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Zuotin, a ribosome-associated DnaJ molecular chaperone

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Correct folding of newly synthesized polypeptides is thought to be facilitated by Hsp70 molecular chaperones in conjunction with DnaJ cohort proteins. In *Saccharomyces cerevisiae*, *SSB* proteins are ribosome-associated Hsp70s which interact with the newly synthesized nascent polypeptide chain. Here we report that the phenotypes of an *S.cerevisiae* strain lacking the DnaJ-related protein Zuotin (Zuo1) are very similar to those of a strain lacking Ssb, including sensitivities to low temperatures, certain protein synthesis inhibitors and high osmolarity. Zuo1, which has been shown previously to be a nucleic acid-binding protein, is also a ribosome-associated protein localized predominantly in the cytosol. Analysis of *zuo1* deletion and truncation mutants revealed a positive correlation between the ribosome association of Zuo1 and its ability to bind RNA. We propose that Zuo1 binds to ribosomes, in part, by interaction with ribosomal RNA and that Zuo1 functions with Ssb as a chaperone on the ribosome.
Keywords: DnaJ/molecular chaperone/ribosome/ RNA binding/Zuo1

Introduction

Molecular chaperones are ubiquitous proteins which bind to proteins in non-native conformations, thus facilitating the cellular processes of protein folding and translocation across membranes (Hartl, 1996). Chaperones are involved in the biogenesis of many proteins from the time of their synthesis until the time they reach their native conformation in the appropriate cellular compartment. The period spent as a nascent chain on a ribosome during synthesis is a particularly crucial time in the life of a protein. Trigger factor, a peptidyl-prolyl isomerase found in *Escherichia coli* (Hesterkamp *et al.*, 1996) and Ssb, an Hsp70 of *Saccharomyces cerevisiae* (Nelson *et al.*, 1992; Pfund *et al.*, 1998), are ribosome-associated chaperones which interact with nascent chain and likely play an important role in early protein-folding events.

Molecular chaperones rarely function alone; rather they function together in complex pathways (Johnson and Craig, 1997). The most well-studied chaperone partnership is one found in *E.coli* between DnaK, a chaperone of the Hsp70 class, and DnaJ, an Hsp40. Both genetic and biochemical evidence indicates a functional partnership between these two chaperones (Georgopoulos *et al.*, 1994; Bukau and Horwich, 1998). Strains with mutations in *dnaJ* or *dnaK* have similar phenotypes including slow growth at temperatures >30°C and failure to replicate lambda phage DNA (Sell *et al.*, 1990). DnaJ and DnaK can both bind to unfolded or partially unfolded polypeptides. In some cases, DnaJ is thought to target substrate proteins to DnaK (Gamer *et al.*, 1992, 1996; Wall *et al.*, 1995). The binding of DnaK to unfolded polypeptides is modulated by the binding of ATP to its N-terminal ATPase domain. However, in addition to binding unfolded proteins, DnaJ interacts with DnaK, stimulating its ATPase activity (Liberek *et al.*, 1991). This enhanced ATPase activity increases the rate of conversion of the ATP- to the ADP-bound form of DnaK. Since the ADP-bound form of DnaK binds more stably to unfolded polypeptides than the ATP-bound form, DnaJ's association with DnaK acts to stabilize the interaction between DnaK and its substrate polypeptide (Schmid *et al.*, 1994). All DnaJ-type proteins (called DnaJs throughout) contain a signature J domain which is required for interaction with DnaK (Karzai and McMacken, 1996; Szabo *et al.*, 1996), but vary in the number and type of additional domains. Therefore, while all DnaJs may not bind unfolded proteins, all are expected to interact with an Hsp70 via their J domain. Other domains may be involved in binding unfolded polypeptides or targeting the DnaJ to a particular cellular location.

Eukaryotes have multiple Hsp70s and DnaJ proteins. Only a few partnerships between these various DnaJs and Hsp70s have been elucidated. For example, it has been demonstrated that the DnaJ-like protein auxilin is necessary for localization of Hsc70 to clathrin-coated baskets (Ungewickell *et al.*, 1995). In the recently completed sequence of the *S.cerevisiae* genome, 14 genes encoding Hsp70 homologs and 18 genes encoding potential DnaJ proteins have been identified (Zuber *et al.*, 1998). These chaperones are localized to different cellular compartments, including the endoplasmic reticulum, mitochondrion and cytosol. In the yeast endoplasmic reticulum (ER), the DnaJ, Sec63, targets the Hsp70, Kar2, to its site of action on the lumen side of the ER, where both participate in the translocation of proteins across the ER membrane (Brodsky and Schekman, 1993; Corsi and Schekman, 1997). In the cytosol of yeast, two classes of Hsp70s have been well characterized: *SSA* and *SSB* proteins (Craig *et al.*, 1994). The four *SSA* proteins are involved in translocation of proteins into the mitochondria and ER, regulation of the heat shock response and protein folding. Ydj1, a cytosolic yeast DnaJ, has genetic interactions with Ssa1 (Becker *et al.*, 1996) and also functions in the translocation of preproteins (Atencio and Yaffe, 1992; Caplan *et al.*, 1992). Additionally, Ydj1 has been shown to stimulate the ATPase activity of Ssa1, suggesting a functional partnership (Cyr and Douglas, 1994).

The *SSB* family members, composed of the nearly identical Ssb1 and Ssb2 (referred to collectively as Ssb), have been of particular interest because of their association with ribosomes (Nelson *et al.*, 1992). Strains lacking the Ssbs are viable, but cold-sensitive for growth and sensitive to certain protein synthesis inhibitors including aminoglycosides such as paromomycin and hygromycin B. This hypersensitivity to protein synthesis inhibitors and ribosome association suggests a role for Ssbs in protein translation. In fact, Ssb can be crosslinked to the nascent chain, suggesting a role for these Hsp70s in translation and early folding events (Pfund *et al.*, 1998). Thus far, a DnaJ-partner for Ssb has not been identified. Because another yeast cytosolic DnaJ, Sis1, has been localized to the ribosome (Zhong and Arndt, 1993), it has been the only suggested candidate. However, *sis1* mutants do not show any phenotypes similar to those demonstrated by strains lacking Ssb.

The subject of this report is the DnaJ protein of *S.cerevisiae*, Zuotin (Zuo1). Zuo1 has been identified *in vitro* as a tRNA and Z DNA-binding protein (Zhang *et al.*, 1992; Wilhelm *et al.*, 1994). Like all DnaJ proteins, it contains a classic J domain. Zuo1 also contains a domain rich in charged amino acids with an overall net positive charge. Here we show that Zuo1 associates with both translating and non-translating ribosomes, likely via an interaction with rRNA. Deletion of *ZUO1* results in the same phenotypic effects as deletion of *SSB1* and *SSB2*, suggesting that Zuo1 is the DnaJ partner for Ssb, acting to chaperone nascent chains on the ribosome.

Results

ssb and zuo1 mutants have similar phenotypes

To understand the function of the DnaJ homolog, Zuo1, the phenotypes of a *Zuo1* deletion strain (Δ*zuo1*) were analyzed. Compared with a wild-type strain, growth of the deletion strain was severely impaired at temperatures below 30°C, particularly at 18°C, and in the presence of 75 μg/ml of the translation-inhibiting drug, paromomycin (Figure 1). Previously, we reported that strains lacking the Ssb family of Hsp70s exhibited the same phenotypes (Nelson *et al.*, 1992; Figure 1). In addition, both the *zuo1* and *ssb1ssb2* mutant strains showed impaired growth in the presence of 1 M NaCl. To investigate possible synthetic interactions between a *zuo1* mutation and the *ssb1* and *ssb2* mutations, a strain containing all three mutations was constructed. The phenotypes of the triple mutant were very similar to those of strains having the *zuo1* or *ssb1ssb2* mutations alone (data not shown), indicating a lack of additive effects of the mutations.

Zuo1 is predominantly a cytosolic, not a nuclear protein

DnaJ proteins and Hsp70s have been shown to function together in several cellular compartments (Zuber *et al.*, 1998). Zuo1 had been found previously in nuclear extracts (Zhang *et al.*, 1992; Wilhelm *et al.*, 1994). Since the Hsp70 Ssb has been identified as a cytosolic protein, we re-examined the localization of Zuo1 in cells. Microscopic analysis using a fusion of green fluorescent protein (GFP) with the carboxyl end of Zuo1 was used to determine the cellular localization of Zuo1. This Zuo1–GFP fusion is functional as it rescues the cold-, NaCl- and paromomycin-sensitive phenotypes of a Δ*zuo1* deletion strain and is expressed from the construct as a protein of the expected mobility (data not shown). As can be seen from Figure 2, the Zuo1 fusion is predominantly in the cytosol, showing little fluorescence in the nucleus, whose position is indicated by 4,6-diamidino-2-phenylinodole (DAPI) staining. Fluorescence is also absent from the vacuole. Therefore, we conclude that the majority of Zuo1 in the cell is in the cytosol.

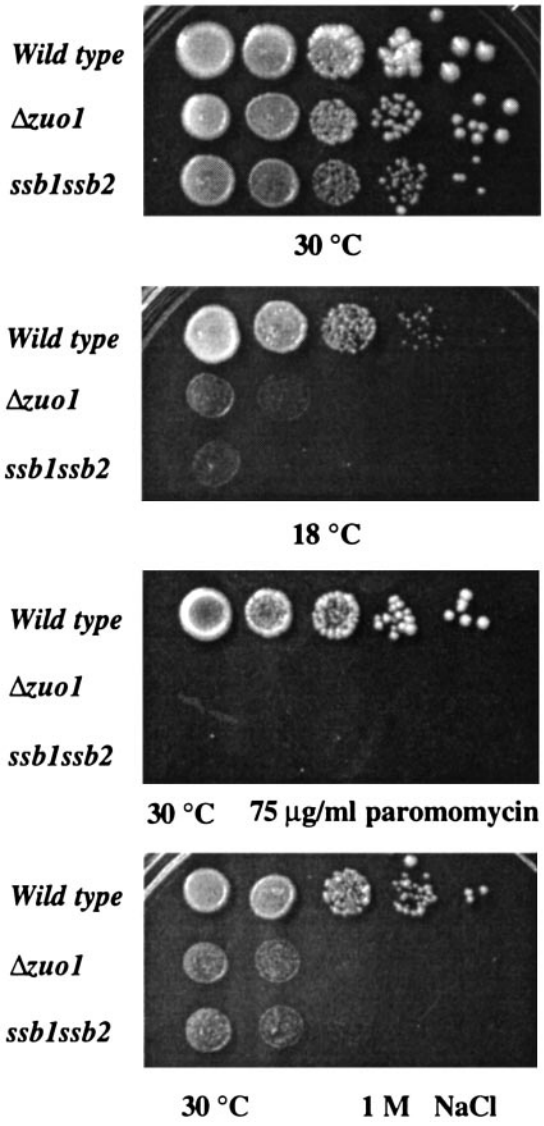

Fig. 1. Δ*zuo1* and *ssb1 ssb2* have similar phenotypes. Cells were grown overnight, counted, and an equal number of cells were spotted as a 10-fold dilution series on YPD plates containing the indicated additions and at the temperatures indicated. Plates were incubated for 4 days, except those at 18°C which were incubated for 5 days.

Zuo1 is associated with ribosomes

Since Ssb has been shown to bind ribosomes, we tested the possible interaction of Zuo1 with ribosomes. Crude cell lysates were prepared and centrifuged through sucrose density gradients. Fractions of the gradients were run on an SDS–polyacrylamide gel and immunoblotted using Zuo1-specific antiserum. The migration of Zuo1 coincided

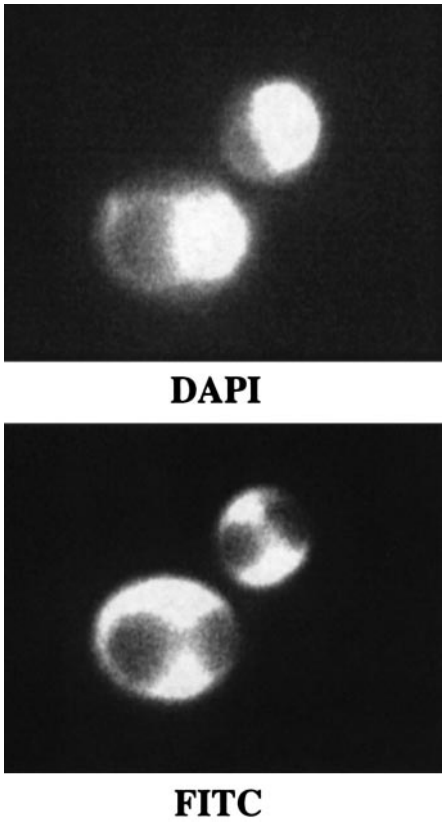

Fig. 2. Cellular distribution of Zuo1–GFP fusion. A Δ*zuo1* strain was transformed with a plasmid carrying a *ZUO1*–GFP fusion gene. Nuclei were localized by staining with DAPI (upper panel). FITC fluorescence of the *ZUO1*–GFP in the same cells is shown in the bottom panel.

closely with the polysome profile and the migration of ribosomal protein L3 (Figure 3A). We employed RNase treatment to degrade the mRNA which links translating ribosomes together into polysomes. After this treatment, the ribosomes migrated with a velocity of 80S, as indicated by both the profile and the migration of ribosomal protein L3. Similarly, Zuo1 sedimented with a velocity no higher than 80S after RNase A treatment. These results indicate that Zuo1 is associated with a high-molecular weight cellular component that contains RNA.

To determine whether the RNase-sensitive Zuo1-containing complexes were ribosomal complexes, we used a strain containing a temperature-sensitive mutation in *PRT1* to alter the migration of polysomes (Feinberg *et al.*, 1982). Prt1 is a component of translation initiation factor eIF-3 (Naranda *et al.*, 1994) required for the initiation of translation. After a 20 min shift to the restrictive temperature of 37°C, the ribosomes of the temperature-sensitive *prt1-1* strain failed to reinitiate translation and accumulated as non-translating 80S monosomes (Figure 3B). Consequently, Zuo1 co-migrated with the 80S peak (Figure 3B). However, temperature shift of the wild-type control strain had no significant effect on the polysome profile or the migration of Zuo1 in the gradient (data not shown). The coincidence of migration of Zuo1 with polysomes and with the 80S peak in the *prt1-1* mutant after shift to the non-permissive temperature strongly suggests that Zuo1 is associated with ribosomes.

No Zuo1 was detected in the soluble fraction after shift

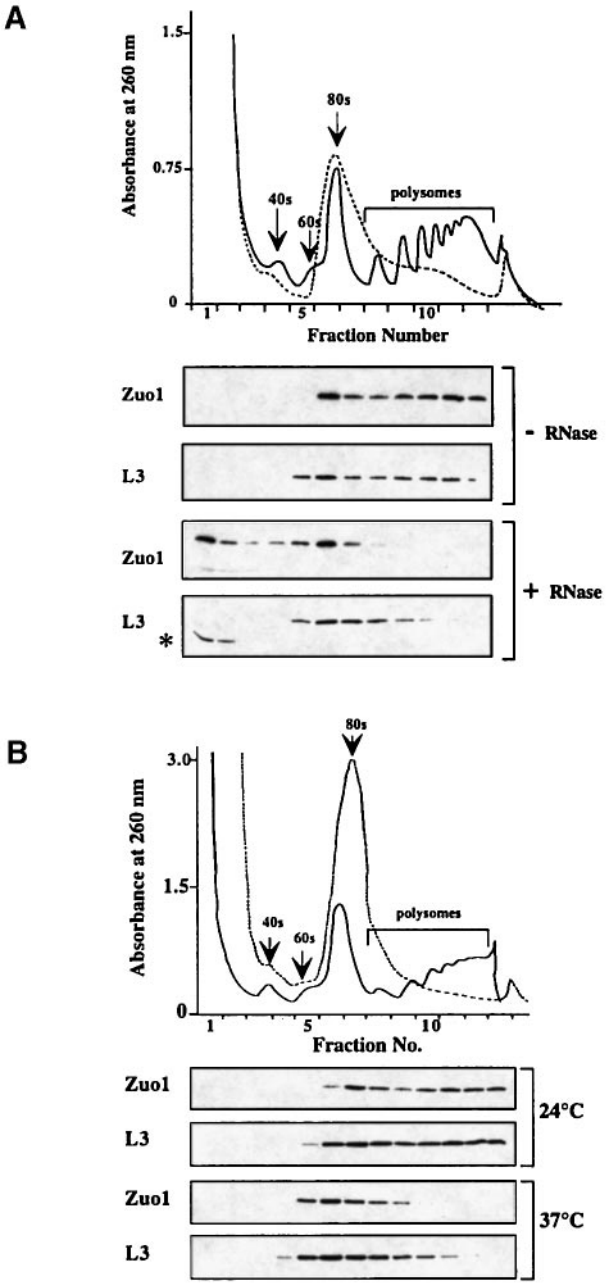

Fig. 3. Zuo1 co-migrates with ribosomes. Extracts were prepared from cells and run through sucrose gradients. Fractions were collected and subjected to immunoblotting with antibodies specific for Zuo1 or the ribosomal protein L3. (**A**) An aliquot of an extract from wild-type cells was treated with RNase to convert polysomes to monosomes. Tracing of OD_{260} of sucrose gradient: solid line, untreated; broken line, RNase treated. The band indicated by an asterisk is a cross-reacting protein unrelated to L3. (**B**) Extracts were prepared from *prt1-1* cells either maintained at 24°C or grown at 24°C and shifted to 37°C for 20 min prior to harvest. Tracing of OD_{260} of sucrose gradient: solid line, 25°C; broken line, 37°C.

of *prt1-1* to the non-permissive temperature (Figure 3B). In contrast, as can be seen in Figure 3A, ~50% of Zuo1 was released into the soluble fraction upon RNase treatment. We found the amount of Zuo1 in association with ribosomes to be variable unless RNase inhibitors were used during preparation of cellular extracts (data not shown). Together, these results suggested to us that the

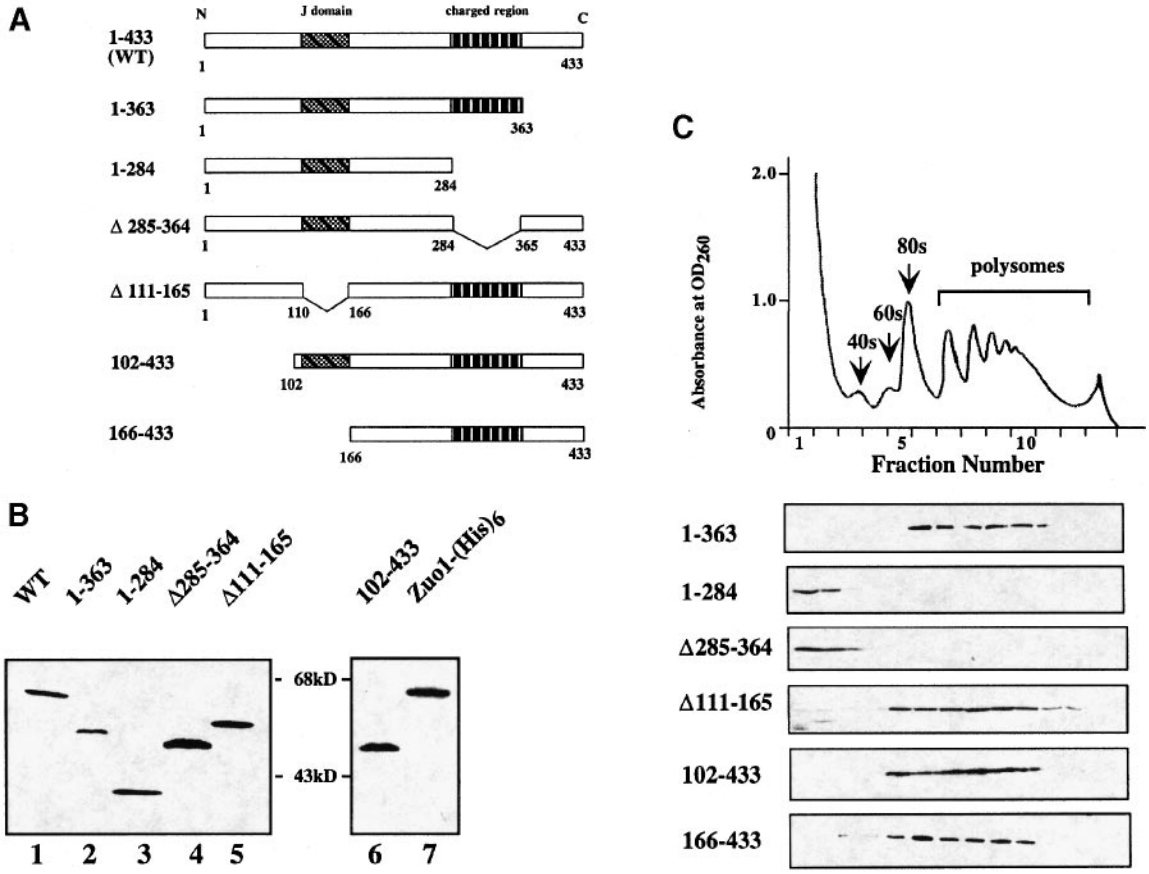

Fig. 4. Co-migration of Zuo1 mutant proteins with polysomes. (**A**) Diagram of *zuo1* mutants used in this study. All mutants which contain the N-terminus of Zuo1 were cloned into vectors allowing expression under both their own promoters and into N-terminal His-tagged versions under the control of the glycerol-3-phosphate dehydrogenase promoter (GPD) (see Materials and methods). The two N-terminal deletion mutants were cloned only into the His-tagged GPD vector. (**B**) Relative level of expression of Zuo1 mutants. The Zuo1 mutants and wild-type were transformed into a Δ*zuo1* strain. Equal amounts of extracts made from transformants grown at 30°C were subjected to electrophoresis and immunoblotted using a Zuo1-specific antibody in cases where proteins were under the control of the Zuo1 promoter (left panel) or an antibody specific for the N-terminal His-tagged proteins (right panel). (**C**) Extracts were prepared from Δ*zuo1* cells expressing wild-type or mutant Zuo1 and separated on sucrose gradients. The gradients were collected and the OD_{260} monitored. All patterns were similar; a representative sample is shown for mutant 1–284. Fractions were collected, proteins separated by SDS–PAGE, and subjected to immunoblotting using Zuo1-specific antibody or an antibody specific for the N-terminal His-tagged protein.

association of Zuo1 with ribosomes is particularly dependent on an intact RNA species.

The charged region of Zuo1 is required for ribosome association

To determine regions of Zuo1 required for ribosome binding, a series of deletion mutations in *ZUO1* were constructed (Figure 4A). In addition, internal deletions were made of Zuo1 which removed the conserved J domain (aa 111–165) and the highly charged region (aa 285–364). Wild-type *ZUO1* and each mutant were also tagged at the N-terminus with six histidine codons (His tag) to aid in purification of the encoded proteins. Each mutant construct was transformed into a Δ*zuo1* strain and tested for the expression of Zuo1 protein by immunoblot analysis (Figure 4B). In most cases, antibodies specific for the N-terminal 70 amino acid region of Zuo1 were used. In the case of N-terminal deletion mutants, which did not contain the epitope against which the Zuo1-specific antibody was raised, an antibody which reacted to the amino acid segment adjacent to the N-terminal His tag was utilized. The level of mutant proteins in the cell extracts were at, or slightly above, that of the wild-type protein with the exception of 1–363, which was found consistently to be at somewhat reduced levels.

To test whether the mutant Zuo1 proteins interact with ribosomes, extracts from the transformants carrying the mutants were centrifuged through sucrose density gradients (Figure 4C). All the Zuo1 mutant proteins were found to co-migrate with ribosomes with the exception of 1–284 and Δ285–364. Both of these mutants lack the highly charged region. These results suggest that the charged region located between amino acids 285 and 364 is important for the interaction of Zuo1 with the ribosome.

The charged region of Zuo1 is important for interaction with RNA

Zuo1 had been shown previously to be a tRNA- and Z-DNA-binding protein (Zhang *et al.*, 1992; Wilhelm *et al.*, 1994). Since light RNase treatment partially disrupted the interaction of Zuo1 with ribosomes, we tested whether the ability of mutant Zuo1 proteins to associate with ribosomes correlated with their ability to bind RNA. The Zuo1–RNA association was tested using a Northwestern binding assay (Wilhelm *et al.*, 1994). The mutant proteins described above and wild-type Zuo1 were partially

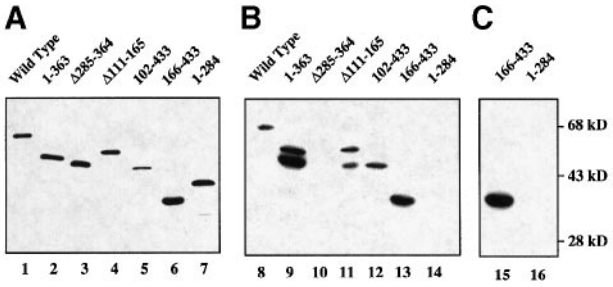

Fig. 5. Binding of Zuo1 mutant protein to RNA. N-terminal His-tagged Zuo1 mutant and wild-type proteins were partially purified from yeast as described in Materials and methods. Equivalent amounts were separated on several gels and transferred to nitrocellulose filters. (**A**) One filter was subjected to immunoblot analysis to determine the position of the Zuo1 proteins using an antibody specific for the N-terminal His-tagged protein. (**B**) The second filter was incubated in renaturing buffer and subsequently with ^{32}P-labeled Phe tRNA, washed and exposed to X-ray film. (**C**) The third filter, containing the 163–433 and 1–284 truncation mutant proteins, was incubated in renaturation buffer and subsequently with ^{32}P-labeled rRNA.

purified by virtue of the affinity of N-terminal His residues for Ni^{2+} (see Materials and methods). The partially purified proteins were separated by electrophoresis, transferred to nitrocellulose and incubated to allow renaturation. The immobilized proteins were then incubated with ^{32}P-labeled tRNA. The migration of the Zuo1 proteins during electrophoresis was monitored by immunoblotting a duplicate gel using an antibody specific for the extreme N-terminus (Figure 5A).

Consistent with previously published results (Wilhelm *et al.*, 1994), wild-type Zuo1 protein bound tRNA (Figure 5B). Several of the mutants also bound tRNA, as indicated by bands at the expected positions. The lower band of the doublets observed in lanes 2 and 4 of Figure 5B corresponds to degradation products of the mutant proteins. These degradation products were detected by the Zuo1-specific antibody (data not shown), but not the antibody specific for the extreme N-terminus (Figure 5A), indicating that they lack the extreme N-terminus of the Zuo1 protein.

No binding to tRNA was observed, even after very long exposures, to the C-terminal truncation lacking the last 149 amino acids or the deletion lacking amino acids 285–364 (Figure 5B, lanes 14 and 10). Both these mutant proteins lack the charged region. The same results were obtained using ^{32}P-labeled rRNA as a probe in Northwestern binding assays (Figure 5C; data not shown), indicating that Zuo1 can bind to ribosomal RNA as well as tRNA and that the absence of the charged region causes a dramatic impairment of Zuo1–RNA binding. Considering these results and those shown above, we conclude that the charged region is important for both RNA binding and interaction with the ribosome.

Analysis of regions of Zuo1 essential for function

To determine regions of Zuo1 required for function *in vivo*, the series of deletion mutants of *ZUO1* were transformed into a Δ*zuo1* strain. The resulting transformants were then tested for their ability to grow at a variety of temperatures, in the presence of paromomycin and 1 M NaCl (Figure 6). Cells containing Zuo1 which lacks the C-terminal 70 amino acids showed no growth defect compared with wild-type under any of the conditions tested. However, deletions lacking the N-terminal 102 amino acids, amino acids 111–165 which contain the J domain, or amino acids 284–363 that contains the charged region were incapable of rescuing either the cold-, paromomycin- or salt-sensitive phenotypes. Therefore, the charged region appears to be necessary not only for binding to the ribosome, but also for function of Zuo1. The N-terminal region and the J domain are also necessary for Zuo1 function in the cell, but neither for binding to RNA *in vitro*, nor for interaction with the ribosome.

Stability of the Zuo1 interaction with ribosomes

The interaction of Ssb with the translating ribosomes has been shown to be very stable, remaining associated with ribosomes after incubation in 1 M KCl (Pfund *et al.*, 1998). To determine the stability of the Zuo1–ribosome interaction, cell extracts in which translation had been 'frozen' by the addition of cycloheximide prior to harvest were centrifuged through 0.5 M sucrose cushions containing various concentrations of KCl ranging from 10 to 200 mM. Equivalent amounts of the material that pelleted through the sucrose cushion and the supernatant were immunoblotted (Figure 7A). As expected, the ribosomes were pelleted as indicated by the absence of the ribosomal protein L3 in all the supernatant fractions. However, the Zuo1–ribosome interaction was sensitive to KCl. We observed an equal distribution in the supernatant and pellet fractions in the presence of 150 mM KCl, and complete loss from the ribosomal pellet in 200 mM KCl.

Ssb interaction with non-translating ribosomes was tested using the *prt1-1* strain and found to be less stable than with translating ribosomes. All Ssb is found in the supernatant fractions when the concentration of KCl in the sucrose cushion is >200 mM (Pfund *et al.*, 1998). To test whether the Zuo1 interaction with non-translating ribosomes may differ from its interaction with translating ribosomes, we tested the stability of the Zuo1–ribosome interaction in the presence of KCl in the *prt1-1* mutant at the non-permissive temperature. After 20 min at 37°C, Zuo1 showed a stability similar to that found with the wild-type strain; for example, there was approximately an equal distribution of Zuo1 between supernatant and pellet at 150 mM KCl in both *prt1-1* and wild-type cells (Figure 7A). These results suggest that Zuo1 interacts similarly with translating and non-translating ribosomes, and therefore its interaction with ribosomes is not dependent on the presence of the nascent polypeptide chain.

Ssb1 interacts with ribosomes in the absence of Zuo1

Since some DnaJ-type proteins target Hsp70s to their sites of action, we tested whether the association of Ssb with ribosomes was dependent on the presence of Zuo1. Extracts from wild-type and Δ*zuo1* cells treated with cycloheximide were centrifuged through sucrose cushions. Typically, ~50% of the Ssb is found associated with ribosomes in such an experiment when extracts from wild-type cells are analyzed, and the interaction is unaffected by KCl concentrations up to 1 M (Nelson *et al.*, 1992; Pfund *et al.*, 1998; Figure 7B). Ssb in extracts from Δ*zuo1* was also in the ribosome-containing pellet fraction even after centrifugation through 1 M KCl (Figure 7B). Therefore, by this measure, the interaction of Ssb with translating

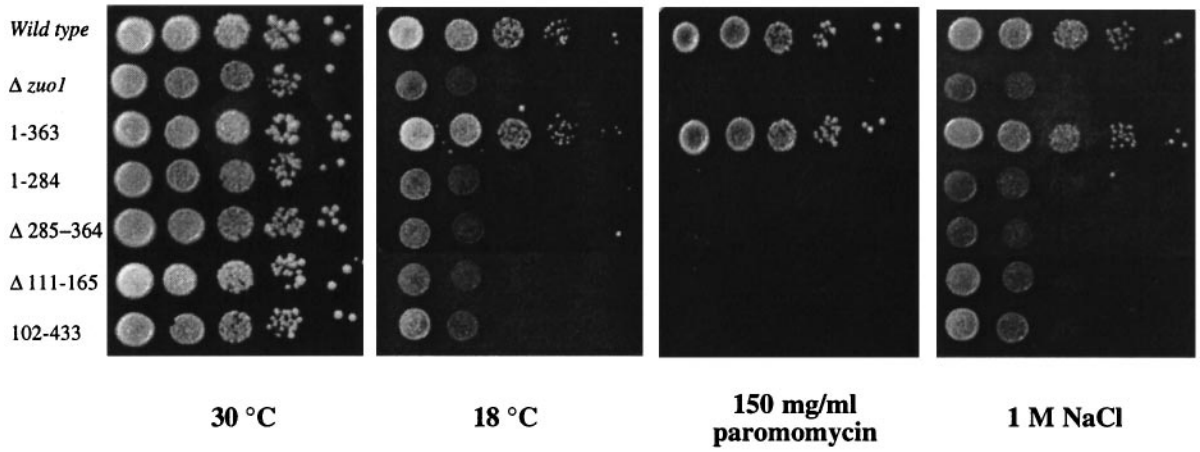

Fig. 6. Ability of *ZUO1* mutants to complement the *ZUO1* mutant phenotypes. Cells were grown overnight, counted, and an equal number of cells were spotted as a 10-fold dilution series on YPD plates containing the indicated additions and at the temperatures indicated. Plates were incubated for 4 days (no addition, 18° and 30°C), 3 days (paromomycin) and 5 days (1 M NaCl). N-terminal His-tagged wild-type and 1–363 were able to rescue the three phenotypes as well as the non-tagged proteins (data not shown).

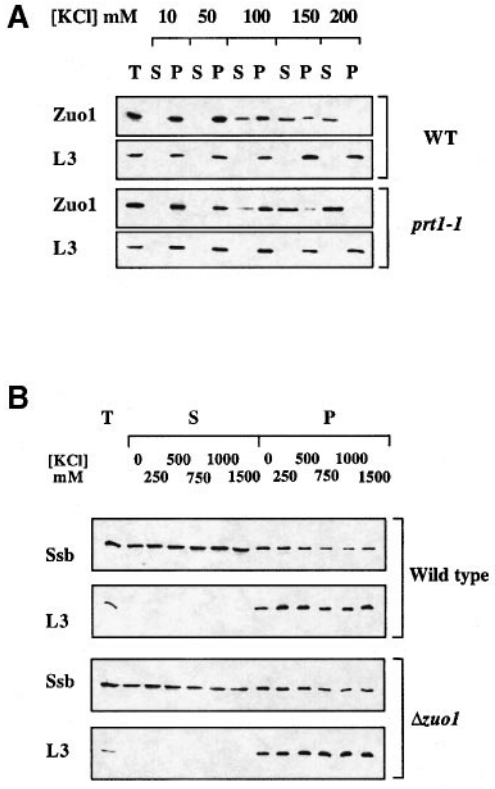

Fig. 7. Stability of Zuo1 and Ssb interactions with ribosomes. Cell extracts were prepared and spun through sucrose cushions containing various concentrations of KCl as indicated. Equivalent portions of the supernatant (S) and resuspended pellet (P) fractions, as well as the original extract (T) were run on SDS–PAGE and immunoblotted. (**A**) Extracts were prepared from wild-type (WT) and *prt1-1* cells. *prt1-1* cells were grown at 25°C, and shifted to 37°C for 20 min prior to harvest. Antibodies raised against Zuo1 and ribosomal protein L3 (L3) were used. (**B**) Extracts were prepared from wild-type (WT) and Δ*zuo1* cells. Ssb and ribosomal protein L3 (L3)-specific antibodies were used.

ribosomes is unaffected by the absence of Zuo1. The interaction of Ssb with non-translating ribosomes in the absence of Zuo1 was also tested by using the *prt1-1* strain. The Ssb–ribosome interaction showed similar salt sensitivities in extracts from *prt1-1* and Δ*zuo1 prt1-1* cells harvested at the non-permissive temperature (data not shown), suggesting that the interaction of Ssb with non-translating ribosomes is also not dependent on the presence of Zuo1.

Discussion

From the data presented here we conclude that Zuo1, a DnaJ-type molecular chaperone, is predominantly a cytosolic protein found in association with ribosomes. The similarities in the phenotypes of *zuo1* and *ssb* mutants indicate that Zuo1 and Ssb are a chaperone pair functioning in the early stages of protein biogenesis on the ribosome.

Zuo1: an RNA-binding, ribosome-associated DnaJ

Zuo1 was previously identified as a Z-DNA- and tRNA-binding protein (Zhang *et al.*, 1992; Wilhelm *et al.*, 1994), indicating an ability to interact with a variety of nucleic acid species. In the cell, however, Zuo1 appears to be nearly exclusively associated with ribosomes. In our analysis of Δ*zuo1* mutants, the capacity to bind nucleic acid is correlated with its ability to associate with ribosomes. Moreover, the internal, highly charged region is required for both ribosome association and for RNA binding. Therefore, we favor the idea that Zuo1 interacts with ribosomes, at least in part, by virtue of its association with ribosomal RNA. Purified Zuo1 interacts with a variety of nucleic acids; its specificity for ribosomes *in vivo* is probably due to additional interactions with ribosomal proteins. While to our knowledge no other chaperones are localized to their site of action via interaction with RNA, the interaction of ribosome-associated proteins with RNA is not without precedent. Other ribosome-associated factors such as elongation factor 2 interact with ribosomes via RNA–protein interactions (Holmberg and Nygard, 1994).

The 80 amino acid charged region of zuotin has an overall positive charge, with 37% of the residues being arginines or lysines and 20% being aspartic and glutamic acid residues. In the original report (Zhang *et al.*, 1992), this charged region was called the H1 region because of

its 21% identity with histone H1. However, this level of identity was based primarily on the fact that both sequences have a predominance of lysine residues. Since the data presented here indicate an association with ribosomes via an interaction with RNA, we compared the charged region with the sequence of known RNA-binding proteins. Other than the predominance of positively charged residues which would facilitate binding of Zuo1 to the negatively charged backbone of RNA, no similarities with other RNA-binding proteins (Mattaj, 1993; Draper, 1995) were found that would suggest a particular mechanism of interaction.

The deletion analysis of Zuo1 indicates that regions other than the charged region are required for function *in vivo*. Not surprisingly, a deletion which encompasses the conserved J domain is unable to rescue any of the Δ*zuo1* mutant phenotypes, suggesting that interaction with Hsp70 is important in Zuo1's *in vivo* role. The C-terminal region of Zuo1 appears dispensable, at least under the conditions tested. However, a deletion at the N-terminus up to the J domain results in a stable, but non-functional protein which is able to associate with ribosomes. This region, which bears no resemblance to any other known DnaJ-type protein, might be important for binding to unfolded polypeptides or play some unknown role. Alternatively, lack of the N-terminus might prohibit proper folding of another domain such as the J domain and thus indirectly affect function. A better understanding of Zuo1 function awaits analysis of base substitution mutants.

Zuo1 interacts in a similar manner to translating and non-translating ribosomes as judged by the sensitivity of the interactions to salt. This similarity is in contrast to the interaction of other known ribosome-associated chaperones. The Hsp70 Ssb, the prolyl isomerase trigger factor and signal recognition particle bind much more stably to translating than to non-translating ribosomes (Hesterkamp *et al.*, 1996; Powers and Walter, 1996; Pfund *et al.*, 1998). Zuo1 is not the first DnaJ-type molecule found associated with ribosomes. A small proportion of DnaJ of *E.coli* is found associated with ribosomes (Hendrick *et al.*, 1993; Kudlicki *et al.*, 1996). Like Zuo1, DnaJ's interaction does not appear to be dependent on the nascent chain as it is not released after puromycin treatment. However, unlike Zuo1, which is nearly quantitatively associated with ribosomes, only a minor portion of the DnaJ of the cell can be found ribosome-associated. Sis1, an essential yeast DnaJ, has also been found associated with ribosomes (Zhong and Arndt, 1993) and has been implicated in translation initiation as it appears to be associated preferentially with small polyribosomes. However, no genetic or biochemical data suggest a functional interaction between Sis1 and the Hsp70 Ssb in translation.

Ssb and Zuo1 as an Hsp70–DnaJ chaperone pair

Both Zuo1 and Ssb associate with ribosomes. As Ssb has been shown to be 2- to 4-fold more abundant than ribosomes (Pfund *et al.*, 1998) and the ratio of Ssb to Zuo1 has been determined to be ~4:1 (data not shown), if Zuo1 and Ssb function together there is sufficient Zuo1 within the cell to function with Ssb on every ribosome. Genetic data strongly suggest this possibility. Both Δ*zuo1* and *ssb1ssb2* strains are cold-, aminoglycoside- and salt-sensitive. These similarities between the *ZUO1* and *SSB* mutant phenotypes are reminiscent of the similarity between *dnaK* and *dnaJ* mutant phenotypes (Sell *et al.*, 1990). A strain lacking both Zuo1 and Ssb has the same phenotype as the individual mutants. In addition, overexpression of Zuo1 did not rescue the phenotypes of an *ssb1ssb2* strain, nor did overexpression of Ssb affect a Δ*zuo1* strain (data not shown). In the simplest scenario, Ssb and Zuo1 would function together in an obligatory way such that the absence of Zuo1 would have the same consequence as the absence of Ssb. Using the same reasoning, the lack of both types of proteins would be expected to have the same effect as the absence of either alone.

Mechanistically, how Zuo1 and Ssb functionally interact remains unresolved. Two other DnaJs have been shown to target Hsp70s to their site of action. For example, the DnaJ Sec63 of *S.cerevisiae* is an integral membrane protein which binds Kar2, an Hsp70 of the ER lumen, localizing it to the translocation pore (Brodsky and Schekman, 1993; Corsi and Schekman, 1997). In addition, the DnaJ-type protein auxilin, a cofactor required for the uncoating of clathrin-coated vesicles of mammalian cells by Hsc70, binds clathrin and thus recruits Hsc70 to vesicles (Ungewickell *et al.*, 1995). However, in a Δ*zuo1* strain, Ssb associates with both translating and non-translating ribosomes with the same salt resistance as in a wild-type strain.

Therefore, we do not favor a model by which Zuo1 targets Ssb to the ribosome; we prefer other possibilities upon consideration of the studies of DnaK and DnaJ. Based on the apparent increased affinity of DnaK for substrate protein in the presence of DnaJ (Gamer *et al.*, 1992), it is thought that DnaJ interacts first with substrate, targeting it to DnaK (Wall *et al.*, 1995). This interaction, in addition to the stimulation of the ATPase activity of DnaK by DnaJ through the J domain (Wall *et al.*, 1994; Jordan and McMacken, 1995) increases the affinity of the DnaK for unfolded protein. Therefore, Zuo1 and Ssb may function together with Zuo1 regulating the interaction of Ssb with nascent chains either by targeting the nascent chain to Ssb or/and by stimulating its ATPase activity. However, an understanding of how Zuo1 and Ssb partner to facilitate protein synthesis and early stages of protein folding awaits further exploration including a determination of whether Zuo1 binds directly to the nascent chain and an understanding of the effects of ribosome binding on biochemical properties of Ssb and Zuo1.

While Zuo1 and Ssb are ribosome-associated, another Hsp70–DnaJ pair, Ssa and Ydj1, are found in the soluble fraction. Zuo1/Ssb may function in early stages of protein folding while Ydj1/Ssa function later with some newly synthesized polypeptides 'passed' from one class of Hsp70 to another. Dissection of the complex functional interactions between these and other chaperones in the cytosol will lead to a more realistic appreciation of the dynamic protein folding pathways in the cell.

Materials and methods

Yeast strains

The yeast strains used in this study were DS10: *MAT***a**, *lys1, leu2-3, 112, ura3-52, his3-11,15 trp1-*Δ*1;* JN212: *MAT***a**, *lys1, leu2-3, 112, ura3-52, his3-11,15, trp1-*Δ*1, ssb1::LEU2, ssb2::HIS* (Nelson *et al*., 1992); prt1-1:

*MAT***a**, *prt1-1*, *ura3-52*, *leu2-3,112 ade1* (Hartwell and McLaughlin, 1968); and WY17: *MAT***a**, *lys1*, *leu2-3*, *112*, *ura3-52*, *his3-11,15 trp1-Δ1 Δzuo1::HIS3*.

Plasmid constructions and antibody generation

Five truncated or deleted fragments of *ZUO1* were made by PCR and cloned into pRS315/pRS316 plasmids (Sikorski and Hieter, 1989) for *in vivo* expression or p415GPDHisB/p416GPDHisB plasmids for purification. p415GPDHisB/p416GPDHisB were constructed by Jill Johnson in this lab. by cloning the *Xba*I–*Hin*dIII fragment of pRSETB (Invitrogen, Carlsbad, CA) containing six histidines and the Anti-Xpress™ antibody (Invitrogen) epitope into the same sites of p415GPD/p416GPD (Mumberg *et al.*, 1995) to allow N-terminal His tag fusion protein to be expressed under the control of the GPD promoter and detected by the Anti-Xpress™ antibody. The mutant *ZUO1* genes include the following: 1–363 encodes a protein containing the first 363 amino acids of Zuo1 plus two amino acids (Ser-His) at the C-terminus, 1–284 encodes a protein containing the first 284 amino acids of Zuo1 plus four amino acids (Arg-Pro-Lys-Gly) at the C-terminus, Δ285–364 encodes a Zuo1 protein in which amino acids 285 to 364 are deleted [three amino acids (Arg-Pro-Lys) are inserted at the deletion site], Δ111–165 encodes a Zuo1 protein containing an internal deletion from amino acid 111 to 165, while 102–433 and 166–433 encode proteins in which the N-terminal 101 or 165 amino acids are removed, respectively.

The Δ*zuo1::HIS3* mutation present in the yeast strain WY17 used in these studies contains the *HIS3* gene in place of sequences –105 to +1223. A *Cla*I–*Xba*I fragment (with the *Xba*I site blunted) containing the entire *HIS3* gene was cloned into the *Bst*BI (105 bp upstream of the ATG of *ZUO1* coding region) site and the third *Sty*I site (blunted) of the *ZUO1* coding region which leaves 75 bp of the C-terminal coding sequence. A *Sal*I–*Sac*I fragment containing this Δ*zuo1::HIS3* construction was transformed into the yeast strain DS10 to make a Δ*zuo1* strain (WY17) by one-step disruption.

A GFP C-terminal fusion to Zuo1 was constructed using the pGFP–C-Fus plasmid (Niedenthal *et al.*, 1996). An *Xba*I–*Xho*I fragment containing the entire coding region for *ZUO1* was obtained from pET21d–ZUO1 and cloned into pGFP–C-Fus digested with *Xba*I and *Sal*I (partially). pET21d–ZUO1 was constructed by introducing *Nco*I and *Xho*I sites to the N-terminus and C-terminus of *ZUO1*, respectively, by PCR and cloning into the same sites of the pET21d vector (Novagen, Inc., Madison, WI).

To generate Zuo1-specific antibodies, a *Bam*H1 site was introduced near the initiating ATG of *ZUO1*. A *Bam*HI–*Sal*I fragment containing the codons for the N-terminal 70 amino acids of Zuo1 was cloned into same sites of pGEX–KG (Guan and Dixon, 1991) to allow expression of a GST–Zuo1 fusion protein in an *E.coli* strain PK101 (*dnaK*$^-$*dnaJ*$^-$) (Kang and Craig, 1990). This GST–Zuo1 fusion protein was used as an immunogen to generate anti-Zuo1 polyclonal antibody in rabbits.

Preparation of yeast extracts and polyribosome characterization

As previously described (Nelson *et al.*, 1992), yeast lysates were prepared from strains grown in YPD to an OD_{600} of 0.7–0.9 at 30°C, unless otherwise indicated. To pellet the ribosomal fraction, 10–20 OD_{260} units of lysate were applied to the top of a 2 ml sucrose cushion, 0.5 M sucrose in CB buffer (20 mM HEPES pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol and 2 mM β-mercaptoethanol) plus variable concentrations of KCl as indicated, and spun at 50 000 r.p.m. for 3 h at 4°C in a TLA100.3 rotor (Beckman, Fullerton, CA). Pellets were resuspended in 100 μl CB buffer and supernatants were acetone-precipitated. Equivalent amounts of supernatant and pellet were subjected to SDS–PAGE, transferred to nitrocellulose (Hybond-C, Amersham Corp., Arlington Heights, IL), and immunoblotted for Zuo1, the ribosomal protein L3 (L3) or Ssb using the ECL detection system (Amersham, Arlington Heights, IL).

To fractionate the polyribosomes, 20 OD_{260} units of lysate were applied to the top of a 4.4 ml 10–47% sucrose gradient in CB buffer and centrifuged for 90 min at 54 000 r.p.m. at 4°C in a SW55Ti rotor (Beckman). Gradients were monitored at 260 nm to detect monosomes and polysomes and 0.4 ml fractions were collected to be used for immunoblots as described above. RNase-free sucrose (Sigma Chemical Co., St Louis, MO) was used to make the cushion and gradient solutions. RNAguard (Pharmacia Biotech, Piscataway, NJ) was added to the cell lysates and the cushion and gradient solutions at 1:1000 dilution just prior to centrifugations.

Purification of His-tagged Zuotin

Both His-tagged wild-type Zuo1 and Zuo1 deletion mutant proteins to be used for Northwestern blots were prepared from yeast as follows. Yeast strains expressing only wild-type or mutant Zuo1 were grown in the appropriate drop-out media at 30°C. Pelleted cells were resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris pH 7.8, 0.1% Triton X-100, 1 mM PMSF, 5 μM pepstatin A) and disrupted in a French press. 2 ml of a 50% slurry of Ni^{2+}-charged His-Bind^R Resin (Novagen, Madison, WI) was added to 3 ml of each cleared lysate, and tubes were rotated slowly at 4°C for 60 min. The resin was washed twice in binding buffer. Bound proteins were eluted by incubating in 1 ml wash buffer, with each consecutive wash containing an increase of 50 mM imidazole, to elute proteins in a stepwise fashion. Zuo1-rich fractions were identified by Western blot, dialyzed against 10 mM Tris pH 7.5 and concentrated in a Speed Vac Concentrator (Savant, Instruments, Inc., Holbrook, NY).

Northwestern blots

Northwestern blot analysis of Zuo1 was performed essentially as previously described to detect Zuo1 binding to tRNA (Wilhelm *et al.*, 1994). Equivalent amounts of wild-type or mutant proteins were separated in a 10% SDS–PAGE. Proteins were transferred to nitrocellulose and membrane-bound proteins allowed to renature by incubation in renaturation buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1× Denhardt's) with gentle rocking at 4°C. The nitrocellulose filter was then incubated with 2×10^6 c.p.m. 5′-end ^{32}P-labeled Phe tRNA or ^{32}P-labeled rRNA in renaturation buffer for 60 min at room temperature. Yeast Phe tRNA was obtained from Sigma (# R4018). rRNA was purified from ribosomes isolated from prt1-1 cells incubated at 37°C prior to harvest. After washing three times in 10 mM Tris pH 7.5, 50 mM NaCl, the membrane was dried and exposed to X-ray film.

Microscopy

The Δ*zuo1* strain containing the Zuo1–GFP fusion was grown in minimal media lacking uracil at 30°C to an OD_{600} of 0.5, at which time 1 mg/ml of DAPI was added to stain the nuclear DNA. After 1 h growth in the dark, equivalent amounts of cell culture and 1% low-melting point agarose (Sea Plaque; FMC Products, Rockland, ME) made in minimal medium lacking uracil were mixed, spotted on a microscope slide, and covered with a coverslip. Preparations were examined using a Zeiss (Germany) Axioplan 2 fluorescence microscope and images were captured by digital camera (QED Imaging Inc., Pittsburgh, PA).

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