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The Yeast hsp70 Homologue Ssa Is Required for Translation and Interacts with Sis1 and Pab1 on Translating Ribosomes*

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The 70-kDa heat shock proteins are molecular chaperones that participate in a variety of cellular functions. This chaperone function is stimulated by interaction with hsp40 proteins. The *Saccharomyces cerevisiae* gene encoding the essential hsp40 homologue, *SIS1,* appears to function in translation initiation. Mutations in ribosomal protein L39 (*rpl39*) complement loss-of-function mutations in *SIS1* as well as *PAB1* (poly(A)-binding protein), suggesting a functional interaction between these proteins. However, while a direct interaction between Sis1 and Pab1 is not detectable, both of these proteins physically interact with the essential Ssa (and not Ssb) family of hsp70 proteins. This interaction is mediated by the variable C-terminal domain of Ssa. Subcellular fractionations demonstrate that the binding of Ssa to ribosomes is dependent upon its C terminus and that its interaction with Sis1 and Pab1 occurs preferentially on translating ribosomes. Consistent with a function in translation, depletion of Ssa protein produces a general translational defect that appears similar to loss of Sis1 and Pab1 function. This translational effect of Ssa appears mediated, at least in part, by its affect on the interaction of Pab1 with the translation initiation factor, eIF4G, which is dramatically reduced in the absence of functional Ssa protein.

The 70-kDa heat shock proteins are molecular chaperones that participate in a variety of cellular functions. hsp70 proteins bind hydrophobic stretches on unfolded proteins, aiding in their folding and oligomerization and in translocating proteins across membranes (reviewed in Refs. 1 and 2). This chaperone function of hsp70 is stimulated by interaction with hsp40 proteins. ATP-bound hsp70 proteins transiently bind hydrophobic stretches on unfolded substrates, and their intrinsic ATPase activity is stimulated by hsp40 proteins, which increases the stability of the hsp70-peptide interaction (1). The hsp70-hsp40 interaction is conserved from bacteria to humans (2).

In *Saccharomyces cerevisiae*, there are at least 14 different hsp70 proteins grouped into five defined subclasses. The two cytosolic hsp70 subfamilies, *SSA* and *SSB*, share 60% amino acid identity but cannot functionally substitute for one another (2). The essential *SSA* subfamily contains four members (referred to collectively as Ssa). Ssa1 and -2 are constitutively expressed, while Ssa3 and -4 are heat-inducible (3). Ssa is important in the folding and membrane translocation of nascent peptides, nuclear import, microtubule formation, and the transcriptional response to heat shock (1, 4–7). The Ssb1 and Ssb2 proteins (referred to collectively as Ssb) are associated with ribosomes and appear to function in binding nascent peptides during translation elongation (8, 9). The *SSB* subfamily is not essential, but deletion of both members results in a cold-sensitive phenotype (9). A yeast hsp40 protein, Sis1, is also ribosome-associated (10). Deletion of *SIS1* is lethal, and a yeast strain that expresses a temperature-sensitive Sis1 protein, *sis1-85*, demonstrates a defect in translation initiation at the nonpermissive temperature (10, 11). Due to its association with ribosomes, Sis1 has been proposed to function with Ssb (12). However, Sis1 does not stimulate the ATPase activity of Ssb but does stimulate that of Ssa (Ref. 13; reviewed in Ref. 14).

The temperature sensitivity of sis1–85 is suppressed by a functional deletion of ribosome L39 protein (*rpl39*/*spb2*) as well as ribosomal protein L35 (*rpl35*/*sos1*), providing further evidence for a translational function for Sis1 (10). Interestingly, a deletion of *rpl39* also suppresses the lethal deletion of another essential translation factor, poly(A)-binding protein (*PAB1*), suggesting that these proteins functionally interact (15). Pab1 binds the 3′-terminal poly(A) tract within an mRNA and also plays a role in the stabilization of RNA messages (reviewed in Ref. 16). Pab1 also serves to bring the 5′- and 3′-ends of mRNA in close proximity by binding the initiation scaffold protein eIF4G (17). This has been shown to be essential for efficient translation initiation as well as mRNA stability (16, 18, 19).

While the functional significance of the genetic relationship between *PAB1* and *SIS1* is unclear, the shared phenotype of Δ*pab1* and *sis1–85* genes suggests a potential interaction between the two in translation initiation. However, as demonstrated here, there is no detectable physical association between Pab1 and Sis1. In contrast, Ssa interacts with both Sis1 and Pab1, and these interactions are specific for the Ssa subfamily of hsp70s. As previously shown for the interaction between hsp40 and hsp70, the interaction of Ssa with Pab1 is mediated via the variable C-terminal domain of Ssa rather than the peptide binding domain (20). Like Sis1, Ssa associates with ribosomes, and a temperature-sensitive strain of *SSA* (*ssa1–45*) demonstrates a severe translational defect (10, 21). Depletion of Ssa decreases the association of Pab1 with eIF4G, suggesting that the translational effect of Ssa is mediated at

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TABLE I
Yeast strains

Strain	Genotype	Source
JN54	*MAT***a** *his3–11, 3–15 leu2–3, 1–112 lys2 trp1-Δ1 ura3–52*	E. Craig[a]
JB67	*MATα leu2–3, 2–112 his3–11 ura3–52 trp1-Δ1 lys2 ssal-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Becker *et al.* (21)
JN49	*MATα his3–11, 3–15 leu2–3, 1–112 lys2 trp1-Δ1 ura3–52 ssa1–3 ssa2–2*	Craig and Jacobsen (29)
MW141	*MATα, his3–11,3–15 leu2–3, 2–112 lys2 trp1-Δ1 ura3–52 ssa1::HIS3 ssa2::LEU2 ssa4::URA3, pGAL:SSA (TRP1)*	Deshaies *et al.* (34)
F294	*MAT***a** *ura3–52 leu2–3, 2–112 ade1 prt1–1*	A. Hinnebusch[b]
CY457	*MAT***a** *ura3–1 leu2–3, 2–112 his3–11, 3–15 ade2–1 ssdl-d2 can1–100 sis1::HIS3* NH_2*-HA SIS1* on *LEU2CEN* plasmid	Luke et al. (11)

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least in part by its effect on the interaction of these two proteins.

EXPERIMENTAL PROCEDURES

Yeast Methods—The yeast strains used in this study are listed in Table I. Yeast cultures were grown as indicated using either synthetic medium plus 2% glucose supplemented with the appropriate additives or complete medium (22). Yeast were grown in the appropriate medium at either 25 or 30 °C to log phase. Where indicated, yeast were shifted to 37 °C by resuspending the cell pellet in prewarmed 37 °C medium and incubated at 37 °C for the prescribed time. For depletion experiments, yeast were grown in complete medium with 2% galactose and then washed three times and shifted to complete medium with 2% glucose for 7 h before harvesting. The *URA3 CEN4* plasmids containing the *SSA1* and *SSB1* fusion constructs were previously described (12). These were transformed into JN54 by standard lithium acetate transformation procedure (22). The C-terminal deletion of *SSA* was created by engineering a stop codon by PCR mutagenesis immediately 3′ to the *Xho*I site used for construction of the chimeras (12).

Fractionation of Ribosomes—All procedures were performed at 4 °C except where indicated. Yeast cells from 50 ml of log phase culture were pelleted, treated for 1 min with 10 μg/ml cycloheximide (Calbiochem), and repelleted. Lysates were made by bead beating the yeast for 4 min, with intermittent cooling on ice, in polysome buffer (PB[1]; 100 mM KCl, 2 mM magnesium acetate, 20 mM HEPES, pH 7.4, 14.4 mM β-mercaptoethanol, 100 μg/ml cycloheximide). This mixture was centrifuged at 5000 rpm for 8 min, and the supernatant was removed. 5–10 A_{254} units were loaded onto a 16.2-ml 10–50% sucrose gradient containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.4, and 2 mM dithiothreitol. Lysates were sedimented in a Beckman SW28.1 rotor at 27,000 rpm for 4.5 h. Gradients were collected with continuous monitoring at 254 nm using an ISCO UA-5 absorbance detector and 1640 gradient collector. Samples run over 10–25% gradients were sedimented in a Beckman SW28.1 rotor at 20,000 rpm for 16.5 h. Pellets were resuspended in polysome buffer, and supernatant fractions were either collected as above or pooled where indicated.

For cycloheximide treatment of cells, 100 μg/ml cycloheximide was added to the yeast medium concomitant with a shift to 37 °C. RNase treatment of the yeast lysate was performed by adding 300 μg/ml RNase A to the PB, followed by regular lysis. High salt treatment was performed by centrifuging lysates in a sucrose gradient containing 500 mM KCl. For immunoprecipitation from gradient fractions, lysates were centrifuged through a 10–25% sucrose gradient for 16 h at 23,100 rpm. The polysome pellet was washed and resuspended for immunoprecipitation in sucrose gradient buffer. Nonribosomal fractions (fractions 1 and 2) were collected and directly immunoprecipitated. Fractions containing the 40, 60, and 80 S ribosomes were pelleted using an SW50 rotor at 49,700 rpm for 7.5 h. Pellets were washed and resuspended for immunoprecipitation in sucrose gradient buffer.

Immunoprecipitations—Lysates were made by resuspending cell pellets in immunoprecipitation buffer (150 mM KCl, 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton, 0.01% SDS) and bead beating for 4 min with intermittent cooling on ice. Lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were transferred to tubes containing 20 μl of protein A beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and either 1 μl of HA-12CA5 (D. Templeton, Case Western Reserve University, Cleveland, OH), PAB 1G1 (M. Swanson, University of Florida, Gainesville, FL), SIS 66932 (23), SSA 1173 (24), or SSB 30733 (9), as indicated. Where noted, 10 μl of lysate was removed and added to an equal volume of Laemmli buffer for analysis by Western blot. Immunoprecipitates were rotated overnight at 4 °C, washed four times with immunoprecipitation buffer, and resuspended in Laemmli sample buffer. Immunoprecipitates from gradient fractions were normalized to 10% sucrose by resuspension of the pellets with sucrose gradient buffer plus 10% sucrose.

Western Blots—For immunoprecipitates, samples were boiled for 5 min, and equal amounts of each sample were separated on 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon (Millipore Corp., Bedford, MA) in 0.01 M CAPS and 10% methanol using a semidry electroblotter (Owl Scientific, Woburn, MA) at 400 mA for 45 min. Immunoprecipitates from the Ssa and Ssb fusion proteins were separated on an 8% SDS-polyacrylamide gel electrophoresis containing a 20:1 acrylamide/bisacrylamide ratio. These gels were transferred to Immobilon for 45 min at 400 mA.

Proteins collected from sucrose gradient fractions were trichloroacetic acid-precipitated, washed twice in 100 mM Tris, pH 8.0, acetone (1:5, v/v), and solubilized by boiling in Laemmli buffer for 5 min. Pellets from 10–25% gradients were resuspended in polysome buffer for immunoprecipitation or in Laemmli buffer. Equal amounts of each fraction were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred as above to Immobilon (Millipore Corp.).

Membranes were blocked in 5% milk in 1× PBS-T (PBS plus 0.02% Tween 20; Sigma) and probed with the following antibody dilutions in 5% milk, 1× PBS-T: HA-12CA5 (1:2000), PAB 1G1 (1:5000), SSA 1173 (1:2000, except in Figs. 1*E* and 2, where a dilution of 1:600 was used), SSB 30733 (1:2000, except in Figs. 1*E* and 2, where a dilution of 1:600 was used), GST 1:2000 (Amersham Pharmacia Biotech), SIS 66932 (1:1000), Hsp70 535 (1:1000; E. Craig, University of Wisconsin, Madison, WI), or eIF4G (1:1000) (17). Western blots were washed three times for 5 min each in 1× PBS-T. HA-12CA5 and PAB 1G1 antibodies were detected with a goat anti-mouse antibody conjugated to horseradish peroxidase (ICN Biomedical Inc., Aurora, OH), diluted 1:5000 in 5% milk in 1× PBS-T. SSA 1173, SSB 30733, SIS1 66932, Hsp70 535, and eIF4G antibodies were detected with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Cappel, Durham, NC) diluted 1:4000 in 5% milk in 1× PBS-T. GST antibodies were detected with a rabbit anti-goat antibody conjugated to horseradish peroxidase (Sigma) diluted 1:8000. Western blots were washed as above and developed using chemiluminescence with ECL reagents (Amersham Pharmacia Biotech).

Slot Blots—RNA was extracted according to standard procedures from 17 A_{600} of *ssa1–45* yeast or wild-type yeast grown at 25 °C and 30 min following shift to 37 °C (22). Volumes throughout the RNA extraction were kept equivalent so that the final RNA content represented RNA extracted from an equivalent A_{600} of yeast. Equal volumes of RNA were diluted as indicated in diethylpyrocarbonated-treated water, 50% formamide, 6.5% formaldehyde, 5X SSC, and bound to nitrocellulose (Schleicher and Schuell) using the Minifold II slot blot apparatus (Schleicher and Schuell). Total mRNA abundance was determined by probing with cDNA made from whole cell RNA from *SSA* cells as previously described (22, 25). Blots were developed by autoradiography.

^{35}S in Vivo Labeling—Four 10-ml cultures of *SSA* and *ssa1–45* yeast were grown at 25 °C overnight until mid-log phase. At zero time, half of the cultures were centrifuged and resuspended in YEPD prewarmed to

[1] The abbreviations used are: PB, polysome buffer; CAPS, 3-(cyclohexylamino)propanesulfonic acid; GST, glutathione *S*-transferase; HA, hemagglutinin.

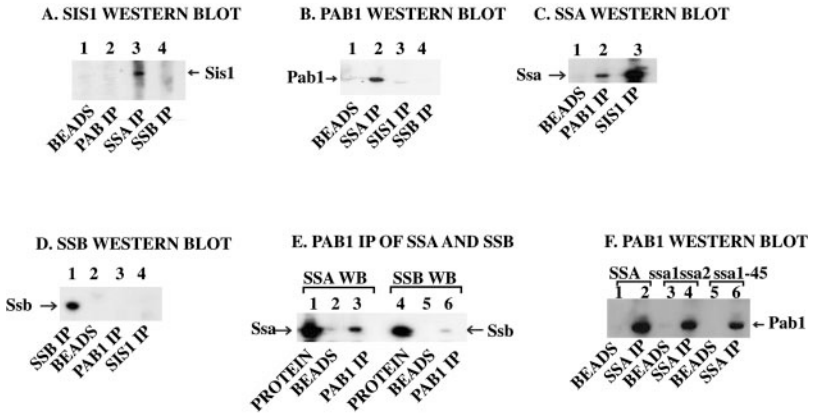

FIG. 1. **Ssa interacts with Sis1 and Pab1.** *A*, Ssa1 coimmunoprecipitates Sis. Lysates were prepared from *SSA* yeast. Proteins were immunoprecipitated (*IP*) with protein A beads or beads plus Pab1, Ssa, or Ssb antibodies. Immunoprecipitates were probed for Sis1 by immunoblotting. *B*, Ssa coimmunoprecipitates Pab1. Proteins were immunoprecipitated using Ssa, Sis1, or Ssb antibodies and analyzed for Pab1 as described above. *C*, Pab1 and Sis1 coimmunoprecipitate Ssa. Proteins were immunoprecipitated using Pab1 or Sis1 antibodies and probed for the presence of Ssa as described above. *D*, Ssb does not coimmunoprecipitate with Pab1 or Sis1. Proteins were immunoprecipitated with Ssb, Pab1, or Sis1 antibodies and probed for Ssb as described above. *E*, Pab1 immunoprecipitates Ssa and not Ssb. Proteins were immunoprecipitated with Pab1 antibody and probed for Ssa or Ssb as described above. An aliquot of the lysate was used as a control for protein expression and loaded in *lanes 1* and *4*. *F*, multiple members of the Ssa family coimmunoprecipitate Pab1. Lysates from the *SSA*, *ssa1ssa2*, and *ssa1–45* yeast were immunoprecipitated with the Ssa antibody and probed for the presence of Pab1 as described above.

37 °C. At 0, 10, 30, and 60 min, individual cultures were labeled with 10 μCi of [^{35}S]methionine (1175 Ci/mmol; PerkinElmer Life Sciences) for 2 min. Triplicate samples of 0.5 ml were removed, an equal volume of ice-cold 25% trichloroacetic acid was added, and the samples were vortexed. Samples were stored on ice for 30 min, and the precipitated proteins were collected onto Whatman GF/C glass fiber filters by vacuum filtration and washed with 5% trichloroacetic acid and 95% ethanol. Amino acid incorporation into acid-precipitable material was determined by scintillation counting in a Beckman LS6000 SE liquid scintillation counter. Final values were corrected for A_{600} readings.

In Vitro Binding Studies—Bacterial strains containing histidine-tagged Pab1 (26), pGEX2TSSA1-RI, or pGEX2T-A1C1ΔB1 (E. Craig, University of Wisconsin, Madison, WI) were grown overnight at 37 °C. The His-Pab1 was purified as previously described (27). The GST fusion proteins were purified as previously described (28) and eluted from glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) in 10 mM glutathione (Sigma). The eluate was incubated with either Talon metal affinity resin (CLONTECH, Palo Alto, CA) alone or His-Pab1 immobilized on Talon resin for 1 h at 4 °C. The resin was washed three times in wash buffer (100 mM KCl, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.1% Triton X-100, 100 μM bovine serum albumin, 10% glycerol, 2 mM imidazole). Samples were then either treated or mock-treated with 2 μg of RNase A for 20 min at 26 °C. Samples were washed twice, and the resin was resuspended in wash buffer with 200 mM imidazole. His-Pab1 was eluted for 15 min at 26 °C, and the eluate was removed. The eluate was separated on 12% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting.

RESULTS

Pab1 and Sis1 Interact with the hsp70 Protein Ssa—PAB1 and *SIS1* share a genetic interaction with *RPL39*, and their gene products are ribosome-associated proteins that function in translation. Therefore, we investigated whether Pab1 and Sis1 proteins interacted, as determined by coimmunoprecipitation. Immunoprecipitations were performed from the wild type yeast strain JN54 (herein called *SSA*) using either a Pab1 antibody or Sis1 antibody (Fig. 1). The immunoprecipitates were analyzed by Western blot with either the Sis1 (Fig. 1*A*) or Pab1 (Fig. 1*B*) antibodies. Pab1 was not detected in the Sis1 immunoprecipitate, and similarly, Sis1 was not detected in the Pab1 immunoprecipitates. Thus, at the limit of resolution of these immunoprecipitations, Sis1 and Pab1 do not directly interact.

hsp40 proteins function together with an hsp70 partner, and interaction between Sis1 and Pab1 may be indirect and mediated via an hsp70 partner. Therefore, coimmunoprecipitation studies were performed to determine whether Sis1 or Pab1 bound either Ssb or Ssa. Immunoprecipitations were performed and analyzed as above using either Sis1 (Fig. 1*A*), Pab1 (Fig. 1*B*), Ssa-specific (Fig. 1*C*), or Ssb-specific antibodies (Fig. 1*D*). These experiments demonstrated that Ssa, but not Ssb, coimmunoprecipitated with Sis1, and similarly Pab1 coimmunoprecipitated Ssa but little detectable Ssb. In reciprocal immunoprecipitations, Pab1 coimmunoprecipitated with Ssa but not Ssb (data not shown). To ensure that the differential association of Ssa and Ssb with Pab1 was not due to differences in total abundance of these hsp70 homologues, an aliquot of lysate was analyzed by Western blotting for Ssa and Ssb, concurrent with the Pab1 immunoprecipitates (Fig. 1*E*). As demonstrated here, the Pab1-Ssa interaction appears to be 8–10-fold stronger than the Pab1-Ssb interaction (as determined by densitometry) when the total amount of these proteins is taken into account. This preferential binding of Ssa to Pab1 was confirmed by further studies (Fig. 2 and data not shown). Thus, there appears to be a specific interaction of Ssa hsp70 proteins with Pab1 and Sis1.

To determine whether Pab1 bound all Ssa family members, immunoprecipitations were performed with lysates from strains lacking different family members: *SSA*; JN49, which lacks Ssa1 and Ssa2 (herein called *ssa1ssa2* (29)); and JB67, whose only source of Ssa is a temperature-sensitive *SSA1* (herein called *ssa1–45*) (Fig. 1*F*). Pab1 coimmunoprecipitated with Ssa from both the *ssa1ssa2* and *ssa1–45* strains, indicating that Pab1 binds to Ssa family members 1, 3, and 4. Although we do not have definitive data on Ssa2, it is likely that Pab1 also binds this protein.

Pab1 Binds to the C-terminal Domain of Ssa—To identify the Pab1 binding site on Ssa, we took advantage of the fact that Pab1 did not bind Ssb and analyzed Ssa/Ssb fusion proteins by immunoprecipitation. Each fusion contains an ATPase domain, a peptide binding domain, and a C-terminal domain from one of these two hsp70 protein subclasses (12). Each fusion is named for the domains they contain (*i.e.* AAB contains the ATPase and peptide binding domain from Ssa1 and the variable domain from Ssb1). Pab1 was immunoprecipitated from *SSA* yeast transformed with one of the six fusion constructs, and immunoprecipitates were analyzed by Western blot using either Ssa (Fig. 2*A*) or Ssb (Fig. 2*B*) antibodies. As a protein expression control, aliquots of each of the lysates were analyzed by Western blotting (Figs. 2, *A*, *lanes 1–3*, and *B*, *lanes 1–4*). All six of the fusion proteins, as well as the native hsp70, were expressed

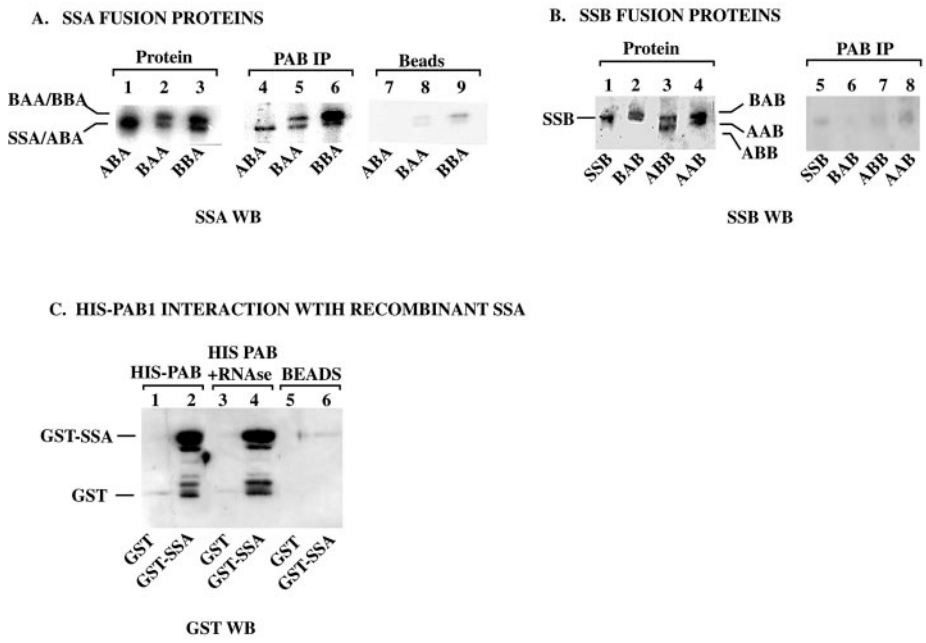

FIG. 2. **The interaction between Pab1 and hsp70 proteins is specific to the Ssa C terminus.** *A*, fusion proteins containing the C terminus of Ssa coimmunoprecipitate with Pab1. Lysates from the *SSA* strain transformed with either the *ABA*, *BAA*, or *BBA* fusion protein constructs were immunoprecipitated with the Pab1 antibody or beads alone as a control. Prior to immunoprecipitation, an aliquot of the lysate was removed as a control for protein expression (*left panel*). Immunoprecipitates (*middle panel*) and control immunoprecipitates (*right panel*) were probed for Ssa by immunoblotting. All *figures* represent equal exposure times. *B*, fusion proteins containing the C terminus of Ssb do not coimmunoprecipitate with Pab1. Lysates from the *SSA* strain transformed with either the *BAB*, *ABB*, or *AAB* fusion protein constructs were immunoprecipitated with Pab1 antibody. Prior to immunoprecipitation, an aliquot of the lysate was removed as a control for protein expression (*left panel*). Immunoprecipitates were probed for Ssb by immunoblotting (*right panel*). The *right panel* represents a 10-fold longer exposure time than the *left panel*. *C*, His-Pab1 associates with a recombinant C-terminal fragment of Ssa. Recombinant Ssa fusion proteins were purified and eluted from glutathione beads and incubated with immobilized histidine-tagged Pab1 or resin alone. Samples were either mock- or RNase A-treated and then eluted with 200 mM imidazole from the resin and analyzed by Western blotting (*WB*) with anti-GST antibody.

to similar levels and were recognized by the appropriate antibody. The variable C-terminal domain of Ssa was necessary and sufficient for Pab1 association, as seen by the coimmunoprecipitation of BBA, and not AAB, with Pab1 (Fig. 2*A*, *lane 6*). In contrast, fusion proteins containing the C terminus of Ssb only weakly coimmunoprecipitated with Pab1, despite the fact that all of these proteins were expressed at similar levels (Fig. 2*B*, *lanes 5–8)*. We assume that the ABA fusion protein also coimmunoprecipitated with Pab1, but because it comigrated with wild type Ssa, the association of ABA with Pab1 could not be assessed (12).

The above experiments demonstrated a specific interaction between the C-terminal domain of Ssa and Pab1 *in vivo*. To confirm that this was due to a direct interaction between these two proteins and not simply coprecipitation with a common ribonucleoprotein complex, an Ssa C-terminal fragment was fused to GST, expressed in bacteria, purified on a glutathione column, and eluted. As a control, GST was used. Recombinant histidine-tagged Pab1 was purified, and eluates from the GST or GST-Ssa purification were applied to either metal affinity resin alone or metal affinity resin with immobilized Pab1 (Fig. 2*C*). Additionally, these pull-downs were either treated with RNase A or left untreated. GST alone did not bind to immobilized Pab1, but the C-terminal fragment of Ssa bound Pab1 in the presence or absence of RNase A treatment. In addition, another GST-Ssa fragment, encompassing a shorter C-terminal fragment, also bound to immobilized Pab1 to a similar degree (data not shown). As demonstrated here, the Pab1-Ssa interaction is not dependent on RNA but appears due to a direct protein-protein interaction between Pab1 and Ssa. Additionally, neither the addition of excess poly(A) mRNA to immunoprecipitates nor *in vivo* depletion of poly(A) mRNA blocks the Ssa-Pab1 association (data not shown). Finally, since Ssa and Pab1 coimmunoprecipitate from cytosolic fractions that do not contain detectable mRNA or ribosomes (see Fig. 4*B* and data not shown), the interaction of these two proteins appears direct.

Ssa Proteins Are Found in Association with Translating Ribosomes—Since both Sis1 and Pab1 are ribosome-associated proteins and interact with Ssa, we determined whether Ssa associated with ribosomes. Lysates from *SSA* yeast were analyzed by sedimentation through 10–50% sucrose gradients, and the distribution of ribosomes in the gradient was determined by absorbance at 254 nm. Proteins were recovered from the fractionated lysate, and the presence of Ssa in the fractions was analyzed by Western blotting using antibodies that recognized Ssa proteins but not Ssb. As seen in Fig. 3*A*, the Ssa proteins (*top panel*) were most abundant in the top of the gradient (fractions 1 and 2). However, a significant portion sedimented at the same position as ribosomal subunits and translating polysomes. This is comparable with that seen with Ssb, Sis1, and Pab1 (Fig. 3*A*, lower panels).

Previous experiments suggested that the most rapidly sedimenting Ssa proteins were not bound to ribosomes, since their sedimentation was unaffected by RNase A (12). To determine whether the Ssa proteins found in the polysome fractions were polysome-associated, lysates from *SSA* yeast were treated with RNase A prior to fractionation. This reduced the amount of material that sedimented in polysome fractions and increased the amount in 80 S fractions (Fig. 3, compare *A* and *B*). Coincident with this, the amount of Ssa protein was reduced in polysomal fractions, paralleling results for Ssb, Sis1, and Pab1 (Fig. 3*B*, *lower panels*). Thus, under the conditions used here, the rapidly sedimenting Ssa proteins are part of an RNase-sensitive particle.

As an independent means of confirming Ssa association with

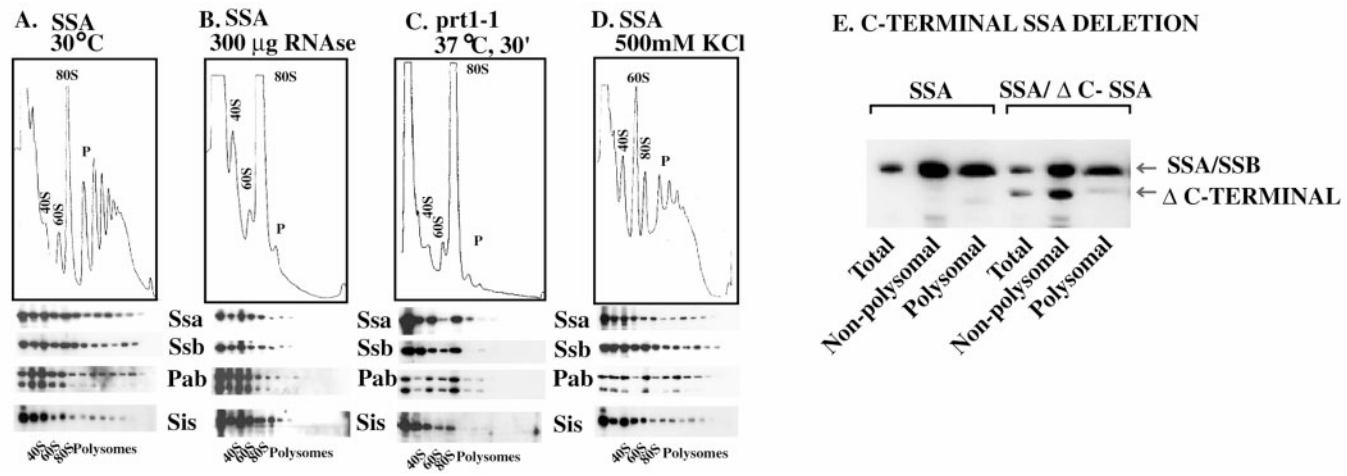

FIG. 3. **Ssa proteins are associated with ribosomes.** *A*, Ssa associates with polysomes. Lysate prepared from *SSA* yeast was fractionated through a sucrose gradient and collected with continuous monitoring of UV absorbance at 254 nm. The positions of the 40, 60, and 80 S peaks are indicated. *P* indicates the presence of the first polysome fraction. Total proteins from each fraction were collected and probed for the presence of either Ssa, Ssb, Pab1, or Sis1 by immunoblotting. Blots are displayed in the *lower panels*. *B*, Ssa association with polysomes is RNase-sensitive. Lysate was treated with RNase A prior to sedimentation. Fractionated proteins were collected as above and probed for the presence of Ssa, Ssb, Pab1, and Sis1 by immunoblotting. *C*, Ssa abundance in polysome fractions is directly related to polysome content. The *prt1–1* strain was grown at 25 °C until mid-log phase and then shifted to 37 °C for 30 min. Proteins from gradient fractions were collected and probed for Ssa, Ssb, Pab1, and Sis1 as described above. *D*, Ssa association with polysomes is stable to 500 mM KCl. Lysate from *SSA* yeast was fractionated through sucrose gradients containing 500 mM KCl. The presence of Ssa, Ssb, and Pab1 in the gradient fractions was determined by immunoblotting. *E*, the C terminus of Ssa is required for its interaction with polysomes. Lysates prepared from *SSA* yeast or *SSA* yeast transformed with a C-terminal deletion of *SSA* were loaded onto 10–25% sucrose gradients. Nonpolysomal fractions were combined, and 1% of the total extract, 5% of the nonpolysome fraction, and 20% of the pelleted polysomes were probed for Ssa using the hsp70 antibody described under "Experimental Procedures."

polysomes, strains temperature-sensitive for translation initiation factors *prt1–1* (F294) and eIF4E *(cdc33)* were utilized (Fig. 3*C* and data not shown). Under permissive conditions, Pab1, Sis1, Ssa, and Ssb exhibit a polysomal distribution, similar to that seen in the *SSA* yeast (data not shown). At the nonpermissive temperature (37 °C for 30 min), both of these strains showed a loss of polysomes and accumulation of 80 S ribosomes. A concomitant loss of Ssa from the polysome fractions was also observed, similar to the results for Pab1, Sis1, and Ssb (Fig. 3*C*). Association of Ssa, as well as the other proteins, with polysomes was stable to sedimentation in 500 mM KCl (Fig. 3*D*), suggesting that all of these proteins are present on translating ribosomes.

The C-terminal domain of Ssa appears required for its interaction with Pab1. To determine whether this domain also mediated Ssa association with polysomes, a C-terminal truncation was created at the juncture between the peptide binding domain and the C terminus as defined in the chimeras (12). This truncated protein was expressed in *SSA* yeast and total extract, nonpolysomal, and polysomal fractions isolated. ΔC terminus Ssa was identified in these fractions by Western blotting (Fig. 3*E*). In contrast to the full-length Ssa, the C-terminal truncation of Ssa dramatically reduced its association with polysomes. Thus, an intact C terminus is required not only for efficient binding of Ssa to Pab1 but also for the association of Ssa with polysomes.

Ssa Interacts with Sis1 and Pab1 on Translating Polysomes but Not on 40 S Subunits or 80 S Ribosome Couples—While both Sis1 and Ssa are abundant in the cytoplasm and associate with ribosomes, if Ssa functions as the hsp70 partner for the translational functions of Sis1 then their interaction should be detectable on ribosomes. To determine whether this was the case, cell lysates from *SIS1-HA* yeast were fractionated through 10–25% sucrose gradients. Polysomes and associated proteins are found in pellets, while the cytoplasm and 40, 60, and 80 S fractions remain in the supernatant. While interaction of Sis1 with Ssa was observed in the most slowly sedimenting subribosomal fraction, the interaction appeared strongest in polysomes, as judged by the amount of coimmunoprecipitating material (Fig. 4*A*). In addition, since both Ssa and Sis1 are present in 80 S fractions, the absence of a detectable interaction in this fraction provides evidence that the Sis1-Ssa interaction is specific for translating ribosomes and not simply due to coprecipitation with ribosomes (30).

Similar studies were performed to characterize the site of Pab1 interaction with Ssa. Using the *SSA* strain, Pab1 immunoprecipitates from subribosomal, polysomal, 40 S, 60 S, and 80 S fractions were analyzed by Western blotting with the Ssa and Pab1 antibodies (Fig. 4*B*). While Ssa and Pab1 interacted in the subribosomal fraction, there was an equivalent interaction on polysomes. Similar to the Ssa-Sis1 interaction, the Ssa-Pab1 interaction was not detected on either ribosomal subunits or in translationally inactive 80 S ribosomes despite the fact that these proteins were present in these fractions (see also Fig. 3*A*) (30). Thus, physical interaction of Ssa with both Pab1 and Sis1 appears to occur preferentially on translating ribosomes.

Depletion of Ssa Affects Translation—The temperature-sensitive mutant, *sis1–85*, exhibits a translational defect (10). Because Sis1 activates the ATPase activity of Ssa and we have shown that Ssa interacts with Sis1 on translating ribosomes, we hypothesized that Ssa was the hsp70 partner to Sis1 in translation initiation. To determine whether Ssa had a role in translation, we utilized the temperature-sensitive *ssa1–45* yeast strain. Incorporation of [^{35}S]methionine into newly translated proteins was measured during a 2-min pulse label at 0, 10, 30, and 60 min after shift to the nonpermissive temperature (Fig. 5*A*). While in the wild type *SSA* strain a minor decrease in translation rate occurred at 10 and 30 min (reduced to 84 and 71%, respectively, of wild type), complete recovery occurred by 60 min postshift. In contrast, translation rate was significantly reduced by 10 min postshift in the *ssa1–45* strain (reduced to 62% as compared with the control *ssa1–45* at 25 °C) and continued to decrease to 9.7% of control at 60 min postshift.

Since heat shock affects transcription and has been implicated to play a role in mRNA stability as well (31–33), we

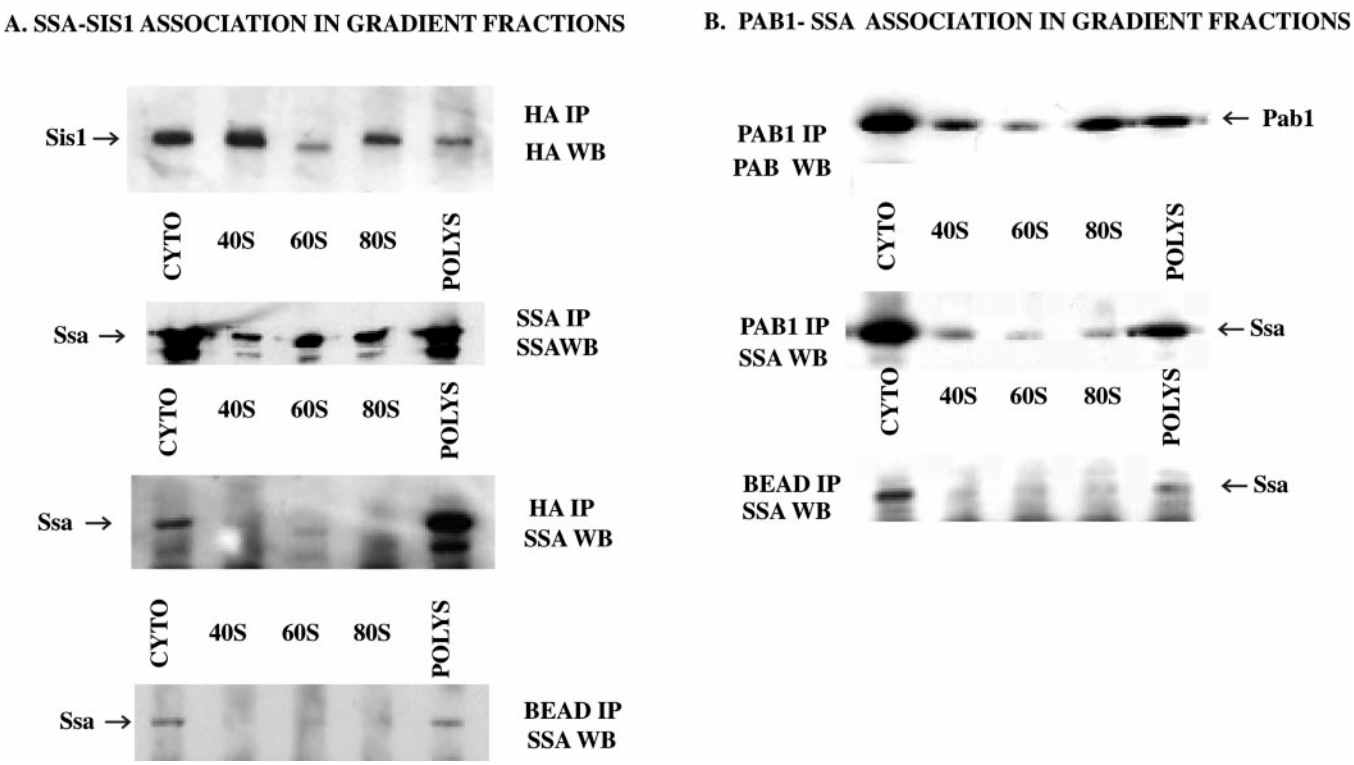

FIG. 4. **Ssa associates with Sis1 and Pab1 in both nonribosomal and polysomal fractions.** *A*, Ssa coimmunoprecipitates with Sis1 from polysomes. Lysates prepared from the *SIS1-HA* strain were fractionated in 10–25% sucrose gradients. Proteins were collected from the soluble cytoplasm (*CYTO*), 40 S, 60 S, 80 S, and polysome (*POLYS*) fractions; immunoprecipitated (*IP*) with the HA antibody (*top* and *third panels*), Ssa antibody (*second panel*), or beads alone (*bottom panel*); and probed for the presence of Sis1 (*top panel*) or Ssa (*panels 2–4*). The *bottom two panels* represent equal exposure times. *B*, Ssa coimmunoprecipitates with Pab1 from polysomes. Lysates prepared from the *SSA* strain were fractionated onto 10–25% sucrose gradients. Proteins were collected from the soluble cytoplasm (*CYTO*), 40 S, 60 S, 80 S, and polysome (*POLYS*) fractions. Proteins were mmunoprecipitated with Pab1 antibody (*top* and *middle panels*) or beads alone (*bottom panel*) and probed for Pab1 (*top panel*) or Ssa (*middle* and *bottom panels*) as described above. The *middle* and *bottom panels* represent equal exposure times. *WB*, Western blot.

determined whether the decrease in translation rate was due to a decrease in mRNA abundance. RNA was extracted from equivalent amounts of *ssa1–45* yeast at the permissive temperature and 30 min following temperature shift. Total cellular mRNA was quantified by slot-blot hybridization with ^{32}P-labeled cDNA made from total cellular mRNA. As demonstrated in Fig. 5*B*, there was no significant decrease in mRNA abundance following temperature shift. Therefore, the reduction in translation seen at this time appears due to an effect on the translational machinery and not a lack of mRNA.

Sucrose gradients were used to further define the nature of the translational defect in the *ssa1–45* yeast. (Fig. 5*C*). At the permissive temperature, the *SSA* and *ssa1–45* strains had a similar distribution of ribosomes. However, following shift to the nonpermissive temperature, the *ssa1–45* strain demonstrated a rapid loss of polysomes with an accompanying increase in 80 S ribosomes. The 60% decrease in polysome content at this time paralleled the decrease in translation rate, as determined by radioactive methionine incorporation (Fig. 5*A*). With additional time of incubation, a further decrease occurred, and by 60 min the loss of polysomes was nearly complete (Fig. 5*C* and data not shown). These results are similar to that seen with Sis1 depletion, suggesting that these proteins both affect translation initiation (10).

To ensure that the sustained decrease in translation observed following temperature shift of the *ssa1–45* strain was not simply an exaggerated heat shock response, Ssa protein was depleted by use of a strain that contained a triple *SSA1,2,4* knockout covered by a plasmid that expressed *SSA1* under the control of the galactose promoter (herein called *pGAL-SSA1*) (34). Following 7 h of growth in glucose medium, translation was assessed as above. Similar to the *ssa1–45* strain, a reduction in polysome content occurred, and this was accompanied by an increase in 80 S ribosomes (Fig. 5*D*). This change is made more dramatic by the fact that shifting yeast from galactose to glucose increases translation rate, with an accompanying increase in polysomes and a decrease in 80 S ribosomes (Ref. 35 and data not shown).

The effect of Ssa depletion on the relative abundance of polysomes and 80 S ribosomes was consistent with a defect in translation initiation (10, 36). If Ssa depletion preferentially affected initiation, then cycloheximide should prevent the redistribution of ribosomes into inactive 80 S complexes by increasing the elongation phase of translation or by blocking run-off of translating polysomes. As demonstrated in Fig. 5*E*, this was the case, since concurrent exposure to cycloheximide at the nonpermissive temperature prevented polysome depletion and 80 S ribosome accumulation. Thus, the primary translational defect of Ssa depletion appears to be in initiation.

Depletion of Ssa Affects Pab1 Association with eIF4G—The translational effects of Pab1 are mediated at least in part by its interaction with initiation factor, eIF4G (16–19). We therefore investigated whether Ssa affected this interaction by analyzing Pab1 immunoprecipitates for eIF4G by Western blotting (Fig. 6). Growth at the nonpermissive temperature did not affect interaction of eIF4G with Pab1 in the wild-type *SSA* strain (Fig. 6, *top two panels*). However, in the *ssa1–45* strain, growth at the nonpermissive temperature resulted in a substantial decrease in recovery of eIF4G. Although expression of eIF4G was also reduced in this latter strain, growth at the nonpermissive temperature did not alter eIF4G expression. Thus, interaction of Pab1 and eIF4G is reduced in the absence of functional Ssa.

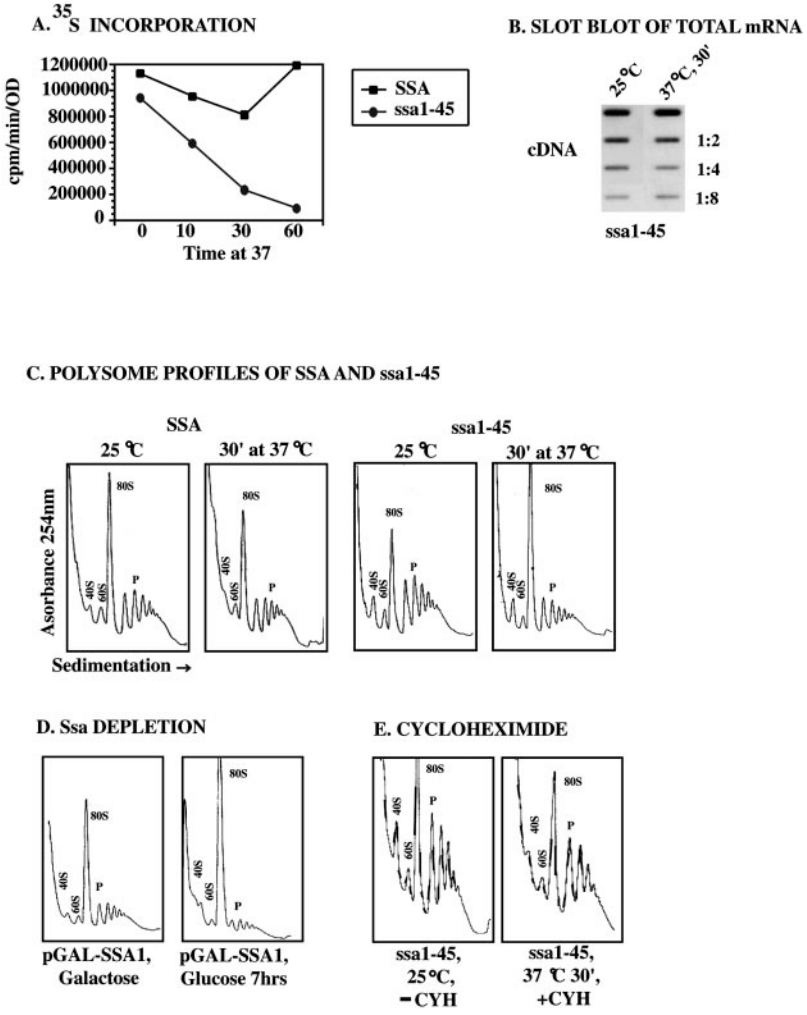

FIG. 5. **Depletion of Ssa causes a defect in translation.** *A*, [35S]methionine incorporation into acid-precipitable peptides. SSA (*squares*) and *ssa1–45* (*circles*) yeast were pulse-labeled for 2 min with [35S]methionine at 25 °C and at 10, 30, or 60 min following shift to 37 °C. Values are in cpm/min/A_{600}. *B*, mRNA content in ssa1–45 yeast. *ssa1–45* yeast were split into two equal aliquots and grown at 25 °C or 37 °C for 30 min. RNA was extracted from an equal A_{600} of each sample, and recovered RNA was loaded on a slot blot at the indicated dilutions. The blot was probed with 32P-cDNA made from total mRNA. *C*, ssa1–45 yeast demonstrate a decrease in polysomes and an increase in 80 S ribosomes at the nonpermissive temperature. *SSA* and *ssa1–45* yeast were grown at 25 °C in YEPD. Half of the culture was shifted to 37 °C for 30 min. Equal amounts of lysate were prepared and loaded onto sucrose gradients. Gradients were collected with continuous monitoring of UV absorbance at 254 nm. The positions of the 40, 60, and 80 S peaks are indicated. *P* indicates the presence of the first polysome fraction. *D*, Ssa depletion inhibits translation. *pGAL-SSA* yeast were grown in medium containing 2% galactose until log phase. Half of the culture was shifted to medium containing 2% glucose for 7 h. Equal amounts of lysate were analyzed by sucrose gradient sedimentation as described above. *E*, cycloheximide prevents the accumulation of 80 S ribosomes and reduction of polysomes. *ssa1–45* yeast were treated with cycloheximide at 25 or 37 °C for 30 min. Lysates were analyzed by sucrose gradient sedimentation, as above.

DISCUSSION

The data presented here demonstrates that the yeast hsp70 homologue, Ssa, has a specific physical interaction on translating polysomes with Pab1, as well as with Sis1. As shown previously for mammalian hsp40 and hsp70 and shown here for Pab1-Ssa, these interactions are mediated by the variable C-terminal domain on hsp70 (20). Ssa appears necessary for translation initiation, since a temperature-sensitive strain of Ssa shows a decrease in polysome content and an accumulation of salt-labile, 80 S ribosomes at the nonpermissive temperature. Depletion of Ssa also reduces interaction of Pab1 and eIF4G, suggesting this to be a target for the chaperone activity of Ssa.

The data presented here provide evidence that Ssa interacts with Pab1. One explanation for this is that Ssa serves as a chaperone for Pab1. However, in that case, Ssa would be expected to interact with Pab1 via its peptide binding domain. Instead, Pab1 binds to the C-terminal variable domain on Ssa. Further, since Sis1 does not bind Pab1, Pab1 appears to be an unlikely substrate for the chaperone activity of the Ssa-Sis1 complex. Hop, another protein that binds to the C-terminal variable domain on hsp70s, stimulates the interaction between substrate-bound hsp70 and hsp90 (20, 37–39). Therefore, Pab1 may function like Hop to mediate the association of Ssa and its partner Sis1 with substrates on ribosomal complexes.

Since Sis1 stimulates the ATPase activity of Ssa, it was not surprising that these proteins coimmunoprecipitated (10, 13). In fact, as demonstrated here, this interaction appears markedly enhanced on polysomes, suggesting that Ssa is the hsp70

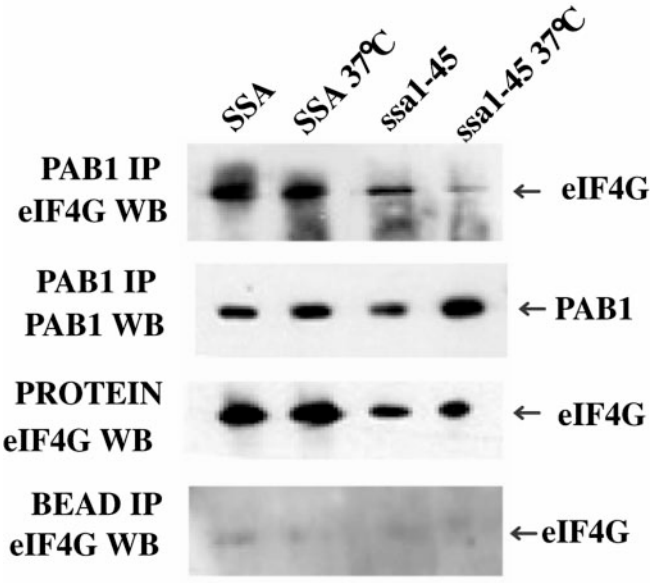

FIG. 6. **Depletion of Ssa affects the eIF4G interaction with Pab1.** *SSA* and *ssa1–45* yeast were grown at 25 °C, and then half of the culture was shifted to 37 °C for 30 min. Equal amounts of lysate were prepared and immunoprecipitated with Pab1 antibody (*top panels*) or beads alone (*bottom panel*). Recovered proteins were analyzed by immunoblotting with antibodies to either eIF4G (*top panel*) or Pab1 (*second panel*). Expression of eIF4G in the cell lines was assessed by removing an aliquot of the lysate prior to immunoprecipitation and probing for the presence of eIF4G as described under "Experimental Procedures" (*third panel*).

partner for previously described translational function of Sis1. However, despite the fact that genetic evidence suggests a functional relationship between Sis1 and Pab1 (10, 15), a physical interaction of these two proteins could not be demonstrated. A Pab1-Sis1 complex may be transient or undetectable by our means. Alternatively, Sis1 and Pab1 may bind Ssa competitively through the Ssa C-terminal domain (20). In addition to its role in stimulating the ATPase activity of hsp70 proteins, the hsp40 partner functions in the substrate specificity and intracellular localization of hsp70 (40–42). Therefore, by binding both Pab1 and Sis1 through its C-terminal domain, Ssa may be first targeted to ribosomal complexes through its interaction with Pab1 and subsequently to a specific substrate by Sis1.

The fact that Sis1 and Pab1 both affect translation initiation and that Ssa associates with Pab1 and Sis1 on ribosomes, led us to investigate whether this latter protein also affected translational initiation. Such a role for Ssa was foreshadowed in experiments investigating the role of Ssa in folding newly synthesized ornithine transcarbamylase (7). Incubation of the *ssa1–45* strain at the nonpermissive temperature resulted in a 90% decrease in ornithine transcarbamylase translation. Utilizing this same temperature-sensitive strain, the experiments reported here demonstrate a similar overall decrease in protein synthesis rate, a reduction in polysomes, and an increase in translationally inactive 80 S ribosomes at the nonpermissive temperature. These results are consistent with a defect in translation initiation. The translational effect of depletion of Ssa is similar to those previously reported following loss of function of both Sis1 and Pab1 (10, 15). Thus, these interacting proteins all function in translation initiation.

Both Ssb and Ssa associate in an RNase-sensitive, salt-stable manner with ribosomes, and both aid in the folding of nascent peptides (1, 8, 9). However, while Ssa and Ssb are related subfamilies of cytosolic hsp70 proteins, they do not complement each other, and *SSA* function is essential, while *SSB* is not (9, 12, 29). Sis1 stimulates the ATPase activity of Ssa but Ssb (13). This work provides further evidence that Ssa and Ssb have distinct functions. These proteins differ dramatically in their ability to associate with Pab1. Depletion of Ssa and Ssb produce dissimilar effects upon translation as assayed by polysome profiles (Refs. 9 and 12 and this study). Current data suggest a role for Ssb in elongation, whereas our data suggest a role in initiation for Ssa (Refs. 9 and 12 and this study). Consistent with this, subtle differences in the polysomal distribution of these two proteins exist, with Ssa being most abundant in small polysomes, while Ssb is most abundant in the largest polysomes. Interestingly, the bacterial translation initiation factor IF2 has chaperone function similar to hsp70 (43), suggesting that chaperone functions may be required at multiple steps during translation.

Pab1 is part of a multiprotein complex that plays a role in translation initiation, and one of its functions is to bind eIF4G, bringing the 5′- and 3′-ends of the mRNA in proximity (16–19). While mutation of the Pab1 binding site on eIF4G is not lethal, this interaction is presumed to be important for cap-dependent translation initiation (19, 26, 44). The data presented here demonstrate that Ssa appears necessary for the association of Pab1 with eIF4G. Although we have not yet been able to unequivocally demonstrate a physical interaction between eIF4G and Ssa (data not shown), this result provides one explanation for the effect of Ssa depletion on translation initiation. However, it may be anticipated that Ssa modulates other, as yet unidentified ribosomal functions (possibly related to rpl39), since the translational effect of heat shock is conserved in bacteria, in which the mechanism of mRNA binding to ribosome is different and does not involve eIF4G.

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