAT*α *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100*). The derived strains include DEHQ1-1 (*MAT*α *qsr1Δ1::HIS3* +pHFF22 [*CEN URA3 QSR1*]), DEQ2 (*MAT*α, *qsr1Δ1::HIS3* +pDEQ2 [*CEN LEU2 QSR1*]), DEQ2-24 (*MAT*α *qsr1Δ1::HIS3* +pDEQ2 [*CEN LEU2 qsr1-24*]), DEH221+ (*MAT*α *qsr1Δ1::HIS3* +pDEGQ2 [*CEN URA3 GAL1-10*$_{UAS}$*:: QSR1*]), MMY3-3A (*MAT***a** *QSR1*), and MMY3-3B (*MAT***a** *qsr1-1*).

Construction of a *qsr1* regional codon randomized library. Long PCR in combination with inverse PCR was used to generate a plasmid library incorporating a 20-amino-acid codon randomized region (5, 11). A partially randomized 81-mer oligonucleotide (coding strand 5′-*GGT TTG AGA AGA GCC AGA TAC AAG TTC CCA GGT CAA CAA AAG ATT ATT TTG TCT AAG AAG* TGG GGT TTC ACC AAC TTG GAC) with codons for amino acids 151 to 170 randomized (italics) and codons for amino acids 171 to 177 unchanged, such that approximately 3,000 clones would contain all single-amino-acid substitutions, was synthesized (5). A second 19-mer wild-type oligonucleotide was made (noncoding strand, 5′-TTC AAC GAC AAC ATC CTT G) in a back-to-back configuration for inverse PCR. The 8-kb plasmid pDEQ2 was used as a template for long-inverse PCR (11), and a library was made by pooling 18,000 colonies and purifying the plasmid DNA.

Plasmid shuffling and isolation of temperature-sensitive *qsr1* alleles. The *LEU2* marked pDEQ2 codon randomized library was transformed into yeast strain DEHQ1-1 in which the *qsr1Δ1* chromosomal deletion is covered by a centromeric *URA3 QSR1* plasmid. Approximately 20,000 *Leu*$^+$ primary transformants were replica plated to synthetic media lacking leucine but supplemented with 5-fluoroorotic acid (1 g/liter) to select for loss of the *URA3* plasmid. Duplicate replica plates were incubated for 3 days at 23 and 37°C. Colonies that failed to grow at 37°C were restreaked, and total DNA from temperature-sensitive clones was used to transform *E. coli*. Purified plasmid clones were reshuffled to confer linkage of temperature sensitivity to the plasmid, and the *QSR1* gene was sequenced to identify mutations.

Growth curves, viability tests, and microscopy. For growth curves, log-phase cells grown at 23°C were inoculated into 40 ml of fresh SC medium at a density of 0.2 to 0.4 absorbance unit at 600 nm and incubated at 23 or 37°C. At the indicated times, 1-ml aliquots were removed in duplicate and the absorbance at 600 nm was plotted with respect to time. For viability tests, log-phase cells grown at 23°C were inoculated into fresh SC or YPD medium at a density of approximately 10^5 cells/ml and shifted to 37°C for up to 6 days. Cell viability was determined by plating onto SC or YPD plates. Wild-type and mutant *QSR1* strains were viewed at a magnification of ×1,200 with a Nikon Optiphot microscope with Normarski optics and photographed at a magnification of ×375 with Kodak T-Max 400 film.

Incorporation of [^{35}S]methionine into total protein. Protein synthesis was quantitated by pulse-labeling the cells and measuring trichloroacetic acid (TCA)-precipitable counts (2). Strains DEQ2 and DEQ2-24 were grown to log phase in SD medium lacking methionine, inoculated into fresh medium to a density of 0.05 absorbance unit at 600 nm, and grown at 23 or 37°C. At selected times, cells equivalent to 0.01 absorbance unit were removed and diluted to 200 μl at 23 or 37°C, and 3 μCi of [^{35}S]methionine in 25 μl was added to duplicate samples. The samples were incubated for 7 min, the incubation was stopped by the addition of 250 μl of 20% TCA, and the reaction mixture was heated at 95°C for 20 min before being chilled on ice. The samples were collected by filtration onto fiberglass filters presoaked in 10% TCA and washed four times, and their radioactivity was measured with a Beckman LS7500 scintillation counter.

Polyribosome and ribosomal subunit analysis. Cells were inoculated into 100 ml of SC medium and harvested when the absorbance at 600 nm of the culture was 0.7 to 0.9. Preparation of cell lysates and polysome analysis were described previously (8, 37). Lysates equivalent to 15 absorbance units at 260 nm were loaded onto 5 to 47% linear sucrose gradients and centrifuged at 220,000 × *g* for 3 h 45 min in a Beckman SW40 rotor. The gradients were fractionated with an ISCO UA-6 absorbance monitor set at 254 nm, and 0.5-ml fractions were collected. In some experiments, cycloheximide and/or $MgCl_2$ was omitted. High-salt sucrose gradients were made by the addition of KCl to 0.5 M, and cell extracts were made to 0.5 M KCl just before being loaded on to the gradient. *E. coli* β-galactosidase (540 kDa) was added to some polysome extracts as a 15S marker and was located in the gradient by assaying for β-galactosidase activity (1). Ratios of 40S to 60S subunits were calculated by determining the corresponding areas under the profiles of absorbance at 254 nm. The 0.5-ml fractions from the polysome gradients were mixed with 50 μl of 6× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (26) and incubated at 55°C for 15 min before being stored at −20°C. Western analysis of Qsr1p and ribosomal protein L3 was as previously described (8).

Subunit joining assays. Ribosomal subunits from yeast strain W303-1B were purified on sucrose gradients (12, 38) and stored as described previously (53). 60S subunits containing mutant qsr1p were purified from MMY3-3B by isolating 80S couples, followed by dissociation in 0.5 M KCl and purification on 10 to 40% sucrose gradients. 60S subunits lacking Qsr1p were obtained from strain DEH221+ grown on dextrose for 16 h, pooled and concentrated as above. 40S subunits from DEH221+ were obtained by pooling only free subunits from 10 to 40% sucrose gradients. Subunit-joining reactions were carried out at 4°C for 1 h in 0.25 ml of buffer E (10 mM Tris [pH 7.5], 8 mM $MgCl_2$, 50 mM KCl, 6 mM 2-mercaptoethanol, 0.1 mM EDTA) containing 0.26 absorbance unit at 260 nm of 40S subunits and/or 0.5 absorbance unit of 60S subunits. Complexes were resolved on 10 to 40% sucrose gradients in buffer E by centrifuging at 275,000 × *g* for 2 h in a Beckman SW60 rotor. The gradients were fractionated as described above.

RESULTS

Isolation of *qsr1* temperature-sensitive mutants. A plasmid-shuffling procedure (43) in combination with mutagenesis of plasmid DEQ2 (*CEN LEU2 QSR1*) was used to isolate mutants with a temperature-sensitive defect in *QSR1*. Initially, plasmid mutagenesis by chemical methods was unsuccessful. Despite screening more than 30,000 clones, we did not obtain *qsr1* temperature-sensitive alleles by chemical mutagenesis of the plasmid. This negative result, when contrasted with the success of the alternative mutagenesis described below, suggests that the limited amino acid substitutions that result from single mutations are unable to render Qsr1p temperature sensitive.

We thus used regional codon randomization (5) to make a *qsr1* mutagenized library for plasmid shuffling. We targeted the mutagenesis to a conserved and highly charged 20-amino-acid region from G151 through K170 in Qsr1p and designed the library so that all possible single-amino-acid substitutions, in addition to a lower frequency of more than one substitution per clone, would be represented. By this approach, we obtained 59 temperature-sensitive clones including 31 unique alleles with an average of 1.8 mutated codons per clone (Table 1). From the distribution of mutations, it does not appear that there are any residues uniquely capable of rendering Qsr1p temperature sensitive. Instead, particular changes appear to be needed, as exemplified by the F159S mutation, which was isolated 12 times with three different codons.

All of the mutants can double several times after a switch to the restrictive temperature of 37°C, and variations in the rate of growth arrest are allele specific. Whether the differences in the degree of growth arrest are due to the nature of the mutations or are a result of compensating changes in the centromeric plasmid copy number is not known. Several attempts at integrating selected alleles into the *QSR1* genomic locus were unsuccessful. None of the mutants gave rise to spontaneous revertants, as evidenced by the lack of "pop-out" colonies. Introduction of wild-type *QSR1* on a second plasmid into yeast strains carrying temperature-sensitive *qsr1* alleles resulted in wild-type growth rates and normal cellular morphology at 37°C, indicating that the mutations are recessive (data not shown).

Strain DEQ2-24 carrying the *qsr1-24* allele has an adjacent double-amino-acid substitution at positions 164 to 165 (KI to WM) and has been used for most of the studies herein, since growth arrest at the restrictive temperature occurs sooner with this mutant than the others. The doubling time of DEQ2-24 at 23°C is 4.5 h compared to 1.75 h for the plasmid-shuffled wild-type control. When DEQ2-24 is shifted to 37°C, several doublings occur, but the growth rate gradually decreases and the cells stop growing (data not shown).

Temperature-sensitive *qsr1* cells are viable and arrest as large, unbudded cells. When examined by microscopy with Normarski optics at the permissive temperature, all of the mutants appeared morphologically the same as the wild-type yeast. When growth was arrested at the restrictive tempera-

TABLE 1. Temperature-sensitive alleles of QSR1[a]

Clone	G	L	R	R	A	R	Y	K	F	P	G	Q	Q	K	I	I'	L'	S	K	K	Changes	Isolates
12									S												1	10
13																	P			G	2	2
11																	G				1	2
10				G	S																2	2
7						D				G											2	1
18										F											1	1
24														W	M						2	1
9						F												G			2	2
34						V				G								G			3	1
42																	D				1	2
2																			G	G	2	1
5												C					K			T	3	3
22																		E			1	4
21													N	P							2	2
35										W											1	3
48								A				H		G							3	1
8			G		V																2	1
14			T	W																	2	1
51												L						T			2	1
25								A								A					2	1
52																	A			V	2	1
57															F						1	1
61														G					W		2	1
63								T													1	1
31																E					1	1
33	L																				1	1
17											T										1	2
39													H	P							2	1
40													G						Q		2	1
46								R													1	1
47																				I	1	1

[a] Amino acids G151 to K170 are shown across the top, with the two nonconserved residues marked with an apostrophe. Missense mutations are shown below each amino acid. On the right are the number of amino acid changes in the mutant clone and the number of times the mutation was isolated. Clones in which cessation of growth at 37°C was the fastest are in the upper group, followed by the group in which growth arrest was not as fast. The third group consists of those considered to be weakly temperature sensitive.

ture, more than 80% of the mutant cells were round, unbudded, and larger than wild-type cells (Fig. 1). A small population consisted of mixed phenotypes of very large and unbudded cells or misshapen aberrant cells, some with an elongated bud. In addition to being large and unbudded, most of the cells have a large vacuole and a single nucleus, consistent with the observation that a variety of protein synthesis mutants exhibit these aberrant morphologies and arrest in the G_0 stage of the cell cycle (2, 18, 36). Furthermore, such G_0-arrested phenotypes are more pronounced on synthetic media than on rich media, as has also been reported by others (19).

Viability of the *qsr1-24* strain was examined by incubating the cells in liquid culture at the restrictive temperature for 1 to 6 days, withdrawing them at 1-day intervals, and incubating them on plates for 3 days at 23°C. By this criterion, 99% of the mutant cells were viable even after 6 days compared to wild-type cells, which further indicates that the *qsr1* mutants are arrested in G_0 at 37°C (data not shown). Colonies did not appear if the plates were incubated at the restrictive temperature.

Protein synthesis is impaired in the *qsr1-24* mutant. To test whether protein synthesis is impaired in the *qsr1-24* strain, cells were grown at the permissive temperature and then pulse-labeled with [^{35}S]methionine after being shifted to 37°C for various times. Prior to shifting to the nonpermissive temperature, the rate of protein synthesis in the *qsr1-24* strain was only 50% of that of the *QSR1* strain (data not shown). After the cells were shifted to 37°C, the rate of protein synthesis decreased progressively with time until it was only 10 to 15% of that of the wild-type cells after 24 h (Fig. 1).

Joining of 40S and 60S subunits is defective in *qsr1* temperature-sensitive mutants. The profile of 40S and 60S ribosomal subunits, 80S monosomes, and polysomes in *qsr1-24* cells was examined by sucrose gradient centrifugation before and after the cells were shifted to the restrictive temperature. Fractions from the gradients were also probed with antibodies to Qsr1p and L3, another 60S ribosomal subunit protein (51). As shown in Fig. 2, a shift to the nonpermissive temperature causes a pronounced decrease in the size of the 80S and polyribosome peaks to the extent that the 80S peak becomes smaller than the 60S peak after 16 h. The decrease in polysome peak heights indicates that the rate of initiation is reduced to a greater extent than is any defect in elongation. Although we cannot exclude the possibility that there is also some defect in elongation, a severe defect in elongation would lead to a backup in mRNA-associated ribosomes due to inefficient completion of nascent polypeptide chains, which would cause an increase in polysome peak sizes, and this is not seen (Fig. 2).

At the permissive temperature, qsr1-24p is present in the 60S, 80S, and polyribosome peaks, as previously reported for wild-type Qsr1p (8). After 16 h at 37°C, only trace amounts of qsr1-24p are seen in the 60S peak, while the amount of L3 protein is relatively unchanged by the temperature shift (Fig. 2D). Decreased amounts of qsr1-24p and L3 protein in the polyribosomes at 37°C correlate with the decrease in polyribosome peak heights. The presence of qsr1-24p in the polyribosome fractions indicates that mutant qsr1-24p can be assembled into translating ribosomes. Within 2 h after a shift to 37°C, all of the phenotypic changes revealed by the polysome gradient are evident, and they become progressively more pro-

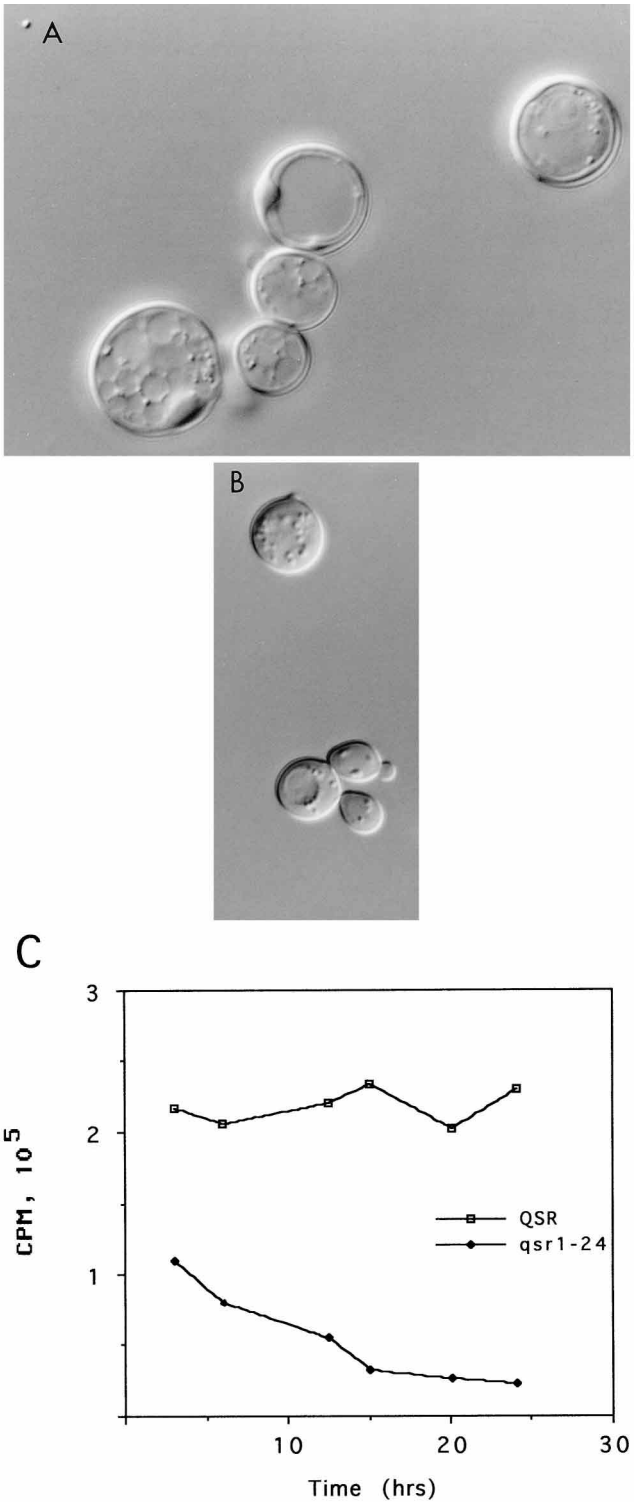

FIG. 1. Cell morphology and rates of protein synthesis in the *qsr1-24* strain. (A and B) Morphology of the *qsr1-24* mutant viewed by Nomarski optics after growth at 23°C (A) and after 48 h at 37°C (B). (C) Rates of protein synthesis by *QSR1* and *qsr1-24* cells. The cells were pregrown at 23°C in SD medium lacking methionine and inoculated into fresh medium. After incubation at 37°C for the indicated times, aliquots of cells were pulse-labeled with [^{35}S]methionine for 7 min at 37°C, followed by TCA precipitation and counting of the incorporated label.

nounced with time (data not shown). When polysomes from several other temperature-sensitive *qsr1* alleles were analyzed on sucrose gradients after growth at 23 and 37°C, they exhibited the same profile as the *qsr1-24* allele (data not shown).

At 37°C a more slowly sedimenting species is present to the left of the 40S subunit in the polysome profiles of both the mutant and wild-type strains (Fig. 2B and D). The nature of this species is not known, but it has been observed by others (6). It migrates midway between 15S and 40S as determined by a 15S β-galactosidase marker (data not shown) and may be a fractional component of the 40S subunit.

The polysome profiles from the *qsr1* mutants differ from those of the *QSR1* cells in that a shoulder is evident on the right-hand or heavier side of the monosome and polysome peaks at 23°C and is more pronounced at the restrictive temperature (Fig. 2C and D). These shoulders, indicated by arrows in the profiles, result from formation of half-mers in the polyribosome population. Such half-mer polyribosomes are a sensitive indication of a defect in a late step of the translation initiation process such that 43S preinitiation complexes bound to mRNA have not joined with a 60S subunit (20, 40). The half-mers are present at 23°C but become progressively more pronounced with time at 37°C.

Half-mers have been attributed either to a defect in the assembly of 60S subunits and thus a stoichiometric imbalance of subunits or to a subunit joining defect. Inhibitors of translation initiation that impair 40S-60S subunit joining result in the formation of half-mers (20, 23, 24). The majority of reported half-mer phenotypes occur in mutants with decreased amounts of 60S subunits and reflect a 60S assembly defect. Most of these mutants have depleted or mutated 60S ribosomal proteins (6, 7, 30, 33, 34, 40, 41). There are fewer examples of half-mer phenotypes in which subunit imbalance is not the cause. A eukaryotic initiation factor 2B (eIF-2B) (*gcd2 gcn3*) double mutant causes a transient half-mer phenotype that is seen only 30 min after the shift to restrictive temperature (14). Elimination of the nonessential 60S ribosomal protein L30 results in the formation of half-mers (3). Deletion of one of two copies of the 60S ribosomal protein gene *SSM1* also leads to half-mer formation (35). In both cases, half-mers occur without a decrease in the number of free 60S subunits.

It appears that the stability or assembly of 60S subunits is not affected by the *qsr1-24* allele, since the ratio of free 40S to 60S subunits (0.45 to 0.65) is relatively constant over time after a shift to the restrictive temperature, and the ratio is typical for wild-type cells. In contrast, a defect in 60S subunit assembly typically causes a severalfold excess of free 40S subunits relative to free 60S subunits (30, 34, 40). The relatively unchanged ratio of subunits in the *qsr1-24* mutant indicates that a subunit joining defect causes the half-mers in this mutant, as opposed to a subunit imbalance.

The progressive decrease in the 80S peak also indicates that subunit joining is defective. The 80S peak is a mixture of both 80S monosomes and 80S "couples", which are an association of 40S and 60S subunits in the absence of mRNA and initiation factors that prevent their association (14, 37). The formation of 80S couples is dependent on Mg and is thought to be the true representation of nontranslating subunits in vivo (21). A typical early block in translation initiation causes most of the 40S and 60S subunits to be in a very prominent 80S couple peak (22). This is not the case in the *qsr1* mutants, where the disassociated 40S and 60S subunits are mainly subunits present at the restrictive temperature (Fig. 2D).

The defect in subunit joining is a result of 60S subunits that lack the qsr1-24 protein. To more accurately examine the ratio

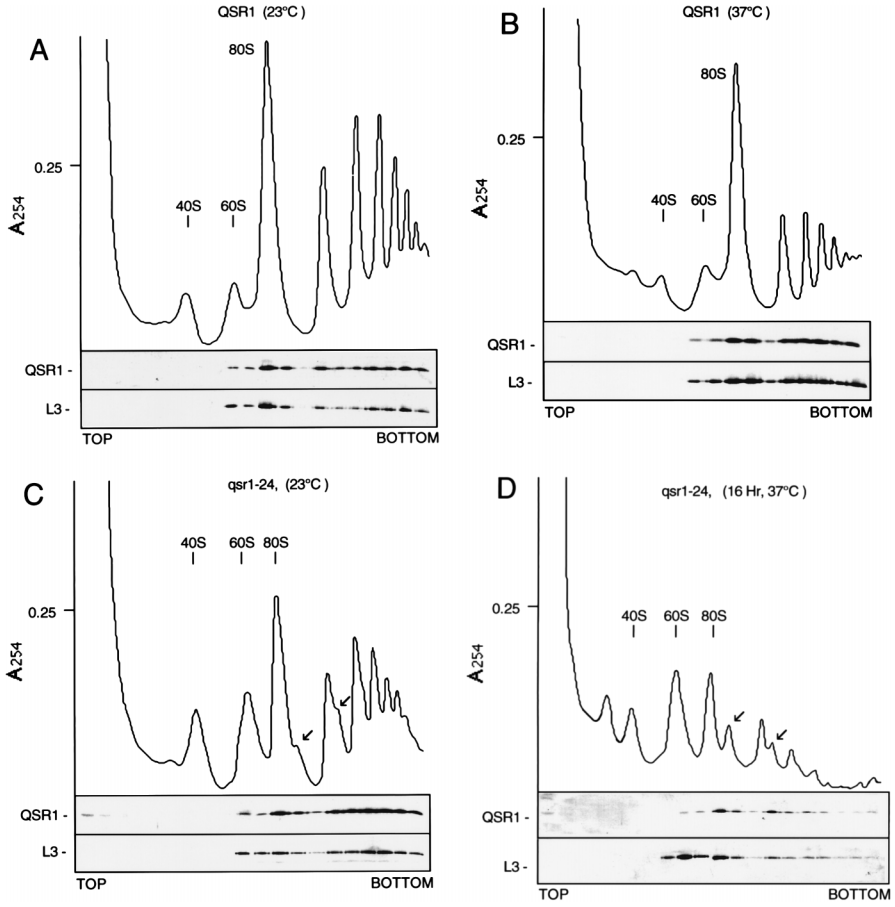

FIG. 2. Effect of temperature on polysome profiles and distribution of Qsr1p in *QSR1* and *qsr1-24* cells. Cells were pretreated with cycloheximide, and lysates were fractionated on 5 to 47% sucrose gradients containing Mg and cycloheximide. The 40S, 60S, and 80S peaks are marked in the absorbance (A_{254}) profile, and arrows mark the presence of half-mer polysome peaks. Immunoblots of gradient fractions are aligned below the tracings to show the corresponding distributions of Qsr1p and L3 protein. The strains and conditions are as follows: DEQ2 (*QSR1*) at 23°C (A) and at 37°C (B) for 16 h; DEQ2-24 (*qsr1-24*) at 23°C (C) and at 37°C (D) for 16 h.

of 40S to 60S subunits and the distribution of qsr1-24p, we ran polysome gradients that lacked Mg, which causes 80S ribosomes to dissociate (14, 37). As shown in Fig. 3, the ratio of 40S to 60S subunits in the *qsr1* mutant at 23°C (0.63) is slightly greater than that in the wild-type cells (0.47). This ratio increases only slightly at the restrictive temperature (0.75), and there is a decrease in the total amount of both subunits. The amount of Qsr1p relative to L3 in these 60S subunits was analyzed by transmission densitometry of underexposed blots and found to be only slightly decreased in the mutant compared to the wild type at 23°C but decreased by 40% at the restrictive temperature (Fig. 3C). This agrees with a 50% decrease of total Qsr1p in whole-cell extracts after 16 h at the restrictive temperature (data not shown). Under these conditions and at both temperatures, some qsr1-24p (less than 10%) is clearly present in the 40S subunit, whereas in the wild-type control the distribution of Qsr1p closely follows that of L3, as previously reported (8). This altered distribution of Qsr1p suggests that the mutated protein may be less stably associated with the 60S subunit in the absence of Mg than in its presence and is binding specifically or nonspecifically to the 40S subunit. It was previously shown that Qsr1p can be extracted from the 60S subunit by 0.5 M KCl in the absence of Mg but not in its presence (8).

The observations that subunit stoichiometry is not dramatically out of balance and that the amount of qsr1-24p associated with the free 60S subunit is decreased after a shift to the restrictive temperature suggest that qsr1-24p is limiting and that only 60S subunits that have qsr1-24p can join with a 40S subunit to make a translating ribosome or an 80S couple. To investigate this possibility, we analyzed the polysomes from the *qsr1-24* strain on gradients in the presence of Mg but without exposing the cells or the lysates to cycloheximide. Omission of cycloheximide leads to polysome runoff during harvest and preparation of cell extracts, such that translating ribosomes can complete peptide synthesis. New rounds of initiation are infrequent, but the newly run-off subunits can assemble into inactive 80S couples (37).

As shown in Fig. 4A, when cycloheximide is omitted at the permissive temperature, a very large 80S peak is formed and the half-mer polysomes disappear. The same result was obtained at 37°C (data not shown). The simplest explanation of this result is that 60S subunits containing qsr1-24p were made available by polysome runoff and joined either a stalled 43S preinitiation complex or a 40S subunit to form an 80S couple, either of which would result in the disappearance of the half-mers. If the presence of qsr1-24p made subunit joining less efficient, it is unlikely that harvest without cycloheximide would rescue the half-mer defect and cause an increase in the size of the 80S peak, especially since the procedure is carried out on ice. The prospect that cycloheximide causes reduced subunit joining in the *qsr1-24* strain is also unlikely, since it is added

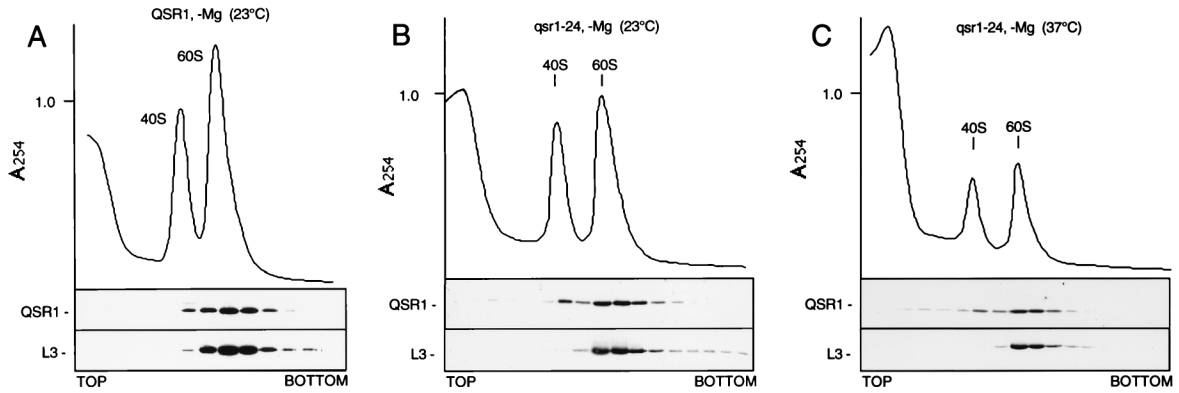

FIG. 3. Analysis of 40S to 60S subunit ratios and distribution of Qsr1p after a shift to the restrictive temperature in low Mg sucrose velocity gradients. Cell lysates were fractionated on 5 to 47% sucrose gradients in which Mg and cycloheximide were omitted. The absorbance profile at 254 nm is shown, with the 40S and 60S subunit peaks marked. Immunoblots showing the distributions of Qsr1p and L3 protein are aligned below the tracings. The strains and conditions are as follows: (A) DEQ2 (*QSR1*) at 23°C, (B) DEQ2-24 (*qsr1-24*) at 23°C, (C) DEQ2-24 (*qsr1-24*) at 37°C (all for 16 h).

only 5 min before harvest, the half-mer phenotype is more pronounced with increasing time at the restrictive temperature, and we could not find an increased sensitivity to different concentrations of cycloheximide in the mutant strain compared to the wild-type strain (data not shown).

To further investigate the content of 80S couples with respect to translating ribosomes, we examined the distribution of 40S, 60S, and 80S ribosomes from the *qsr1-24* cells on high-salt polysome gradients (Fig. 4B). When polysomes are fractionated on sucrose gradients containing 0.5 M KCl, 80S couples dissociate into 40S and 60S subunits but polysomes and 80S monosomes engaged in translation remain stable (14, 28, 38). At 23°C, the relative intensity of the 80S peak in the polysome profile on the high-salt gradient from the mutant cells is reduced compared to that of the mutant cells on a low-salt gradient (compare Fig. 4B and 2C). This demonstrates that many of the 80S ribosomes are inactive couples and that the increase in qsr1-24p detected in the 60S peak (Fig. 4B) must come from such dissociated couples. At 37°C, the intensity of the 80S peak does not change, nor does the amount of qsr1-24p in the 60S subunit peak (compare Fig. 4C and 2D), which means that virtually all of the 80S ribosomes are engaged in translation and are not forming 80S couples. This reinforces the notion that 60S subunits containing qsr1-24p are rate limiting for translation and essential for subunit joining in both translating ribosomes and inactive 80S couples.

The original *qsr1-1* mutant is defective in subunit joining. *QSR1* was isolated by the discovery that *QCR6*, a single-copy nuclear gene encoding a nonessential subunit of the mitochondrial cytochrome bc_1 complex, can rescue a point mutation in a *qsr1-1* mutant (46). We examined the polysome profiles of two sister haploids that are wild type for *QCR6* and differ only in that strain MMY3-3A has a wild-type *QSR1* gene whereas strain MMY3-3B harbors the original *qsr1-1* mutation and grows very slowly. The polysome profile from MMY3-3A is typical of wild-type cells, with a large 80S peak and a progressive increase in the size of polyribosomes (Fig. 5A). In contrast, polysomes from MMY3-3B (*qsr1-1*) exhibit a profile (Fig. 5B) similar to that of the DEQ2-24 strain (*qsr1-24*), in that there are large half-mer peaks and a small 80S peak. Also, there are reduced amounts of qsr1-1p in the mutant 60S subunits, even though the 60S peak from the mutant cells is larger than that from the wild-type sister haploid. The similar biochemical phenotypes of the *qsr1-1* mutant and the temperature-sensitive *qsr1-24* mutant provide further support for the notion that Qsr1p is important for subunit joining.

Depletion of Qsr1p results in a 40S to 60S subunit-joining defect. To further investigate the idea that subunits lacking Qsr1p are defective in subunit joining, we constructed an inducible *QSR1* gene construct such that expression of *QSR1* could be controlled by the *GAL1-10* upstream activating sequence. The GAL_{UAS} was placed in front of a minimal *QSR1* promoter in which the yeast ribosomal protein upstream activating sequences consisting of two tandem RPG consensus sequences (51) and a poly(dA-dT)-rich region are missing. Tetrad analysis of a hemizygous *QSR1* deletion strain harboring the GAL_{UAS}-*QSR1* construct on a centromeric plasmid revealed that on dextrose the plasmid could not rescue the lethality of the *QSR1* deletion, but on galactose the plasmid covered the deletion efficiently (data not shown).

We used a haploid strain, DEH221+, in which the GAL_{UAS}-*QSR1* plasmid covers the chromosomal deletion, to analyze polysome profiles when Qsr1p is depleted. The prominent 80S and polysome peaks present in galactose-grown cells (Fig. 5C) were severely diminished when the cells were transferred to dextrose for 16 h, large half-mers became evident, and the large 60S peak was depleted of Qsr1p relative to L3 (Fig. 5D). The resemblance of this profile to that of the *qsr1-24* and *qsr1-1* mutants shows that Qsr1p is essential for translation, since it is present in translating ribosomes, 60S subunits are still assembled when Qsr1p is depleted, and 60S subunits lacking Qsr1p are unable to form 80S couples.

Analysis of 60S subunit function by a subunit-joining assay. A subunit-joining assay was used to test whether 60S subunits from *qsr1* mutant and wild-type cells can join with wild-type 40S subunits to form 80S couples. As shown in Fig. 6, when 60S subunits (Fig. 6B) and 40S subunits (Fig. 6A) from wild-type cells were mixed, they formed 80S couples (Fig. 6C). Likewise, when 60S subunits containing mutant qsr1p from *qsr1-1* cells (Fig. 6D) were mixed with wild-type 40S subunits, they also formed 80S couples (Fig. 6E).

In contrast, when subunits depleted of Qsr1p (Fig. 6F) were mixed with 40S subunits from wild-type cells, they did not form 80S couples (Fig. 6G). Western blots of purified 60S subunits were used to confirm the depletion of Qsr1p from these subunits (Fig. 7). Subunits lacking Qsr1p formed a small 80S-like peak (Fig. 6F and G). This reflects more complicated alterations of the 60S subunit when Qsr1p is absent and is reminis-

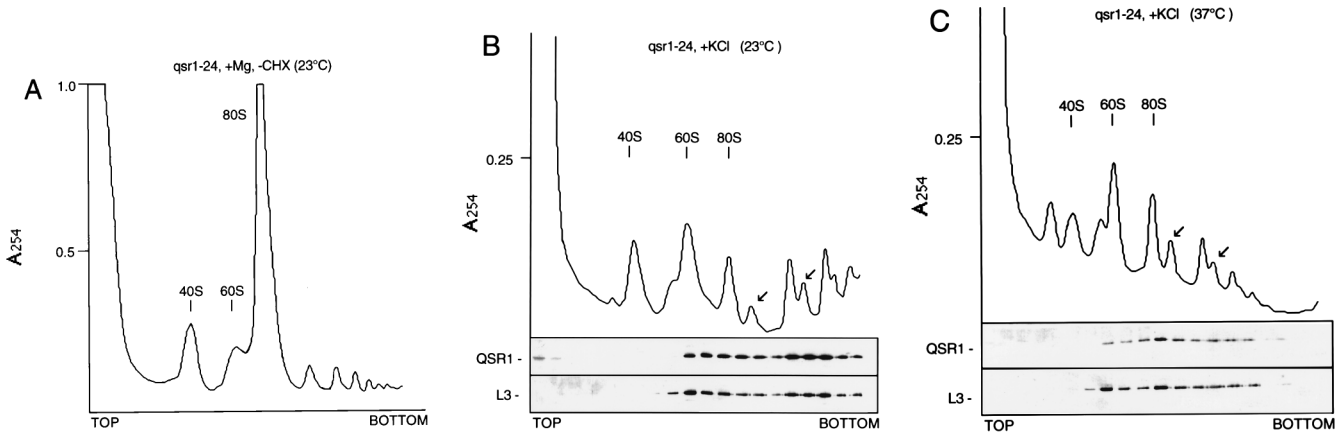

FIG. 4. Polysome profiles showing the formation of 80S couples in the DEQ2-24 strain (*qsr1-24*). (A) Profile from cells grown at 23°C. Cell lysates were prepared without pretreatment with cycloheximide. The 5 to 47% sucrose gradient contains Mg but not cycloheximide. (B and C) Profiles of high-salt gradients from cells grown at 23°C (B) and 37°C (C) and treated with cycloheximide. The 5 to 47% sucrose gradients contained 0.5 M KCl, Mg, and cycloheximide. Half-mers are marked by arrows, and immunoblots of gradient fractions are aligned below the tracings to show the distributions of Qsr1p and L3 protein.

cent of previously reported core particles derived from NH_4Cl-washed 60S subunits that dimerize and are resolved as higher-order complexes in velocity gradients (27). When Mg was omitted from the gradient, the 60S subunit was resolved into a sharp, well-defined peak (Fig. 6H).

Attempts at reconstituting functional 60S subunits by adding purified Qsr1p to these inactive subunits have been unsuccessful. We have been unable to express the full-length Qsr1p in bacteria due to extensive codon bias in the amino terminus of the protein, and purification of Qsr1p from salt disrupted subunits has been hindered by solubility problems of ribosomal subunit proteins. To generate 60S subunits lacking Qsr1p, cells requiring galactose to express *QSR1* are grown on dextrose. These cells obligatorily become compromised in protein synthesis. To rule out a pleiotropic loss of ribosomal proteins in these translationally compromised cells, we isolated free 40S subunits from these cells and showed that these subunits were completely functional in the subunit-joining assay (data not shown). Although we cannot exclude the possibility that the effects of Qsr1p depletion are indirectly manifested through the loss of other required proteins, the subunit-joining assay confirms that 60S subunits containing mutant qsr1p are capable of efficient subunit joining whereas subunits depleted of Qsr1p are not.

DISCUSSION

Qsr1p is a recently discovered *S. cerevisiae* ribosomal protein (8) that is homologous to the rat L10 ribosomal protein (4). To investigate the function of Qsr1p in yeast, we isolated 31 unique temperature-sensitive alleles by codon randomization. The temperature-sensitive alleles had an average rate of 1.8 mutated codons per clone (3 triple, 15 double, and 13 single), whereas sequencing 30 random clones revealed an average substitution rate of 1.08 mutated codons per clone. This comparison indicates that there was a selection for more than one substitution to generate a temperature-sensitive *qsr1* allele. Moritz et al. found it difficult to generate temperature-sensitive alleles to *RPL16* (32), and two of the four alleles they obtained had two substitutions, one of which was an adjacent double substitution, an extremely rare occurrence for standard hydroxylamine plasmid mutagenesis. If ribosomal proteins are relatively resistant to temperature lability by single-mutation events, regional codon randomization should be a useful means for mutagenizing other yeast ribosomal proteins for which few temperature-sensitive mutants exist.

The ability of the *qsr1* mutants to divide several times and survive at the nonpermissive temperature for extended periods is a characteristic exhibited by ribosomal assembly mutants. Temperature-sensitive alleles of *RPL16* and mutants which are conditional for the synthesis of *RPL16*, *rp59*, and RNA polymerase I exhibit a delayed growth arrest (32, 33, 49). Growth is not immediately inhibited when nucleolar ribosome assembly factors U14 and *NOP1* are depleted (45, 52). In such instances, it is believed that ribosomes made before a temperature shift or promoter shutoff are not defective and, because of their intrinsic stability, are diluted in the partitioning process of cell division. The observation that protein synthesis decreases in unison with a decreased rate of growth suggests that a similar dilution of functional ribosomes occurs in the *qsr1* mutants. The increased size of the *qsr1* cells at the restrictive temperature indicates that cell surface growth is not initially blocked but, rather, is preceded by a block in bud formation or DNA replication.

A unique aspect of the *qsr1* mutants is that 60S subunits lacking Qsr1p could be detected on polysome gradients and purified. The vast majority of 60S ribosomal protein mutants are defective for assembly of the entire subunit (6, 7, 32–34, 40, 41, 51), whereas there is only a slight increase in the ratio of 40S to 60S subunits in the *qsr1* mutants. The observation that core 60S particles are assembled without Qsr1p is consistent with the finding that Qsr1p is a more peripheral ribosomal protein that can be removed from 60S subunits with 0.5 M KCl if Mg is omitted. More integral proteins such as L3 remain with the core 60S particles under these conditions (8).

After a shift to the nonpermissive temperature, there is a progressive reduction in 80S and polysome peak sizes with a subsequent increase in the formation of half-mer polyribosomes in the temperature-sensitive mutants. Half-mers are caused by a late-stage translational initiation defect in which 43S preinitiation complexes are attached to an mRNA that has at least one translating 80S ribosome on it but has not yet joined with a 60S subunit (20, 40). The small half-mer peaks visible at the permissive temperature show that a subunit-joining defect exists but is not detrimental enough to cause a block in cell division. The amount of Qsr1p associated with free 60S subunits is smaller at the restrictive temperature, yet

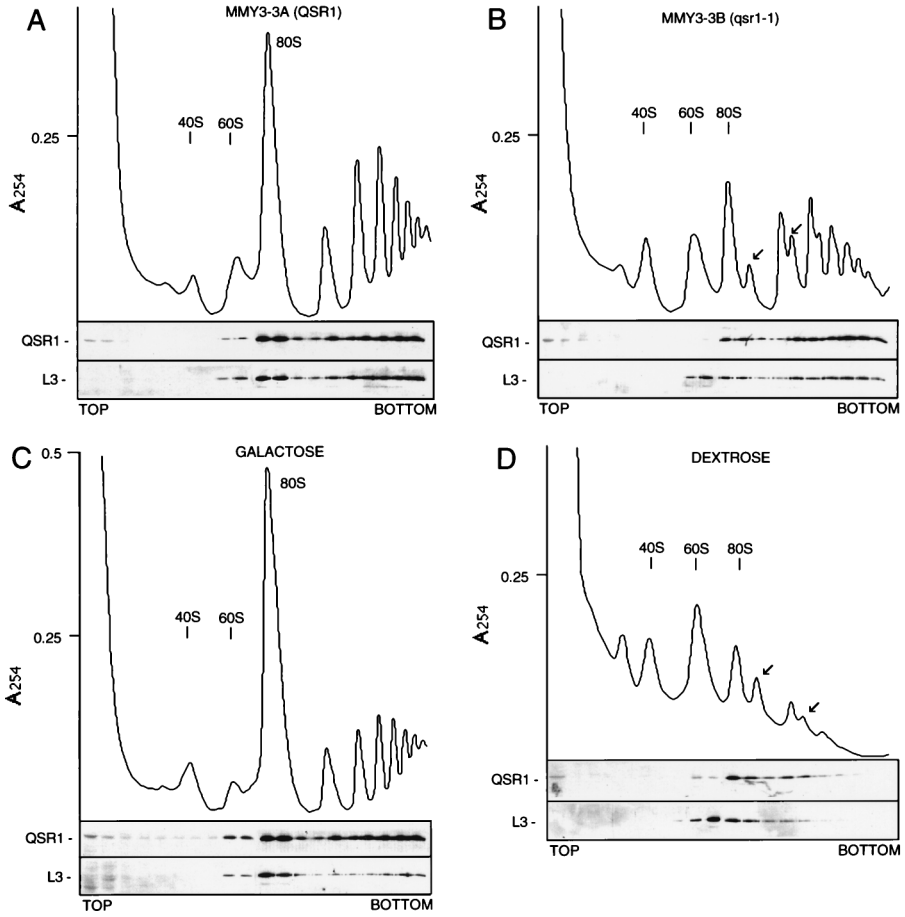

FIG. 5. Polysome profiles and distribution of Qsr1p in two non-temperature-sensitive mutant strains. Strains were grown at 30°C and treated with cycloheximide before lysis, and lysates were fractionated by centrifugation on 5 to 47% sucrose gradients containing Mg and cycloheximide. (A) Strain MMY3-3A (*QSR1*); (B) Strain MMY3-3B (*qsr1-1*); (C) Strain DEH221+, in which Qsr1p was induced by growth on galactose; (D) strain DEH221+ grown on dextrose for 16 h.

Qsr1p is still present quantitatively in translating ribosomes, which suggests that it is essential for translation.

Half-mer polyribosomes are most commonly caused by a defect in the assembly of 60S subunits, resulting in severalfold-fewer 60S subunits available for joining (6, 7, 32–34, 40, 41, 51). The observation that 60S subunit assembly is only slightly affected in the *qsr1* mutants demonstrates defective subunit joining, rather than an imbalance of subunit stoichiometry. This is supported by several lines of evidence which show that a subunit-joining defect results when 60S subunits are devoid of Qsr1p and are not able to form either translating 80S ribosomes or 80S couples. First, the amount of Qsr1p associated with the free 60S subunits decreases after the temperature shift, while the original *qsr1-1* mutant displays this polysome profile constitutively. Second, omission of cycloheximide leads to complete disappearance of the half-mers and emergence of a large inactive 80S peak. This is explained by the runoff of translating ribosomes providing 60S subunits harboring Qsr1p to join 43S preinitiation complexes (half-mers) or free 40S subunits. Third, high-salt gradients show all of the 80S peak to be translating monosomes at the restrictive temperature and a mixture of translating monosomes and 80S couples at the permissive temperature. At the permissive temperature, there is an increase in the amount of Qsr1p associated with the 60S subunits, which results from dissociated 80S couples. Fourth, depletion of Qsr1p by promoter shutoff results in pronounced half-mers and in decreased 80S and polysome peaks, and the free 60S subunits are devoid of Qsr1p whereas the translating ribosomes harbor Qsr1p. Finally, in vitro subunit-joining assays of purified subunits demonstrate that 60S subunits with mutant protein can form an 80S couple whereas 60S subunits lacking Qsr1p cannot.

Defective subunit joining in the *qsr1* mutants results from impaired assembly of Qsr1p onto the 60S subunit. The resulting 60S subunits lacking Qsr1p would dilute the pool of 60S subunits harboring Qsr1p, which would account for the gradual decrease in the rates of growth and protein synthesis for the *qsr1-24* strain. A similar decrease in growth rate and altered polysome profile occurs when the GAL_{UAS}-controlled *QSR1* is shut off. If the shift to the nonpermissive temperature released Qsr1p from the ribosome and this occurred uniformly, the abrupt cessation of protein synthesis would result in an abrupt growth arrest and altered polysome profile.

In the polysome gradients from both the mutant and wild-type strains, a small amount of Qsr1p is detectable at the top of the gradient, where the bulk of free proteins do not enter the gradient. This is most probably Qsr1p that is stripped from the 60S subunits during extraction or centrifugation, since the half-life of unassembled yeast ribosomal proteins is a few minutes (48). If Mg is omitted from the gradients, some Qsr1p is associated with 40S subunits, which suggests that Qsr1p may be a 40S docking protein and is associated with its "receptor"

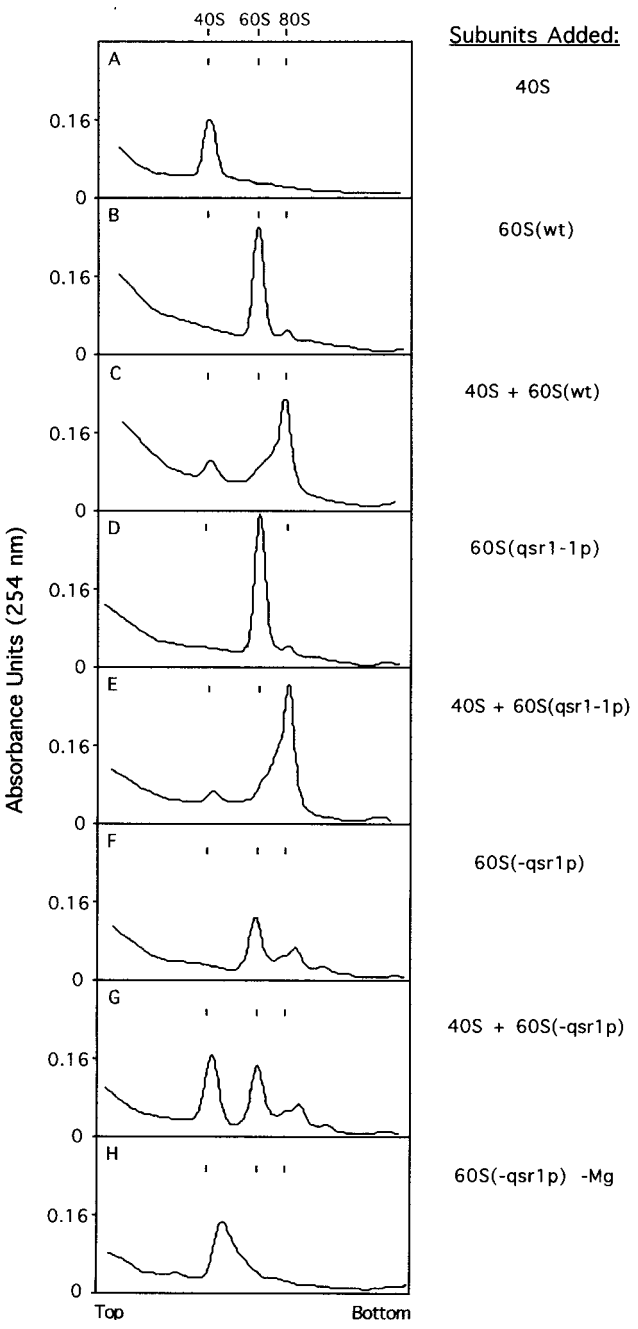

FIG. 6. Profiles from in vitro subunit-joining assays, showing that 60S subunits from *qsr1* mutant cells can join with 40S subunits from wild-type cells to form 80S couples. Samples from subunit-joining reactions were fractionated on 10 to 40% sucrose velocity gradients in the presence of Mg unless otherwise noted. The locations of the 40S, 60S and 80S peaks are indicated. (A) Wild-type 40S alone, (B) wild-type 60S alone, (C) wild-type 40S and 60S, (D) mutant 60S alone from strain MMY3-3B (*qsr1-1*), (E) wild-type 40S and 60S from strain MMY3-3B, (F) 60S alone depleted of Qsr1p from strain DEH221+ grown on dextrose, (G) wild-type 40S and 60S depleted of Qsr1p, (H) same as panel F except that the gradient buffer lacked Mg.

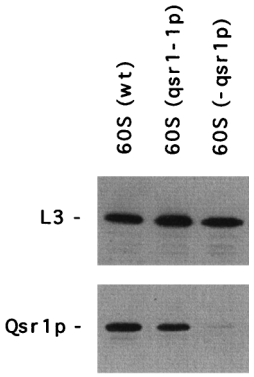

FIG. 7. Western blots of purified subunits from the experiment shown in Fig. 6. Wild-type 60S subunits, mutant 60S subunits from MMY3-3B, and 60S subunits depleted of Qsr1p probed with antibodies to Qsr1p and L3 are shown.

under such conditions. It has been previously demonstrated that Qsr1p is accessible to protease treatment on free 60S subunits but is protected in 80S couples, which suggests that this protein is at the subunit interface (8).

Ribosomes are assembled in the nucleolus, and a large body of work exists on the mechanistic details of rRNA processing, while little is known about the assembly of the proteins to form the large and complicated structure of the ribosome (16, 25, 31, 48, 51). Whether Qsr1p is added to the 60S subunit in the nucleolus or the cytoplasm is not known, but only four ribosomal proteins have been shown to be exchangeable in the cytoplasm after assembly (54), and the N terminus of Qsr1p has a putative nuclear localization signal.

It is also not known how a single chromosomal copy of *QCR6*, which encodes a protein whose only known location is in the inner mitochondrial membrane, can rescue an otherwise lethal missense mutation in a cytoplasmic ribosomal protein. In the present study, we have shown that the *qsr1-1* mutant which is rescued by *QCR6* exhibits the same defect in 60S subunits as the temperature-sensitive *qsr1* mutants, which implies that Qcr6p may play a role outside of the mitochondria and is somehow aiding in the assembly or stability of Qsr1p with the 60S ribosomal subunit. Although attempts to detect Qcr6p outside of the mitochondria in cells where it rescues the *qsr1-1* mutation have thus far been unsuccessful (8), it remains possible that undetectable amounts of Qcr6p transiently participate in assembly of Qsr1p onto ribosomes, either in the nucleolus or in the cytoplasm. Further investigations of the association of Qsr1p with the 60S subunit and on the relationship between Qsr1p and Qcr6p are under way.

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