Mutational Analysis of the Ribosomal Protein Rpl10 from Yeast*

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Yeast Rpl10 belongs to the L10e family of ribosomal proteins and is orthologous to bacterial L16. In the crystal structure of the Haloarcula marismortui large subunit (1, 2), L10e is situated in a deep cleft between the central protuberance and the GTPase-activating center. The assembly of L16 into the bacterial subunit is accompanied by a large conformational change of the subunit (3), consistent with the idea that L16 is necessary for organizing the ribosome structure surrounding its binding pocket. The central core of L10e is a sandwich composed of five β strands and two α helices (2). A similar architecture is observed for L16 in the bacterial ribosome; however, in L16 the central core is reduced to four β strands and two α helices. The solution structure of free L16 from Thermus thermophilus (4), is remarkably similar to that of L16 in the context of the subunit, indicating that L16 does not undergo large conformational changes upon binding to the subunit. Although L10e makes no contacts with other ribosomal proteins, it makes extensive contacts with 23 S rRNA along helices H38 and H89 and minor contacts with 5 S rRNA. H38, also referred to as the “A-site finger” is involved in a bridging interaction with the 30 S subunit, whereas H89 extends from the peptidyl transferase center. Fitting the archaeal ribosome structure to cryo-electron microscopic reconstructions of the yeast ribosome (5) shows that Rpl10 is similarly disposed in the eukaryotic ribosome.

The location of L16 in the large subunit, close to the A-site and the GTPase-activating center, suggests the possibility that it is intimately involved in translation. In fact, bacterial subunits reconstituted without L16 have reduced catalytic activity and are defective for interaction with the small subunit (6, 7). Furthermore, in the crystal structure of the bacterial ribosome L16 makes contacts with the T-loop of the A-site tRNA (8), and cryo-EM structures of bacterial ribosomes show L16 making contacts with initiation factor IF2 (9).

In eukaryotes, ribosomes are assembled in the nucleolus, a subcompartment of the nucleus where transcription of the bulk of ribosomal RNA takes place. Early analysis of the in vivo assembly of ribosomes in yeast led to the conclusion that Rpl10 is loaded into the ribosome at a late step (10) and that the protein exchanges on mature subunits (11). Free Rpl10 is bound by the WD repeat protein Sqt1 and is unstable in temperature sensitive sqt1 mutants (12). Thus, Sqt1 appears to act as a chaperone for free Rpl10. C-terminally truncated Rpl10 proteins are unable to bind stably to the ribosome and are toxic because they titrate Sqt1 away from functional Rpl10 (12, 13). Whether Rpl10 initially loads onto the subunit in the nucleus or cytoplasm has been debated. The export adapter for the large subunit in yeast is Nmd3 (14, 15), a protein that shuttles into and out of the nucleus to mediate subunit export dependent on the export receptor CRM1. Mutations in RPL10 or repression of RPL10 transcription lead to a failure to recycle Nmd3 to the nucleus (16), indicating that the release of Nmd3 from subunits in the cytoplasm depends on Rpl10. Release of Nmd3 also depends on the cytoplasmic GTPase Lsg1, and we have suggested that the GTPase activity of Lsg1 drives the accommodation of Rpl10 into the subunit, facilitating the release of Nmd3 (12, 16). Lsg1 belongs to a small but highly conserved family of circularly permuted GTPases (17). The related circularly permuted GTPase RbgA from Bacillus subtilis is also required for late, and possibly rate-limiting, assembly of L16 into the bacterial large subunit (18).

Here, we have carried out a mutational analysis of Rpl10 to identify mutations that would define different domains impor-
TABLE 1

| Plasmid used in this study | Description | Source |
|---------------------------|-------------|--------|
| pAJ1409                   | URA3-CEN NMD3 | Ref. 22 |
| pAJ11                     | URA3-2p NMD3  | This study |
| pAJ142                    | URA3-CEN NMD3-myc | Ref. 16 |
| pAJ144                    | URA3-2p NMD3-myc | This study |
| pAJ792                    | LEU2-CEN GAL10-RPL10-myc | Ref. 12 |
| pAJ1066                   | LEU2-2a SQF1-ncy | This study |
| pAJ1197#                  | LEU2-CEN RPL10-myc | This study |
| pAJ1199                   | LEU2-CEN RPL10[R153E,R154E]-myc | This study |
| pAJ1200                   | LEU2-CEN RPL10[K38A,K39A,K40A,N187]-myc | This study |
| pAJ1315                   | LEU2-CEN NMD3[R112T,J362T]-myc | Ref. 16 |
| pAJ1319                   | URA3-2p NMD3[R112T,J362T]-myc | This study |
| pAJ1363                   | LEU2-CEN RPL10[K38A,K39A,K40A]-myc | This study |
| pAJ1364                   | LEU2-CEN RPL10[K38A,K39A,K40A,R153E]-myc | This study |
| pAJ1777                   | LEU2-CEN RPL10[A102–112]-myc | This study |

# All of the rpl10 mutants not listed here were derived from pAJ1197.

tant for interaction with Sqt1, the ribosome, and for release of NMD3.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—All of the strains were grown at 30 °C in rich medium (yeast extract peptone) or synthetic drop-out medium (20) containing either 2% glucose or 1% galactose as the carbon source. The following strains were used in this study: AJY2104 (MATa kanMX6:GAL1:RPL10 ura3 Leu2 ade2 ade3), made by integration of the GAL1 promoter (21) into the genomic locus of Rpl10, W303 (MATa leu2-3,1123 his3-11 ura3-1 trpl-1 ade2-1 can 1-100 SSD1-d), BY4741 (MATa his3Δ1 leu2ΔO ura3Δ0 met15ΔO), and AJY2141 (MATa SQT1: GFP-HIS3 his3Δ1 leu2ΔO ura3Δ0 met15ΔO).

Construction of Plasmids—Plasmids are listed in Table 1. pAJ1197 (RPL10-myc LEU2-CEN), where myc denotes 13 tandem copies of the c-Myc epitope (21), was made by combining RPL10, as a PCR product amplified with oligonucleotides AJO645 and AJO644 and cut with SstI and BamHI, with the Myc epitope as a BamHI-HindIII fragment from pAJ538 (15) into pRS315. Random loss of function mutants of RPL10 were made by PCR mutagenesis and in vivo recombination in yeast. Forty PCR reactions of twenty cycles were carried out in parallel using Taq DNA polymerase (GeneChoice) with AJO264 and AJO722, and pAJ1197 as the template. The products were pooled and cotransformed into AJY2104 with BsaBI-SalI fragments from pAJ1197 and pAJ1199, respectively, into the same sites of pAJ1197. pAJ1200 was made similarly using the oligonucleotide pairs AJO645 and AJO744, and AJO773 and AJO622. The product was digested with BsaBI and BamHI and ligated into the same sites of pAJ1197.

pAJ1119 (RPL10[R153E,R154E]-myc LEU2-CEN) was made by fusion PCR using the oligonucleotide pairs AJO264 and AJO744, and AJO743 and AJO722 (Table 2). The resulting fragment was then cut with Sall and BamHI and ligated into the same sites of pAJ1197. pAJ1200 was made similarly using the oligonucleotide pairs AJO645 and AJO744, and AJO773 and AJO622. The product was ligated into pAJ1197 as a BsaBI and BamHI fragment. pAJ1363 and pAJ1364 were made by inserting Sall-Pacl fragments from pAJ1197 and pAJ1199, respectively, into the same sites of pAJ1200. pAJ1777 (A102–112) was made by fusion PCR with the oligonucleotide pairs AJO645 and AJO829, and AJO828 and AJO622. The product was digested with BsaBI and BamHI and ligated into the same sites of pAJ1197.

pAJ111 (NMD3 URA3 2μ) was made by moving NMD3 as a Smal-HindIII fragment from pAJ409 (NMD3 URA3 CEN) (22) into pRS426. pAJ414 (NMD3-myc URA3 2μ) was made by inserting a Smal-HindIII fragment from pAJ412 (URAS3-CEN NMD3-myc) (16) to pRS426. pAJ1066 was made by moving SQT1 from pSQTMYC1 (13) as a BamHI fragment into pRS426. pAJ1319 (NMD3[R112T,J362T]-myc URA3 2μ) was made by moving the NMD3-containing HindIII-Eagl fragment from pAJ1315 (16) into the same sites of pRS426.

Western Blotting—Extracts were prepared from 5-ml cultures of AJY2104 containing mutant Rpl10 plasmids grown in Leu– galactose medium. All of the subsequent steps were carried out at 0–4 °C. The cells were washed in 500 μl of extract buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl2), 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin and pepstatin A) and resuspended in 250 μl of extract buffer. An equal volume of glass beads (400 μm) was added, and the samples were vortexed in three 1-min pulses with 2-min intervals on ice. The samples were then centrifuged at 15,000 × g for 10 min, and the supernatant was recovered. The samples were subjected to SDS-PAGE and Western blotting using mouse α-c-Myc (9E10) (Covance), affinity-purified rabbit anti-Rpl8, affinity-purified rabbit anti-Nmd3, affinity-purified rabbit anti-Sqt1, and affinity-purified rabbit anti-Lsg1.

Immunoprecipitations—For immunoprecipitations (IPs)2 of mutant myc-tagged Rpl10 proteins, 25-ml cultures were grown to A600 < 0.5 in Leu– galactose medium. All of the subsequent steps were carried out at 0–4 °C. The cells were harvested, washed with IP buffer (20 mM Tris, pH 7.6, 50 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin and pepstatin A), and resuspended in 250 μl of IP buffer. Extracts were made by glass bead lysis (five 30-s pulses with 2-min intervals on ice) and were clarified by centrifugation at 15,000 × g for 10 min. 1.0 μl of α-c-Myc (9E10) antibody was added to equal A260 units of sample supernatants and rocked for 1 h at 4 °C. 30 μl of a 50% slurry of bovine serum albumin-blocked protein A-agarose beads (Invitrogen) was then added, and rocking was continued for an additional hour. The beads were washed three times with lysis buffer and eluted in 40 μl of Laemmli sample buffer without β-mercaptoethanol. Immunoprecipitation from sucrose gradient fractions was carried out in a similar fashion.

Polysome Analysis—5 ml fresh overnight cultures in Leu– galactose medium were diluted into 100 ml of Leu–glucose medium and cultured until A600 = ~0.3. Cycloheximide was added to 150 μg/ml, and the cells were immediately poured onto ice and collected by centrifugation at 5,000 × g for 5 min. All of the subsequent steps were carried out at 0–4 °C. The cell pellets were washed with 2 ml of polysome buffer (10 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM MgCl2, 6 mM β-mercaptoethanol, 150 μg/ml cycloheximide, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin and pepstatin A), resuspended in one volume of the same buffer and disrupted by vortexing with glass beads. The extracts were clarified by centrifugation at 15,000 × g for 10 min. Equal A260 units of supernatant 2 The abbreviation used is: IP, immunoprecipitation.
were loaded onto continuous 7–47% sucrose gradients in poly-
some buffer without protease inhibitors. After centrifugation
for 2.5 h at 40,000 rpm in a Beckman SW40 rotor, the gradients
were analyzed by absorbance at 254 nm. The proteins were
precipitated from fractions with 10% trichloroacetic acid and
analyzed by SDS-PAGE and Western blotting.

**Microscopy**—Indirect immunofluorescence was performed
as described (23).

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**FIGURE 1. Alignment and structure of Rpl10.**

**A**, Rpl10 amino acid sequence from *Saccharomyces cerevisiae* (*S. cer*) was aligned with other eukaryotic and
darchaeal orthologs (*D. mel*, *Drosophila melanogaster*; *H. sap*, *Homo sapiens*; *H. mar*, *H. marismortui*; *M. jan*, *Methanococcus janischii*; *S. sol*, *Sulfolobus solfataricus*).

**B**, cartoon of the large subunit from *H. marismortui* in crown view, showing the position of L10e relative to the central protuberance (CP) and the GTPase-activating center (GAC).

**C**, amino acids 4–173 of Rpl10 were threaded onto the structure of archaeal L10e from *M. marismortui* using Swiss PDB Viewer. Residues 102–112, corresponding to the central loop of L10e, were not resolved in the crystal structure and are represented as a dashed line, although the position of these residues is not known. Helices 38 and 89 (H38 and H89) and the N terminus of Rpl10 (N) are indicated. Amino acids 174–221 comprise a domain unique
to the eukaryotic Rpl10 proteins for which a structure has not been reported.

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**TABLE 2**

| Name   | Sequence                                      |
|--------|-----------------------------------------------|
| AJO264 | 5′-CGC GAA TCC GAA ACT ACT TAG CAG            |
| AJO512 | 5′-CTC GAT GAA CAA AAG ATT ACT TGG TC          |
| AJO622 | 5′-CAC GAG TCC TTA AGG TTC TGT CTT CTT CAA    |
| AJO644 | 5′-CCG CGA TCC TTA AGG TTC TGT CTT CTT CAA    |
| AJO645 | 5′-CCT CTG CTG TAT CTC TTC ACC GAA            |
| AJO722 | 5′-TCA AOT CTT COT CGG AGA TTA GGT TTT CTT CAG CTT TAA TTA ACC |
| AJO743 | 5′-GAT TGG GCT GCT GCA GCT ACC GTC GAT GAT GTC GAT GTC GAT GTC |
| AJO744 | 5′-AAC TTT TAT CTG GCT TCT TCT AGA AGA CAG ACG ACA AC |
| AJO773 | 5′-GTT TGT TAT CTG GCT GCT GCA GCT ACC GTC GAT GAT GTC GAT GTC |
| AJO828 | 5′-GTT TTA COT ACC AAC AAG GAA GGT ATG AGA AGT AGT AGT GC |
| AJO829 | 5′-CTT GCT TGT TGA TAC GTA AGA CAY GGA AAG GAT GG |

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**Mutational Analysis of Rpl10**

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were loaded onto continuous 7–47% sucrose gradients in poly-
some buffer without protease inhibitors. After centrifugation
for 2.5 h at 40,000 rpm in a Beckman SW40 rotor, the gradients
were analyzed by absorbance at 254 nm. The proteins were
precipitated from fractions with 10% trichloroacetic acid and
analyzed by SDS-PAGE and Western blotting.

**Microscopy**—Indirect immunofluorescence was performed
as described (23).
Threading—The sequence of Rpl10 (amino acids 4–173) was threaded onto the structure of L10e in the 50 S ribosomal subunit from the archaeon H. marismortui (Protein Data Bank structure 1S72) (2) using Swiss-PDBviewer www.expasy.org/spdbv (24). A gap from amino acids 102 to 111 corresponds to the unstructured central loop of L10e.

RESULTS

Mutagenesis of RPL10—We introduced random mutations into the open reading frame of RPL10 using PCR and recombined the PCR products into a c-Myc-tagged expression vector in vivo in yeast. In the strain used for this screen, RPL10 was expressed from its genomic locus but under control of the GAL1 promoter. This strain is inviable under repressing conditions (the presence of glucose) unless functional RPL10 is provided episomally. However, the growth of this strain is indistinguishable from wild type in the presence of galactose (data not shown). Repressing genomic conditional RPL10, instead of selecting against an RPL10-containing vector on 5-fluoroorotic acid, avoided interplasmid recombination, which, in our hands, is a common problem during plasmid shuffle in such screens. In addition, we were able to identify several dominant negative mutants in this screen, probably because the high expression of genomic RPL10 from the GAL1 promoter efficiently outcompeted the dominant negative mutants (data not shown). From this screen, 26 different mutants were obtained. Six contained two mutations, which were subsequently subcloned to isolate the single amino acid changes. In addition to the random mutations, we engineered four directed mutants. In an attempt to disrupt Rpl10 binding to the ribosome without significantly altering the structure of Rpl10, we mutated two regions of basic amino acids that were likely important for electrostatic interaction with tRNA. Within α helix 2 of Rpl10, which contacts helix 89 of 25 S rRNA, we mutated Arg313 and Arg314 each to glutamate. Separately, Lys38, Lys39, and Lys40 in α helix 1, which are modeled to interact with helix 38 of 25 S rRNA, were each changed to alanine. In addition, we made the combined K38A,K39A,K40A,R153E, R154E mutant (referred to as KKKRR). Finally, the mutational analysis identified a cluster of mutations within an internal loop of Rpl10. The corresponding loop in L10e was not resolved in the crystal structure of the 50 S subunit from H. marismortui (2), suggesting that this loop is not structured in the free subunit, consistent with NMR analysis of free L16 (4). To examine the function of this loop, we deleted amino acids 102–112 (Δ102–112). Altogether, we analyzed 39 different mutations (Fig. 1 and Table 3).

Phenotypic Analysis of rpl10 Mutants—Serial dilutions of the rpl10 mutants expressed in AJY2104 (GAL1::RPL10) were plated onto selective plates to examine growth under repressing conditions for genomic RPL10 (Fig. 2). Because we initially screened for slow growth or lethality, we obtained mutants with a spectrum of growth phenotypes. K40E, F136L, and K203M were initially obtained in combination with second mutations and did not appear to contribute significantly to the phenotype of the double mutant (data not shown and Table 3). In Fig. 2 the rpl10 mutants are arranged roughly in the order in which the mutations appear in the gene to aid in identifying mutations with common phenotypes that map close together in primary sequence. The most striking result from this analysis was the cluster of mutations from Ser104 to Met115. These mutations were all lethal and corresponded to an unstructured loop in L10e, indicating that these residues are not needed for any stable contacts with the large subunit. The apparent growth observed in the first two dilutions of some of these mutants represents residual growth supported by preexisting ribosomes. These mutants were not able to form colonies after prolonged incubation (data not shown). Because it was possible that amino acid substitutions within this loop resulted in structural or electrostatic conflicts in the ribosome, we engineered a deletion of this loop that, based on the crystal structure of L10e from H. marismortui, was expected to remove the entire loop without significantly distorting the remainder of Rpl10. Like the point mutations within the loop, the deletion mutant (rpl10Δ102–112) was lethal (Fig. 2).

Rpl10 shows genetic interaction with NMD3, encoding the 60 S subunit export adapter NMD3 (12, 14, 16, 23, 25), and with SQTL1, which encodes a WD repeat protein that interacts with and stabilizes free Rpl10 (12, 13). Because high copy NMD3

| Summary of rpl10 mutants |
|---------------------------|
| Growth in rpl10Δa         | Dominant negativeb | Cellular localizationd |
| Wild type                 | ++ + +             | no                      | Cyt                        |
| Y11N                      | ++                 | no                      | Nuc                        |
| Δ31–32                    | −                   | yes                     | Nuc                        |
| K38A,K39A,K40A            | ++ +               | no                      | Cyt                        |
| K40E,C49R                 | −                   | no                      | NDd                        |
| K40E                      | ++ + +             | no                      | ND                         |
| E45G                      | +                   | no                      | Nuc                        |
| C49R                      | −                   | yes                     | Nuc                        |
| L52S                      | −                   | no                      | ND                         |
| V53G                      | −                   | no                      | Nuc                        |
| Q59R                      | −                   | no                      | Nuc                        |
| S62P                      | +                   | no                      | Cyt/Cyt                    |
| L65S                      | ++                 | +                       | Nuc/Cyt                    |
| N73D                      | ++ + +             | no                      | Nuc/Cyt                    |
| L87S                      | −                   | no                      | Cyt                        |
| R90S,L97S                 | −                   | no                      | Nuc                        |
| R90S                      | + + + +            | no                      | Cyt/Cyt                    |
| L97S                      | −                   | no                      | ND                         |
| K101E,K203M               | −                   | no                      | Nuc/Cyt                    |
| K101E                     | ++ + +             | no                      | Nuc/Cyt                    |
| Δ102–112                  | −                   | yes                     | Cyt                        |
| S104P                     | +                   | no                      | Cyt/Cyt                    |
| C106R                     | −                   | no                      | Cyt                        |
| R110G                     | −                   | no                      | Cyt/Cyt                    |
| L111S,K203M               | −                   | no                      | Cyt                        |
| M1115                     | −                   | no                      | ND                         |
| M1115T                    | −                   | no                      | Cyt                        |
| L115T                     | −                   | no                      | Nuc/Cyt                    |
| L115T                     | −                   | no                      | Nuc/Cyt                    |
| L153T                     | −                   | no                      | Nuc/Cyt                    |
| F136L                     | ++ + +             | no                      | NDd                        |
| S137P                     | −                   | no                      | Nuc                        |
| G151V                     | −                   | yes                     | Nuc/Cyt                    |
| R153E,R154E               | +                   | yes                     | Nuc/Cyt                    |
| K38A,K39A,K40A,R153E,R154E| −                   | yes                     | Nuc                        |
| L163V,E180G               | +                   | ND                      | ND                         |
| L165V                     | ++ + +             | Cyt                     |
| E180G                     | ++ + +             | Cyt                     |
| K203M                     | ++ + +             | ND                      | ND                         |
| F213L                     | ++ + +             | Cyt                     |

* Growth in GAL1::RPL10 strain (AJY2104) under repressing conditions.

+ Scored in the wild-type strain BY4741.

- Determined by IF in BY4741. Nuc, nuclear; Nuc/Cyt, nuclear and cytoplasmic with a nuclear bias; Cyt/Nuc, cytoplasmic and nuclear with a cytoplasmic bias; Cyt, cytoplasmic.

** ND, not determined.
suppresses certain rpl10 mutants by bypassing their defect in recycling Nmd3 (16), we examined the effect of high copy NMD3 on the rpl10 mutants. Surprisingly, only a single mutant, rpl10Q59R, showed significant suppression by high copy wild-type NMD3 (Fig. 2, column 2). We also tested the effect of expressing an nmd3 mutant (NMD3I112TI362T) that has reduced affinity for 60 S subunits and suppresses certain rpl10 mutants that block Nmd3 release in the cytoplasm (16) (Fig. 2, column 3). Nmd3(I112TI362T) was able to suppress various rpl10 mutants. We do not presently understand why high copy NMD3 and NMD3(I112TI362T) suppress rpl10 mutants differentially. It is possible that the release of Nmd3 is coordinated with Rpl10 loading. If so, mutant Nmd3 with weakened affinity for the subunit, but not overexpression of wild-type Nmd3, could allow some defective Rpl10 proteins to load into the subunit. It has been shown previously that C-terminally truncated rpl10 mutants fail to assemble stably with the ribosome and titrate out cellular Sqt1 (12, 13). Because overexpression of SQT1 relieves the dominant effect of these rpl10 mutants, we tested whether overexpression of SQT1 could suppress any of the rpl10 point mutants as an indicator of mutants that are defective for assembly into the ribosome. Because Sqt1 stabilizes free Rpl10, high copy SQT1 is also likely to suppress rpl10 mutations with reduced binding to Sqt1. High copy SQT1 suppressed various rpl10 mutants (Fig. 2, column 4). Interestingly, there was considerable overlap between the mutants suppressed by NMD3I112TI362T and by high copy SQT1, the exceptions being V53G, N73D, and G151V, which were preferentially suppressed by high copy SQT1, and R90S, L97S and KKKRR, which were preferentially suppressed by mutant nmd3. The suppression of rpl10(KKKRR) by NMD3(I112TI362T) was surprising because this mutant Rpl10 does not stably interact with the ribosome. As noted above, the release of Nmd3 may be coordinated with the loading of Rpl10. Mutant Rpl10 that cannot efficiently bind to the ribosome may not be able to trigger the release of Nmd3. However, mutations in Nmd3 that weaken its interaction with the ribosome may alleviate this problem. Finally, we tested all mutants for dominant negative effects when expressed from a low copy vector in wild-type cells (Fig. 3, data not shown, and Table 3).

**FIGURE 2. Growth assays of rpl10 mutants.** AJY2104 was transformed with plasmids expressing the indicated rpl10 mutants. Strains also contained either empty vector, high copy NMD3 (2μ NMD3), high copy NMD3(I112TI362T), or high copy SQT1. 10-fold serial dilutions of cultures grown in selective medium with galactose were spotted onto selective media containing glucose and incubated at 30°C for 3 days. The mutants, listed in the left margin, are arranged approximately in the order in which they occur in the primary sequence of Rpl10. The first two rows show vector (no Rpl10) and wild type (WT, pAJ1197) as controls.
Δ31–32, C49R, L65S, G151V, and KKKRR showed modest effects in this assay, whereas deletion of the internal unstructured loop (amino acids 102–112) was strongly dominant negative. There was no obvious correlation between mutants that were dominant negative and those that were suppressed by NMD3 or SQT1. The slightly reduced 60S subunit levels in the wild-type sample is likely due to a subtle effect of the C-terminal epitope tag.

To examine the effects of the rpl10 mutants on 60S biogenesis and translation, we analyzed the polysome profiles of selected mutants by sucrose gradient centrifugation. The mutant Rpl10 proteins were expressed ectopically in the presence of wild-type Rpl10, to allow similar growth of all cultures. RPL10 expression was then repressed by growth in glucose-containing medium. Because Rpl10 is continuously incorporated into ribosomes during cell growth, the pool of wild-type Rpl10 is rapidly depleted. Under these conditions, the wild-type Rpl10 control showed modest halfmers. However, all of the mutants tested gave profiles similar to the vector control (Fig. 4 and data not shown), indicating that the mutant proteins were not supporting ribosome assembly.

Coimmunoprecipitation and Sucrose Gradient Sedimentation—To test more directly for interactions between the mutant Rpl10 proteins and the ribosome, Nmd3, Sqt1, and Lsg1, we carried out co-IP assays. The mutant Rpl10 proteins were immunoprecipitated from extracts by virtue of a C-terminal oligonucleotide Myc epitope tag. The samples were then analyzed by SDS-PAGE and Western blotting for Rpl8 as a 60S subunit marker, Nmd3, Sqt1, and Lsg1 (Fig. 5). The results from this analysis were complex, although several important conclusions can be drawn. First, the KKKRR mutant, engineered to be defective for ribosome binding, coimmunoprecipitated Sqt1 but not Rpl8, indicating that it was not efficiently incorporated into the subunit. Second, the mutations within the internal unstructured loop all resulted in increased levels of Sqt1 and Nmd3 in the IPs. Deletion of the internal unstructured loop (rpl10Δ102–112) was the only mutant that showed detectable levels of Lsg1.

The interpretation of the IP data is complicated because Rpl10 is present in at least three different species that cannot be resolved in this analysis. These are: free Rpl10 that is not assembled into the 60S subunit (and likely bound to Sqt1); the free 60S species; and 80S ribosomes in the monosome and polysome.
Mutational Analysis of Rpl10

A  

B

FIGURE 6. Immunoprecipitation of selected mutants from sucrose gradients. The indicated mutants were expressed in AUY2104 and grown in selective medium containing galactose. The extracts were prepared and separated on 7–47% sucrose gradients. A, fractions were collected and the absorbance at 254 nm was monitored continuously and fractions were assayed for the presence of mutant Rpl10, Nmd3, Sqt1, and Rpl18. B, fractions 1 and 2, representing the free protein pool, and fractions 5 and 6, representing the free 60 S peak, were pooled separately. Myc-tagged Rpl10 proteins were immunoprecipitated from these pools, samples were separated by SDS-PAGE, and the presence of Sqt1 and Nmd3 was determined by Western blotting. WT, wild type.

fractions. Because Nmd3 is found only in the position of free 60 S, an Rpl10 mutant that is predominantly in free 60 S but deficient from the bulk of 60 S in polysomes will appear enriched for Nmd3 relative to Rpl8, potentially giving the impression of an Nmd3-Rpl10-containing species that is deficient for Rpl8. To determine whether the Sqt1 and Nmd3 that was observed in the mutant Rpl10 IPs were associated with free Rpl10 or Rpl10 in 60 S subunits and to determine the distribution of the mutant proteins between free, 60 S, and 80 S, we analyzed selected mutants by sucrose gradient sedimentation (Fig. 6A). Gradient fractions were analyzed by SDS-PAGE and Western blotting for Rpl10, Rpl8, Nmd3, and Sqt1. We also immunoprecipitated Rpl10 from the free 60 S peak and from the ribosome-free pool at the top of the gradient, followed by Western blot analysis (Fig. 6B). In all of the mutants that we examined, Nmd3 was associated only with the free 60 S peak; a free pool of Nmd3 was not detected. Similarly, Sqt1 was found exclusively at the top of the gradient. Thus, although Rpl10Δ102–112 shows unusually strong signals for Sqt1 and Nmd3 in the IP in Fig. 5, Sqt1 and Nmd3 appear to be in separate species.

The different Rpl10 mutants displayed different sedimentation patterns, indicating different degrees of ribosome association and utilization in translating ribosomes. The KKKRR mutant, for example, which showed very little Rpl8 association but a strong signal for Sqt1 by IP (Fig. 5), was strongly biased toward the top of the gradient with very little signal associated with ribosomes (Fig. 6). More interesting was Rpl10Δ102–112, which was present in free 60 S subunits but was not efficiently incorporated into polysomes (similar results were obtained with Rpl10 L111S; data not shown). This suggests that mutations in the internal loop affect utilization of the 60 S subunit in translation and that the strong dominant negative phenotype of the Δ102–112 mutant may be a functional failure of the subunit in the cytoplasm (see discussion). The high 80 S monosome peak and relatively low polysomes seen in Fig. 6 are typical of cells utilizing galactose as their carbon source.

Cellular Localization of Mutant Proteins—We have previously provided evidence that Rpl10 loads into the nascent 60 S subunit in the cytoplasm (12). However, Rpl10 has been identified in pre-60 S particles affinity-purified from whole cell extracts with epitope-tagged nuclear pre-60 S associated proteins (19). To determine whether any of the mutant proteins would shed light on Rpl10 assembly into the ribosome, we determined their cellular localization by indirect immunofluorescence utilizing their C-terminal epitope tags. The immunofluorescence experiments were carried out in a strain expressing genomic wild-type Rpl10 under control of its native promoter. We were surprised to find that the different mutants showed a range of localization patterns from exclusively nuclear to exclusively cytoplasmic (Fig. 7 and Table 3). For most of the mutants, there was not a clear correlation between localization and other properties of the mutants. However, the mutants that were engineered to disrupt ribosome binding showed instructive. The K38A,K39A,K40A mutant, which is via-

hand, combining the K38A,K39A,K40A mutant with the R153E,R154E, which was lethal and abolished ribosome binding and binds ribosomes, was largely cytoplasmic. On the other hand, combining the K38A,K39A,K40A mutant with R153E,R154E, which was lethal and abolished ribosome binding, led to an almost exclusively nuclear localization (Fig. 7). Furthermore, overexpression of wild-type RPL10, leading to Rpl10 in excess of 60 S subunits, resulted in nuclear accumulation of the protein (data not shown). Thus, Rpl10 that is not bound to ribosomes, resulting from overexpression of wild-type protein or mutations in Rpl10 that impair ribosome binding, accumulates in the nucleus.

We have speculated previously that the Rpl10-binding protein Sqt1, which is highly negatively charged, may act to prevent Rpl10 accumulation in the nucleus by masking sequence that could serve as a nuclear localization signal (12). We tested whether overexpressing SQT1 would prevent the nuclear accumulation of Rpl10. In this case, the quintuple mutant, which is able to bind Sqt1 but not the ribosome, was coexpressed from a low copy vector with either empty vector or with high copy SQT1. We observed no reallocation of mutant Rpl10 from the nucleus (Fig. 8A). Because Sqt1 binds free Rpl10 and free Rpl10 accumulates in the nucleus, we asked whether the accumulation Rpl10 in the nucleus would lead to the accumulation of
Sqt1 in the nucleus as well. Indeed, expression of the quintuple mutant or overexpression of wild-type Rpl10 in a strain expressing a Sqt1-GFP fusion protein resulted in nearly complete relocalization of Sqt1-GFP to the nucleus (Fig. 8B). Thus, the cellular localization of Sqt1 is dependent on Rpl10.

DISCUSSION

An Unstructured Loop of Rpl10 Is Critical for Its Function—In structural determinations of L10e and L16, a highly conserved internal loop is not resolved, indicating that it is disordered and consequently not involved in stable contacts within the large subunit. We were surprised to find a cluster of mutations within this loop that impaired the release of Nmd3. Furthermore, the complete deletion of this loop (rpl10Δ102–112) resulted in a potently dominant negative protein. Sucrose gradient sedimentation indicated that Rpl10Δ102–112, as well as the most strongly affected point mutant (Rpl10 L111S), were incorporated into subunits, but those subunits did not join or stably bind 40 S subunits. Rpl10Δ102–112 was predominantly cytoplasmic and did not cause Nmd3 to accumulate in the nucleus.3 Combining the deletion of amino acids 102–112 with the quintuple point KKKRR, which by itself prevents binding to the subunit, resulted in nuclear accumulation of the protein, loss of 60 S binding, and loss of the dominant negative phenotype.4 Thus, the dominant negative effect of Rpl10Δ102–112 is dependent on the protein being incorporated into the subunit and appears to occur in cytoplasmic subunits. This is further supported by the presence of the cytoplasmic GTPase Lsg1 in the Rpl10Δ102–112-containing particle. Considering the strong Nmd3 signal in immunoprecipitations with these loop mutants, we suggest that Nmd3 is not dissociating from these subunits and that this internal loop of Rpl10 plays a dynamic role in the release of Nmd3. We previously showed that mutations in the cytoplasmic GTPase Lsg1 act in a fashion similar to certain Rpl10 mutants in blocking the release of Nmd3. It is possible that the unstructured loop of Rpl10 acts as an effector for the GTPase of Lsg1, stimulating its function on the subunit.

Recently, it has been reported that defective ribosomal subunits bearing point mutations in either the large or small sub-

3 A. Hofer and A. W. Johnson, unpublished observations.

4 C. Bussiere and A. W. Johnson, unpublished observations.
unit rRNA are subject to degradation in yeast (26). These point mutations presumably escape detection during subunit assembly and are recognized only upon translation in the cytoplasm. The most severely affected mutant ribosomes are observed only as free 60 S subunits and are not observed in 80 S ribosomes or polysomes. This is similar to the behavior of mutant Rpl10 proteins A102–112 and L111S, containing mutations in the internal unstructured loop. It will be of interest to determine whether these rpl10 mutations also lead to increased subunit degradation analogous to what was observed for rRNA mutations. If Rpl10 exchanges on subunits (11), its loading or release could be a checkpoint for ribosome function, possibly initiating decay of the defective ribosomes.

Rpl10 in the Nucleus—Our previous results suggested that 60 S subunits can be exported from the nucleus by Nmd3 without a specific requirement for Rpl10 (12). Furthermore, we showed that subunits bound by Nmd3 had substoichiometric amounts of Rpl10 (12), suggesting that Rpl10 loads into the subunit after export to the cytoplasm by Nmd3. Consequently we were surprised to find that mutant Rpl10 that cannot bind to the ribosome, or Rpl10 that is overexpressed accumulates in the nucleus. If Rpl10 does initially bind to pre-60 S subunits in the nucleus and remains stably associated with the subunit, we should be able to detect Rpl10 in the nucleus under conditions that block export of the ribosome. However, Rpl10 does not appear to be trapped in the nucleus by dominant mutations in Nmd3 that block subunit export (14, 15) by inhibition of Crm1-dependent export by leptomycin B or in a mex67 mutant that also blocks 60 S export (data not shown). If Rpl10 that cannot bind to the ribosome accumulates in the nucleus, one might also expect Rpl10 to accumulate in the nucleus in a mutant that fails to assemble a binding site for Rpl10. Repression of RPF2 leads to the production of pre-60 S particles that lack Rpl10.5 We found that repression of RPF2 led to a strong accumulation of the large subunit reporter Rpl25-eGFP, but no apparent accumulation of Rpl10.4 It seems that either the dynamics of the interaction of Rpl10 with the subunit are more complex than we initially imagined or that free Rpl10 may play a role in the nucleus separate from the ribosome.

RPL10 and Human Disease—Two missense mutations in human RPL10 have recently been identified in two pairs of autistic siblings (28). Although causality has not been established, it is postulated that mutations in RPL10 may contribute to autism. The two mutations that were identified were L206M and H213Q. Klauck et al. (28) went on to show that when they replaced yeast Rpl10 with wild-type or mutant human Rpl10, the mutants had reduced levels of polysomes, indicating a defect in translation. These mutations fall within the C-terminal domain of Rpl10 that is unique to the eukaryotic Rpl10 proteins. We have shown previously that deletion of this domain prevents the stable association of Rpl10 with the 60 S subunit in yeast (12). In our mutational analysis of Rpl10, we identified two point mutations in this region. K203M and F213L (Figs. 1 and 2 and data not shown). K203M, which is only three residues from the position corresponding to human Leu206, was found in combination with two other point mutations. When these mutations were separated by subcloning, K203M did not appear to cause an obvious phenotype on its own or exacerbate the phenotype of other K101E or L111S (data not shown). In contrast, F213L, which corresponds in position to human His213, had a strong growth phenotype and strongly reduced polsosome profile. This mutation was suppressed by mutant Nmd3 as well as high copy Sqt1. Furthermore, IPs with F213L showed a strong signal for Sqt1, consistent with previous results in which deletion of this region led to unstable interaction with the subunit and increased binding to Sqt1. Based on our previous work, showing that Rpl10 is needed for the release of the export adapter Nmd3, we suggest that mutations in this region of Rpl10 affect the dynamics of Rpl10 loading and release of Nmd3. It is conceivable that Nmd3 plays a role in cytoplasmic transport of ribosomes as well as nuclear export and that activation requires localized loading of Rpl10. In neurons, where ribosomes function distally from their site of synthesis in the nucleolus, modest defects in the transport and localized activation of ribosomes may have greater consequences than in other cell types less dependent on these pathways.

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