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Association of Protein Biogenesis Factors at the Yeast Ribosomal Tunnel Exit Is Affected by the Translational Status and Nascent Polypeptide Sequence*[S]

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Ribosome-associated protein biogenesis factors (RPBs) act during a short but critical period of protein biogenesis. The action of RPBs starts as soon as a nascent polypeptide becomes accessible from the outside of the ribosome and ends upon termination of translation. In yeast, RPBs include the chaperones Ssb1/2 and ribosome-associated complex, signal recognition particle, nascent polypeptide-associated complex (NAC), the aminopeptidases Map1 and Map2, and the N^{α}-terminal acetyltransferase NatA. Here, we provide the first comprehensive analysis of RPB binding at the yeast ribosomal tunnel exit as a function of translational status and polypeptide sequence. We measured the ratios of RPBs to ribosomes in yeast cells and determined RPB occupation of translating and non-translating ribosomes. The combined results imply a requirement for dynamic and coordinated interactions at the tunnel exit. Exclusively, NAC was associated with the majority of ribosomes regardless of their translational status. All other RPBs occupied only ribosomal subpopulations, binding with increased apparent affinity to randomly translating ribosomes as compared with non-translating ones. Analysis of RPB interaction with homogenous ribosome populations engaged in the translation of specific nascent polypeptides revealed that the affinities of Ssb1/2, NAC, and, as expected, signal recognition particle, were influenced by the amino acid sequence of the nascent polypeptide. Complementary cross-linking data suggest that not only affinity of RPBs to the ribosome but also positioning can be influenced in a nascent polypeptide-dependent manner.

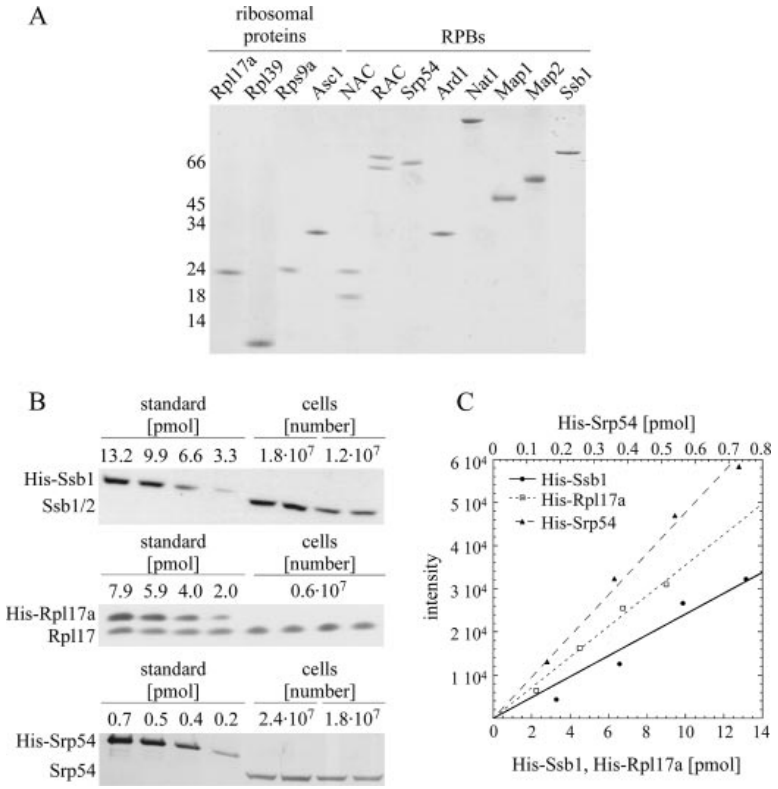


FIGURE 1. **Quantification of RPBs and ribosomal proteins.** *A*, purified RPBs and ribosomal proteins were used as standard proteins. Each 1 μg of the purified protein was separated on a 10% Tris-Tricine gel followed by Coomassie staining. Heterodimeric RAC and NAC were purified from *Saccharomyces cerevisiae*. The Srp54 subunit of SRP and the Nat1 and Ard1 subunits of NatA, Map1, Map2, and Ssb1 were expressed as His_6-tagged versions in *E. coli*. For the quantification of ribosomes two proteins of the small ribosomal subunit (Asc1 and Rps9a) and two proteins of the large subunit (Rpl39 and Rpl17a) were expressed as His_6-tagged versions in *E. coli*. For details see "Experimental Procedures." *B*, quantification via immunoblotting. Total cell extract corresponding to 0.6–2.4 $\times$ 10^7 cells of logarithmically growing wild type yeast was separated on 10% Tris-Tricine gels. Standard proteins were applied to the same gel and were analyzed by immunoblotting using antibodies specifically recognizing the proteins of interest. As an example, immunoblots for the quantification of Ssb1/2, Rpl17, and Srp54 are shown. Note that the purified, His_6-tagged standard proteins have a slightly higher molecular mass. *C*, calibration curves. Densitometric analysis was performed to determine the range of linearity for each standard and to quantify protein concentrations in the total cell extracts. A summary of the results is shown in Table 1 and in Fig. 3*B*.

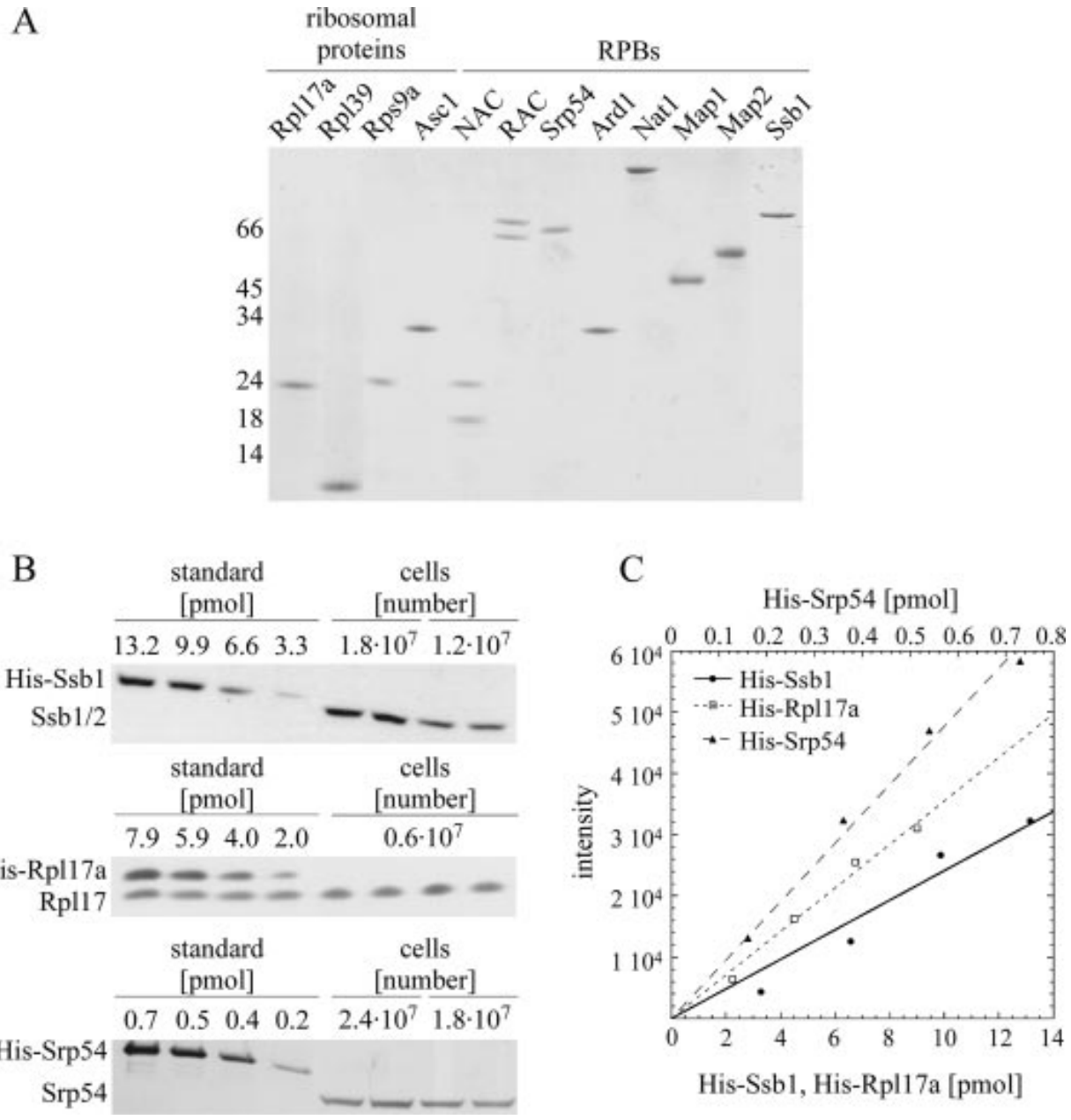

A
ribosomal proteins
RPBs
Rpl17a
Rpl39
Rps9a
Asc1
NAC
RAC
Srp54
Ard1
Nat1
Map1
Map2
Ssb1
66
45
34
24
18
14
B
standard [pmol]
cells [number]
13.2 9.9 6.6 3.3
1.8·10^7 1.2·10^7
His-Ssb1
Ssb1/2
standard [pmol]
cells [number]
7.9 5.9 4.0 2.0
0.6·10^7
His-Rpl17a
Rpl17
standard [pmol]
cells [number]
0.7 0.5 0.4 0.2
2.4·10^7 1.8·10^7
His-Srp54
Srp54
C
His-Srp54 [pmol]
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
intensity
His-Ssb1
His-Rpl17a
His-Srp54
0 2 4 6 8 10 12 14
His-Ssb1, His-Rpl17a [pmol]

TABLE 1

Quantification of RPBs and ribosomes in a logarithmically growing yeast cell

Quantifications were performed as outlined in Fig. 1 and are derived from the analysis of at least three independently grown cultures. Protein/subunit per cell is the number of the respective molecule in a yeast cell. Oligomer per cell is an average of the number of subunits contained in one complex. RPBs per 100 ribosomes is the percentage of RPBs compared to ribosomes in a logarithmically growing yeast cell.

	Protein/ subunit	Protein/subunit per cell	Oligomer per cell	RPBs per 100 ribosomes
Ribosome	Rps9	2.2×10^5	3.15×10^5	
	Asc1	2.6×10^5		
	Rpl39	3.9×10^5		
	Rpl17	3.9×10^5		
Ssb1/2	Ssb1/2	2.80×10^5		89.1
RAC	Ssz1	6.71×10^4	8.61×10^4	27.3
	Zuo1	1.05×10^5		
NAC	αNAC	3.91×10^5		125
SRP	Srp54	7.85×10^3		2.5
Map1	Map1	2.11×10^4		6.7
Map2	Map2	6.21×10^3		2.0
NatA	Nat1	7.66×10^3	7.63×10^3	2.4
	Ard1	7.59×10^3		

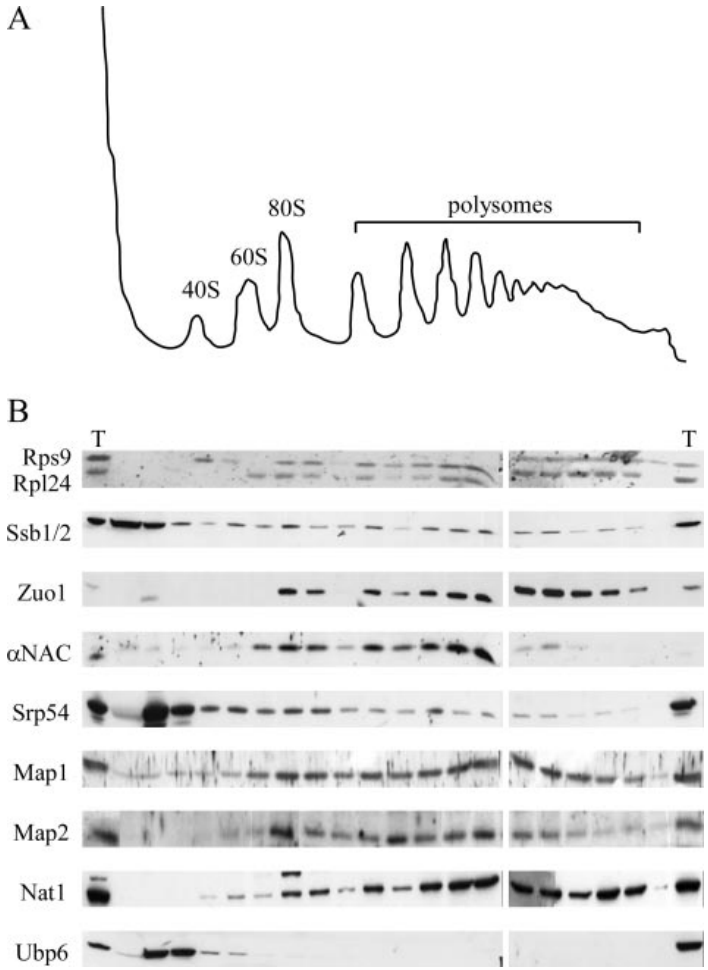


FIGURE 2. **Interaction of RPBs with polysomes.** *A*, ribosome profile of logarithmically growing wild type yeast. Log-phase yeast grown on a rich glucose medium was supplemented with 100 μM cycloheximide prior to harvest in order to stabilize translating ribosomes. Total cell extract was applied to sucrose gradient centrifugation. To localize ribosomal subunits (*40 S*, *60 S*), monosomes (*80 S*), and polysomes, fractionation was monitored at 254 nm. *B*, localization of RPBs in a polysome-rich ribosome profile. Aliquots of the 20 fractions were analyzed by immunoblotting using antibodies as indicated. On each gel 1/20 of the total cell extract (*T*) was loaded as a control. Ubp6 was used as a marker for the localization of cytosolic proteins in the gradient; ribosomal proteins Rps9a (small subunit) and Rpl24a (large subunit) were used as markers for the ribosomal subunits.

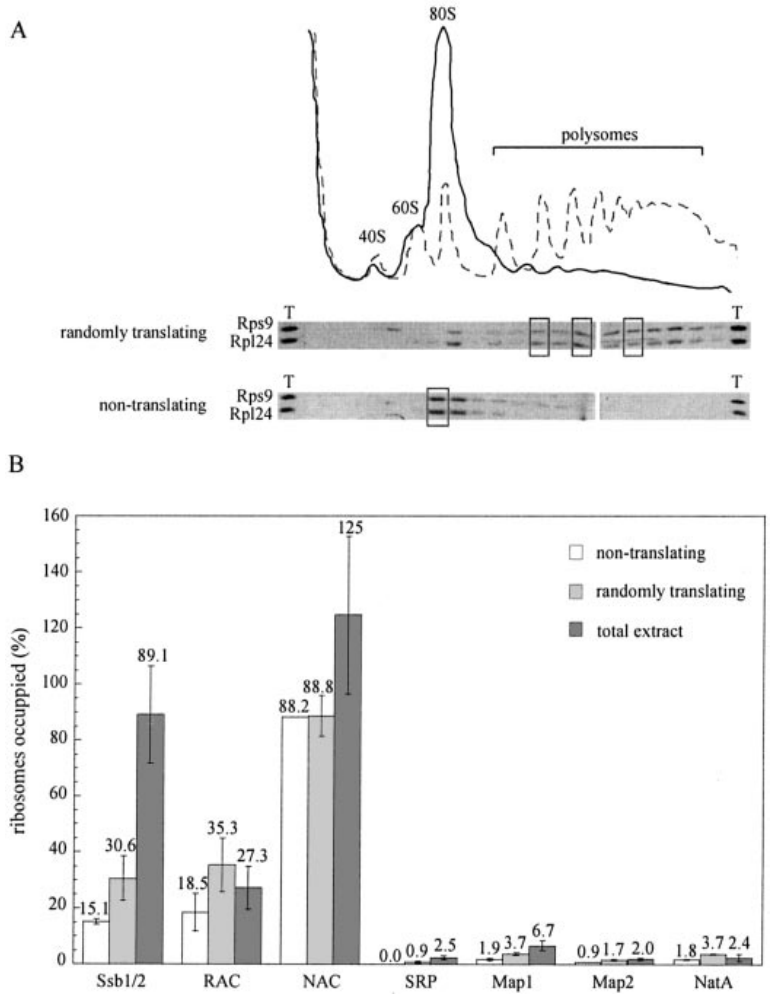


FIGURE 3. **Quantification of RPBs on non-translating and randomly translating ribosomes.** *A*, ribosome profiles of extracts rich in non-translating ribosomes (*solid line*) or randomly translating ribosomes (*dashed line*). Profiles were generated as described under "Experimental Procedures." Fractions were analyzed for the localization of the small ribosomal subunit (*Rps9*) and large ribosomal subunit (*Rpl24*) by immunoblotting. Fractions used for the quantification of RPBs and ribosomes are *boxed*. *B*, RPBs bound to randomly translating or non-translating ribosomes. Aliquots of the boxed fractions shown in *panel A* were analyzed by quantitative immunoblotting. The occupation of ribosomes by RPBs is given in percent. For comparison the number of RPBs/100 ribosomes contained in total extracts is shown (Table 1). *Error bars* indicate the S.D.

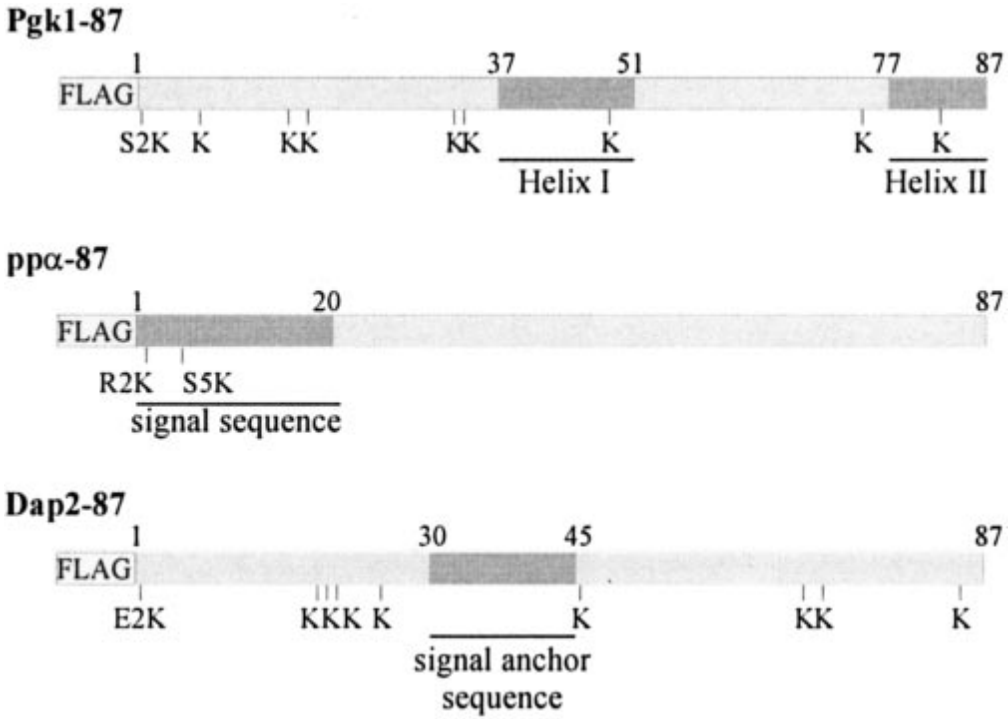


FIGURE 4. **Ribosome-bound nascent polypeptides used as model substrates.** Yeast Pgk1 (3-phosphoglycerate kinase 1) is a monomeric cytosolic protein (39), yeast ppα-factor is the precursor of the secreted pheromone α-factor (40), and yeast Dap2 is a vacuolar type II membrane protein (41). RNCs containing the N-terminal 87 amino acids of Pgk1, ppα-factor, or Dap2 as a nascent polypeptide were used for RPB binding and cross-linking experiments. For the purification of RNCs, nascent polypeptides were fused to an N-terminal FLAG tag (DYKDDDDK). The position of lysines (*K*) that provided primary amino groups for the cross-linking reactions is indicated. For cross-linking experiments untagged versions of the proteins were used in which the amino acid at position 2 was changed to a lysine. Helical regions of Pgk1, the N-terminal signal sequence of ppα-factor, and the signal anchor sequence of Dap2 are indicated.

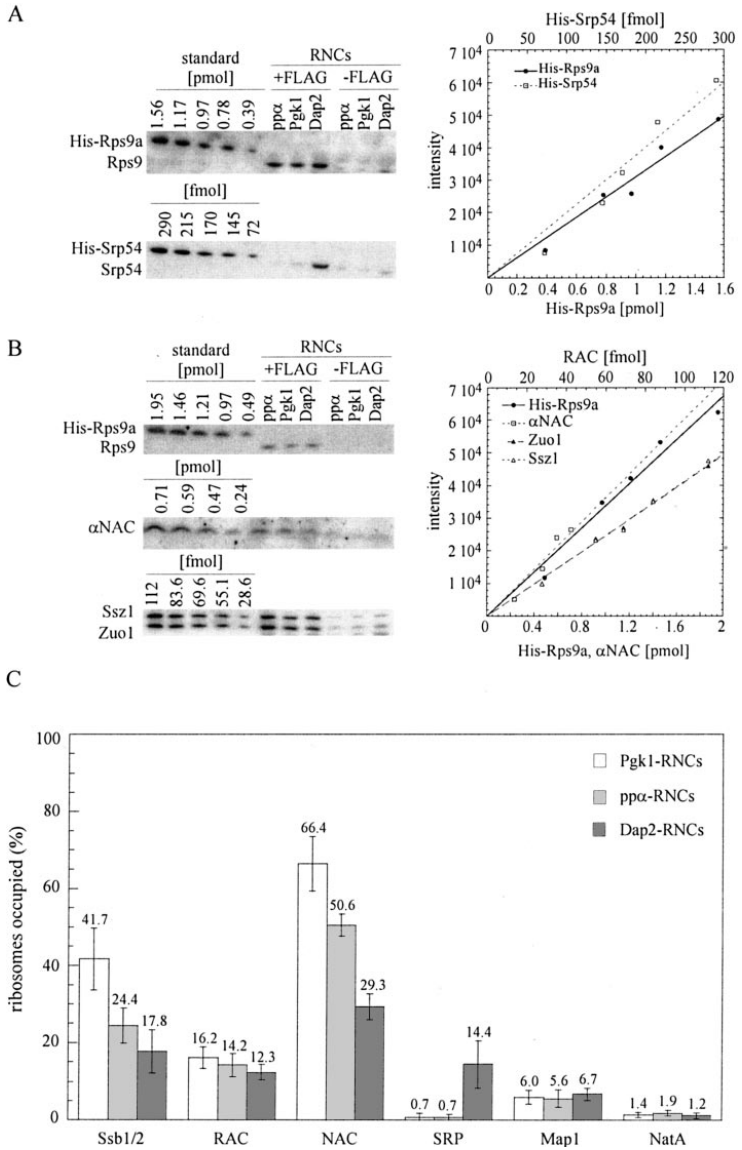
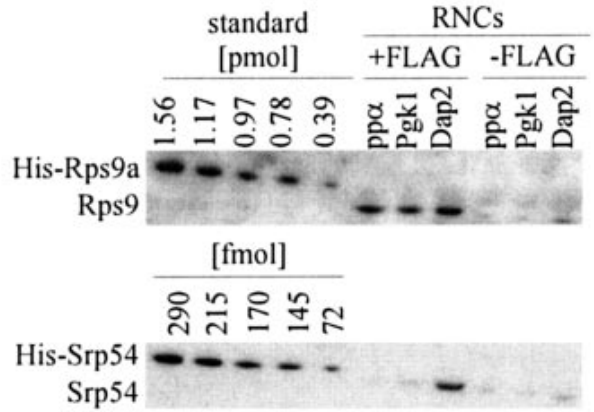


FIGURE 5. **Quantification of RPBs on RNCs engaged in the translation of specific nascent polypeptides.** A yeast translation extract was programmed with truncated mRNA encoding the N-terminal 87 amino acids of Pgk1 (Pgk1–87), ppα-factor (ppα-87), or Dap2 (Dap2–87) fused to an N-terminal FLAG tag (+*FLAG*) or without a tag (−*FLAG*) (see Fig. 4 and "Experimental Procedures"). RNCs carrying FLAG-tagged nascent polypeptides were isolated by native immunoprecipitation using αFLAG-covered beads. RNCs carrying the same nascent polypeptide but lacking the tag served as a control in parallel reactions. Aliquots of the material recovered on αFLAG beads and standard proteins (Fig. 1) were applied to the same Tris-Tricine gel and were subsequently analyzed by immunoblotting. Signals obtained from non-tagged RNCs were subtracted as a background from the signals derived from FLAG-tagged RNCs. Quantification was performed as described in Fig. 1. As examples Rps9a and SRP (*A*) and Rps9a, αNAC, Ssz1, and zuotin (*B*) are shown. *C*, occupation of RNCs with RPBs. The occupation of Pgk1-RNCs, ppα-RNCs, and Dap2-RNCs by RPBs is given in percent. *Error bars* indicate the S.D.

A


standard
[pmol]
RNCs
+FLAG
-FLAG
1.56
1.17
0.97
0.78
0.39
ppα
Pgk1
Dap2
ppα
Pgk1
Dap2
His-Rps9a
Rps9
[fmol]
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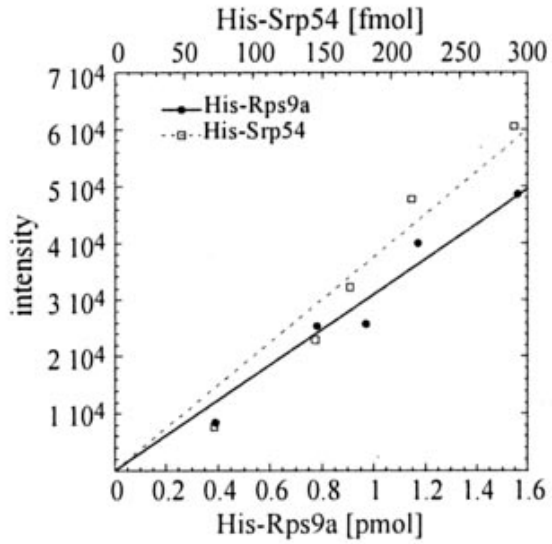
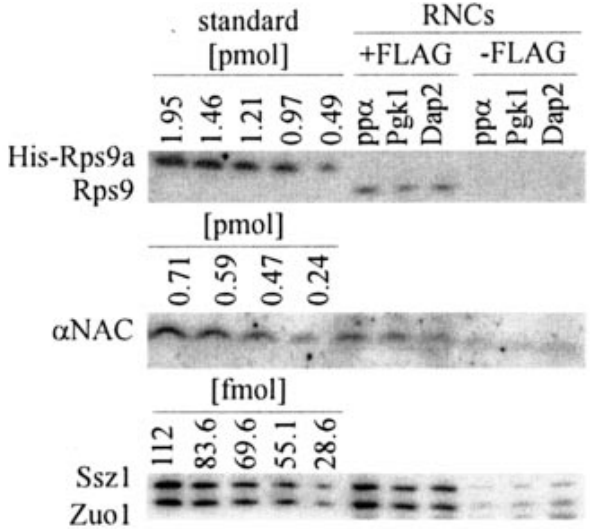
170
145
72
His-Srp54
Srp54


His-Srp54 [fmol]
0
50
100
150
200
250
300
7 10^4
6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
His-Rps9a
His-Srp54
intensity
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
His-Rps9a [pmol]

B


standard
[pmol]
RNCs
+FLAG
-FLAG
1.95
1.46
1.21
0.97
0.49
ppα
Pgk1
Dap2
ppα
Pgk1
Dap2
His-Rps9a
Rps9
[pmol]
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0.24
αNAC
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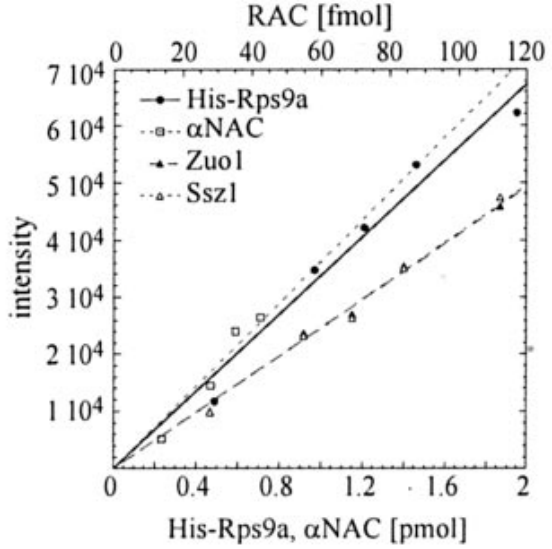
112
83.6
69.6
55.1
28.6
Ssz1
Zuo1


RAC [fmol]
0
20
40
60
80
100
120
7 10^4
6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
His-Rps9a
αNAC
Zuo1
Ssz1
intensity
0
0.4
0.8
1.2
1.6
2
His-Rps9a, αNAC [pmol]

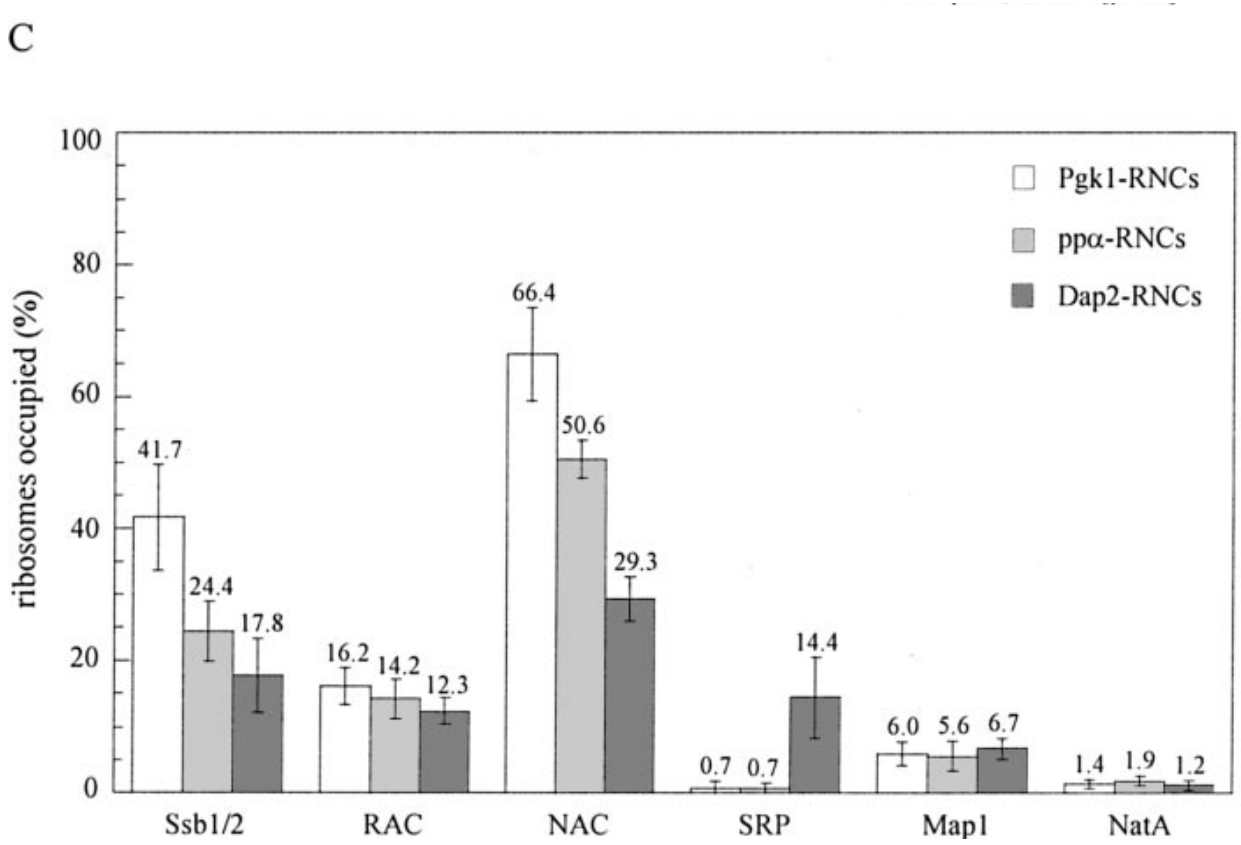


FIGURE 5. **Quantification of RPBs on RNCs engaged in the translation of specific nascent polypeptides.** A yeast translation extract was programmed with truncated mRNA encoding the N-terminal 87 amino acids of Pgk1 (Pgk1–87), ppα-factor (ppα-87), or Dap2 (Dap2–87) fused to an N-terminal FLAG tag (+*FLAG*) or without a tag (−*FLAG*) (see Fig. 4 and "Experimental Procedures"). RNCs carrying FLAG-tagged nascent polypeptides were isolated by native immunoprecipitation using αFLAG-covered beads. RNCs carrying the same nascent polypeptide but lacking the tag served as a control in parallel reactions. Aliquots of the material recovered on αFLAG beads and standard proteins (Fig. 1) were applied to the same Tris-Tricine gel and were subsequently analyzed by immunoblotting. Signals obtained from non-tagged RNCs were subtracted as a background from the signals derived from FLAG-tagged RNCs. Quantification was performed as described in Fig. 1. As examples Rps9a and SRP (*A*) and Rps9a, αNAC, Ssz1, and zuotin (*B*) are shown. *C*, occupation of RNCs with RPBs. The occupation of Pgk1-RNCs, ppα-RNCs, and Dap2-RNCs by RPBs is given in percent. *Error bars* indicate the S.D.

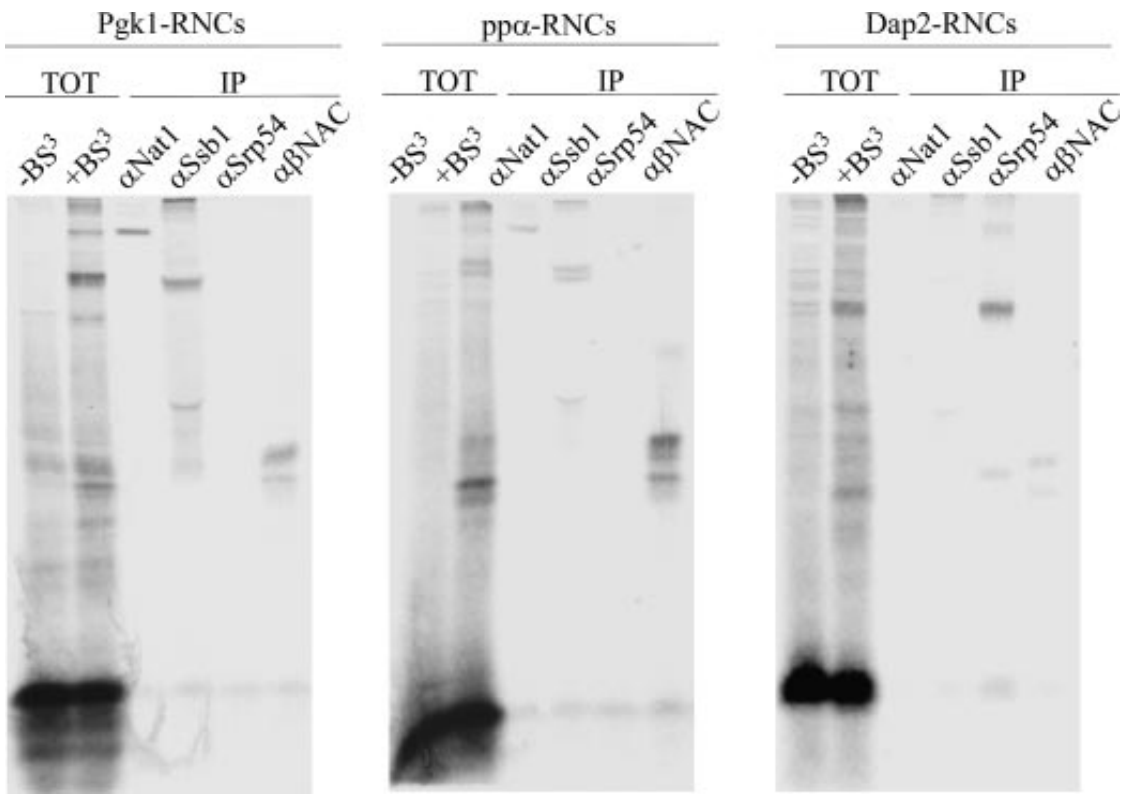


FIGURE 6. **Interaction of RPBs with nascent polypeptides.** A yeast translation extract was programmed with truncated mRNA encoding the N-terminal 87 amino acids of Pgk1 (*Pgk1-RNCs*), ppα-factor (*ppα-RNCs*), or Dap2 (*Dap2-RNCs*) (Fig. 4) in the presence of [^{35}S]methionine. RNCs were isolated by centrifugation through a sucrose cushion and were subsequently incubated either in the absence (*TOT* $-$ *BS^3*) or in the presence (*TOT* $+$ *BS^3*) of the homobifunctional cross-linker BS^3. Aliquots corresponding to 4 $\times$ the material of the TOT $+$ BS^3 were subjected to immunoprecipitations under denaturing conditions (*IP*) with antibodies directed against Nat1, Ssb1, Srp54, and α/βNAC. Samples were run on Tris-Tricine gels and were subsequently analyzed by autoradiography.