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Association of the halobacterial 7S RNA to the polysome correlates with expression of the membrane protein bacterioopsin

(bacteriorhodopsin/archaebacteria/signal-recognition particle/signal sequence)

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ABSTRACT **The sedimentation behavior of the halobacterial 7S RNA and bacterioopsin mRNA was assessed after application of total cell lysates to sucrose gradients. These two RNAs cosedimented predominantly with membrane-bound polysomes, and the quantity of 7S RNA bound to the ribosomes was directly correlated with the expression of bacterioopsin. Puromycin treatment released the 7S RNA from the polysomes, indicating that it is transiently associated with protein translation. We suggest that halobacteria contain a signal-recognition-like particle involved in translation of membrane-associated proteins.**

In mammalian cells the 7S RNA is part of the signal recognition particle (SRP), which mediates cotranslational processing mechanisms for membrane and secretory proteins (1, 2). SRP is a ribonucleoparticle that recognizes the leader sequence of the nascent polypeptide chain as it emerges from the ribosome. The interaction causes a translational arrest that is only released if the complex is targeted to a receptor in the membrane (3). These mechanisms prevent synthesis of hydrophobic proteins in a hydrophilic environment and direct membrane and secretory proteins to their final destinations in the cell. Small ribonucleoproteins resembling SRP have also been found in yeast (*Schizosaccharomyces pombe* and *Yarrowia lipolytica*) and in *Escherichia coli* (4–6), but in contrast to mammalian cells they translocate many proteins posttranslationally; genetic studies do not as yet corroborate a SRP-coupled translocation mechanism (7–9). Genetic analysis in *E. coli* has shown that the 4.5S RNA, which has been identified as a component of the ribonucleoparticle, is acting on translating ribosomes and perhaps in concert with elongation factor G GTP (10). However, it remains unclear whether it is generally obligatory for translation or only participates in translation of a subset of proteins.

Like other archaebacteria, *Halobacterium halobium* possesses a 7S RNA with a possible secondary structure almost identical to that of the mammalian SRP RNA (11–13). Its function is unknown, but as archaebacterial 7S RNA genes can replace the 4.5S RNA gene in *E. coli* (14), it is likely that a signal recognition-like particle exists in halobacteria. In this report, we demonstrate that the halobacterial 7S RNA is associated with ribosomes during translation, and our data suggest that it is specifically involved in translation of membrane proteins.

The main membrane protein in *H. halobium* is bacterioopsin, which serves the cell as a light energy converter (for reviews, see refs. 15 and 16). When oxygen is limiting during the late logarithmic and stationary phase of growth, bacterioopsin becomes the main protein synthesized in the cell (17). Bacterioopsin is synthesized as a precursor with 13 extra N-terminal amino acids (18, 19). The precursor is processed in a two-step mechanism (20, 21), but processing is not necessary for correct folding of the protein. Both forms integrate into the purple membrane with no conformational differences from the mature protein (22). The presequence is unusual and lacks similarities to other prokaryotic and eukaryotic signal sequences (23). This observation raises questions about its functional significance. In this report we show that the N-terminal sequences of six halobacterial integral membrane proteins share a common motif that may be of functional importance.

MATERIALS AND METHODS

RNAzol was purchased from Cinna/Biotecx Laboratories (Friendswood, TX), puromycin was from Sigma, and nylon Hybond membranes and [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) were obtained from Amersham.

Sucrose Gradient Analysis. Cells (700 ml) (ET1001) (24) were grown in complete medium (25) to early (A_{600} = 0.15) or late (A_{600} = 1.15) logarithmic growth phase. Cells were pelleted at 9000 × *g* for 10 min and ≈2 g (wet weight) was gently lysed in 2 ml of lysis buffer (3.4 M KCl/100 mM MgOAc/10 mM Hepes, pH 7.6). The cell lysate was layered on top of a 7–30% sucrose gradient on a cushion of 50% sucrose in lysis buffer and centrifuged for 90 min at 26,000 rpm in an SW 27 rotor at 15°C (26). After centrifugation, tubes were fractionated from the top by pumping a 60% (wt/vol) sucrose solution and 0.8-ml fractions were collected. Samples of individual fractions were diluted 1:10 before determination of A_{260} and A_{410}. The absorbance reading for the membranes was done at 410 nm because this is the absorbance maximum of the membrane protein cytochrome *c*.

In the puromycin experiment, 1l cells were grown to an A_{600} of 0.6, divided into two portions, and incubated with and without puromycin (40 μg per ml of medium) as described by Sumper and Herrmann (17) under normal growth conditions for 20 min. The sucrose gradient was prepared in an SW 40 rotor and 625-μl fractions were collected.

RNA was isolated from individual fractions and analyzed by Northern blots as described below. To visualize the 23S and 16S RNA, gels were stained with 0.1% toluidine blue in 20% (vol/vol) ethanol.

Northern Analysis. Total RNA was extracted with RNAzol according to the manufacturer's instructions from fractions that were diluted with 400 μl of water. The upper phase was further diluted with 2 ml of water, and the RNA was precipitated with isopropanol at a 1:1 ratio. The RNA pellet was dissolved in 300 μl of water and an aliquot of each fraction was electrophoresed on a 1.5% formaldehyde agarose gel (27), followed by transfer to nylon membranes. Radioactive labeling to obtain probes for Northern blots was done by using random primers as described by Feinberg and Vogelstein (28). DNA fragments used to generate probes were

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Abbreviation: SRP, signal-recognition particle.

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the internal *Nae* I/*Xho* I fragment of the bacterioopsin gene, a *Bgl* II/*Pst* I fragment containing the entire *blp* gene (F.G., unpublished data), and the *Sal* I fragment containing the 7S RNA gene described by Moritz *et al.* (12).

Isolation of Free Polysomes. Cell lysate from ET1001 was prepared as described above and loaded onto a step gradient containing 7 ml of 60% and 2 ml of 20% sucrose in lysis buffer (29). The gradient was centrifuged at 35,000 rpm for 22 h at 15°C in an SW 40 rotor; the pellet was resuspended in 300 μl of lysis buffer and applied to the same linear gradient described above. Fractions were analyzed by reading A_{260}.

Quantification of 5S and 7S RNA. Various fractions from the gradient were pooled and centrifuged at 300,000 × *g* for 10 min at 5°C in a TLA-100.1 rotor; the total RNA was extracted by RNAzol as per the manufacturer's instructions. Twenty-five micrograms of total RNA was electrophoresed on an 8% acrylamide gel and visualized by staining with 0.1% toluidine blue in 20% ethanol. The 5S and 7S levels were quantified by scanning the gel with a Joyce–Loebl Ephortec densitometer (626 nm) and integrating the areas under the peaks.

RESULTS

Sedimentation Behavior of Polysomes, 7S RNA, and Bacterioopsin mRNA from Cells at the Early and Late Stages of Logarithmic Growth. To determine whether the halobacterial 7S RNA is a part of the ribosome during translation, total cell lysates from cells at the early and late stages of logarithmic growth were applied to a 7–30% sucrose gradient on a cushion of 50% as described. The gradient was fractionated and analyzed for distribution of the polysomes (Fig. 1*A*), the membranes (Fig. 1*A*), and rRNA species and specific mRNAs (Fig. 2). The position of free polysomes was determined by applying isolated free polysomes to a similar gradient (Fig. 1*B*) and by using a probe for a mRNA encoding a soluble protein in a Northern blot analysis (Fig. 2*D*). This probe corresponds to the *blp* gene, another gene in the bacterioopsin gene cluster (F.G., unpublished data). These control experiments showed that free polysomes sedimented in the middle of the gradient.

At the early growth stage, polysomes were distributed throughout the gradient with a major peak at the bottom of the gradient indicating polysomes that cosediment with the membranes (Figs. 1*A* and 2*A*). Northern hybridization with a 7S RNA-specific probe revealed 7S RNA in the fractions with free and membrane-bound polysomes. However, the strongest hybridization signal coincided with the top of the gradient, indicating that the major part of the 7S RNA is not associated with the ribosome in cells at the early growth stage.

To address the question whether the halobacterial 7S RNA has a general function in translation or acts specifically on ribosomes translating membrane proteins, we took advantage of the fact that, at late logarithmic growth, the halobacterial translation machinery is mainly focused on generating the membrane protein bacterioopsin. Under this condition, the polysomes almost exclusively cosedimented with the membranes as shown by both the A_{260} (Fig. 1*A*) and by visualizing 16S and 23S RNA in an agarose gel (Fig. 2*A*). Thus, the ratio of free to membrane-bound polysomes differed significantly between the early and late logarithmic stages of growth. Northern hybridization with a 7S RNA-specific probe (Fig. 2*B*) revealed the strongest signal at the bottom of the gradient, indicating that the 7S RNA almost exclusively cosedimented with membrane-bound polysomes at the late logarithmic stage of growth. Hybridization with a probe specific for bacterioopsin mRNA paralleled this pattern (Fig. 2*C*). The strongest hybridization signal coincided with the bottom of the gradient.

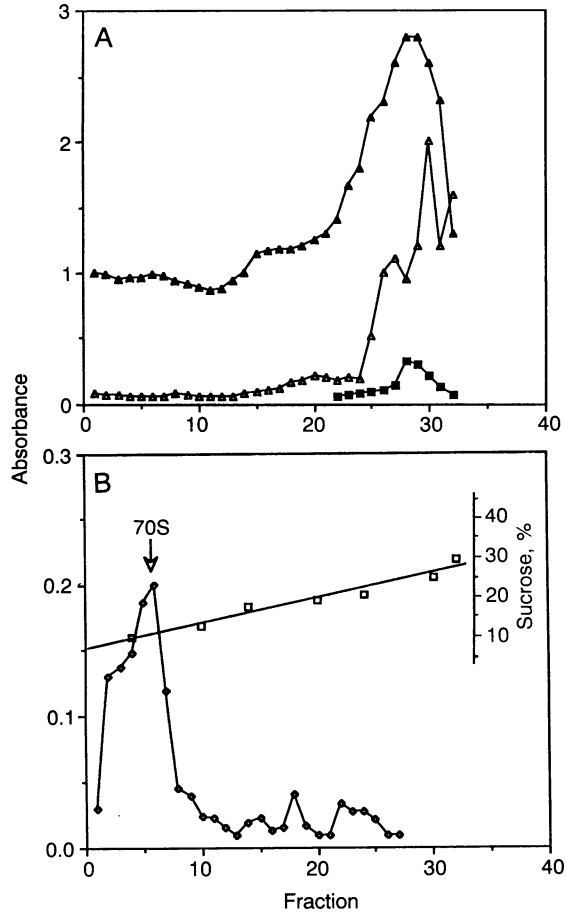

FIG. 1. Sedimentation behavior of polysomes from cells at early and late stages of logarithmic growth. Cell lysates from cells grown to A_{600} of 0.15 and 1.15 were applied to a 7–30% sucrose gradient. Distribution of polysomes and membranes was determined by reading the absorption at A_{260} and A_{410}, respectively, of fractions from the gradient. (*A*) ▲, A_{260}, early growth stage; △, A_{260}, late growth stage; ■, A_{410}, early growth stage. To indicate free polysomes in this gradient, free polysomes were isolated as described and applied to a similar gradient. (*B*) ◇, Isolated free polysomes. □, Density of the gradient.

Sedimentation Behavior of 7S RNA and Bacterioopsin mRNA After Puromycin Treatment. Puromycin is a translation inhibitor that releases the nascent polypeptide chain from the ribosome (30). If the ribosomes are attached to the membranes via the nascent polypeptide chain, the polysomes would be expected to detach from the membranes and sediment as free polysomes in the gradient after puromycin treatment. In addition, if the halobacterial 7S RNA is involved in translation, puromycin treatment should result in release of the 7S RNA from the ribosome and sedimentation on top of the gradient. To determine whether the binding of the polysomes to the membranes is specific and whether the halobacterial 7S RNA is involved in translation, sedimentation analysis was performed after puromycin treatment of cells *in vivo* at the midlogarithmic stage of growth. The gradient was analyzed by isolation of total RNA from the fractions of the gradient followed by electrophoresis on a denaturing agarose gel and Northern hybridization. The stained gel (Fig. 3*A*) shows an increase in rRNA in the middle of the gradient after puromycin treatment, which indicates an increase in free polysomes. In addition, the 7S RNA (Fig. 3*B*) has shifted together with the polysomes to the area of free polysomes and the amount of 7S RNA remaining on top of the gradient has increased. Puromycin treatment also resulted in

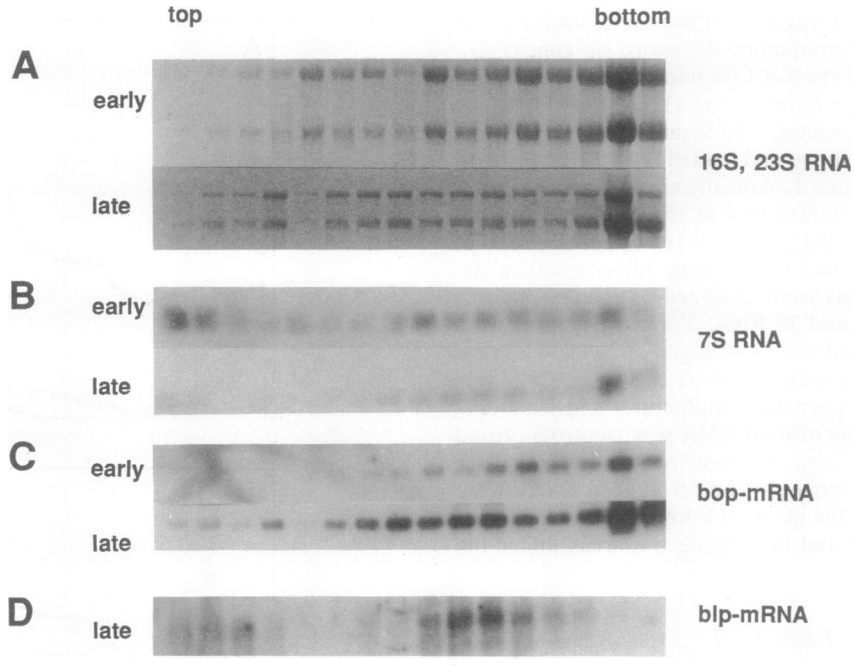

FIG. 2. Sedimentation behavior of 7S RNA and bacterioopsin mRNA at early and late stages of logarithmic growth. Total RNA was extracted from the fractions described in Fig. 1. Every second sample was separated on a 1.5% agarose gel and analyzed by Northern hybridization. (*A*) Gel stained with toluidine blue. (*B*) Northern blot hybridization with a 7S RNA-specific probe. (*C*) Northern blot hybridization with a bacterioopsin (bop) mRNA-specific probe. (*D*) Northern blot hybridization with a blp mRNA-specific probe.

a partial shift of bacterioopsin mRNA to the middle of the gradient (Fig. 3*C*), the region where free polysomes would be expected to sediment. The shift was not complete under these conditions, but length of treatment was kept to a minimum to avoid indirect effects on transcription by inhibition of translation of DNA-dependent RNA polymerase.

Ratio of 5S to 7S RNA Throughout the Gradient. Lysates from cells grown to the late logarithmic stage of growth were fractionated on a sucrose gradient as described above. Total RNA was isolated from pooled fractions and analyzed by electrophoresis on an 8% polyacrylamide gel (Fig. 4). Amounts of 5S and 7S RNA were quantified by scanning the stained bands as described (Table 1). These data indicate that the ratio of 5S to 7S RNA was not constant throughout the gradient. At the bottom of the gradient where most of the membrane-bound polysomes sedimented (i.e., fraction 5; see Table 1 and Fig. 4), about 1/3rd of the ribosomes appeared to be associated with 7S RNA. This amount decreased to 1/6th in the middle of the gradient and to 1/10th at the top of the gradient. The increase in 7S RNA observed at the bottom of the gradient suggests that the 7S RNA is involved not in translation of every protein but in those translated on membrane-bound polysomes.

DISCUSSION

Our data show that the halobacterial 7S RNA comigrated with polysomes in a sucrose gradient, indicating an association between 7S RNA and the ribosome during translation. This association is probably transient since the 7S RNA has

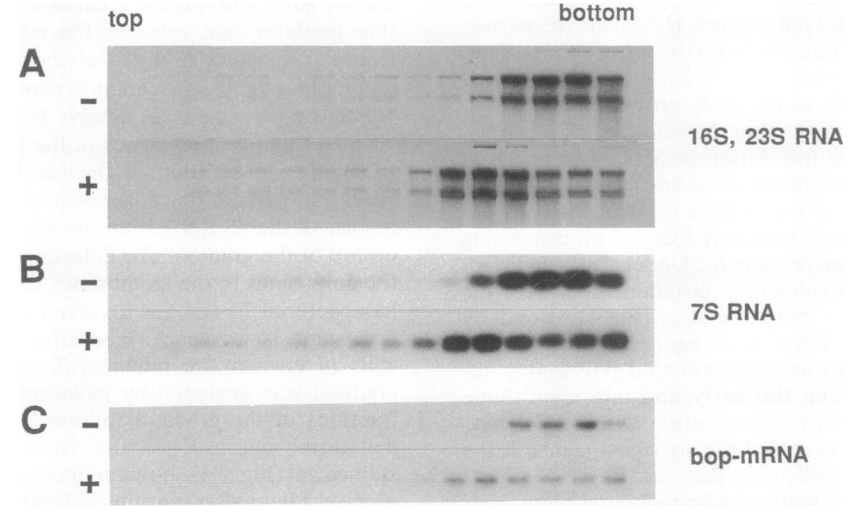

FIG. 3. Sedimentation behavior of 7S RNA and bacterioopsin mRNA after puromycin treatment of cell lysate. Cells at midlogarithmic growth were incubated with (+) and without (−) puromycin and applied to a 7–30% sucrose gradient. Total RNA was extracted from the fractions, separated on a 1.5% agarose gel, and analyzed by Northern hybridization. (*A*) Gel stained with toluidine blue. (*B*) Northern blot hybridization with a 7S RNA-specific probe. (*C*) Northern blot hybridization with a bacterioopsin (bop) mRNA-specific probe.

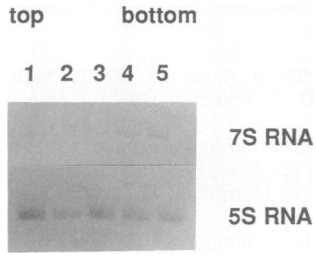

FIG. 4. Ratio of 7S/5S RNA throughout the sucrose gradient. Cell lysates from cells grown to the late stage of logarithmic growth were applied to a 7–30% sucrose gradient. Total RNA was extracted from the following pooled fractions: 6–11 (lane 1), 12–17 (lane 2), 18–23 (lane 3), 24–29 (lane 4), 30–32 (lane 5). Twenty-five micrograms of total RNA was separated on an 8% polyacrylamide gel and stained with toluidine blue.

Table 1. Integrated peak areas of 7S and 5S RNA and ratio of 7S/5S RNA obtained from the gel shown in Fig. 4

Fraction	7S RNA	5S RNA	7S/5S RNA
1	165	1794	0.09
2	128	873	0.14
3	182	1181	0.15
4	499	1168	0.42
5	418	1399	0.29

not been detected as a component of the ribosome (12) and puromycin treatment of cells released the 7S RNA from the polysomes. Thus, the halobacterial 7S RNA appears to participate in translation, but whether it acts in a SRP-like manner by translocating membrane proteins remains to be conclusively determined. However, we believe that it is likely based on the following observations.

First, as shown here, the amount of 7S RNA associated with the polysomes is dependent on the growth stage of the cells. During the early stages of logarithmic growth when the soluble and membrane proteins necessary for growth are synthesized, more free polysomes were detectable in the gradient. Under these conditions, 7S RNA comigrated with polysomes in the gradient; however, the strongest hybridization signal for the 7S RNA correlated with the top of the gradient. These data indicate that only a portion of the 7S RNA is involved in translation. In contrast, the bulk of the 7S RNA comigrated with the polysomes during the late growth stage when the translation machinery is focused on generating the membrane protein bacterioopsin and the majority of the polysomes were associated with the membranes. This correlation of the amount of 7S RNA associated with the polysomes and the expression of bacterioopsin indicates that 7S RNA is necessary for translation of bacterioopsin.

Second, the ratio of 7S to 5S RNA varied throughout the sucrose gradient. On membrane-bound polysomes 1/3rd of all ribosomes appear to be associated with 7S RNA, whereas in the region of free polysomes the amount decreased to 1/6th–1/10th (Table 1). The middle of the gradient most likely contains three distinct populations of polysomes: (*i*) free polysomes responsible for translation of soluble proteins, (*ii*) polysomes translating mRNA of membrane proteins not yet attached to the membranes, and (*iii*) some membrane-bound polysomes associated with smaller fragments of membranes whose sedimentation is not completed under these conditions (26). Less 7S RNA is bound to the ribosome in the region of free polysomes, suggesting that the halobacterial 7S RNA participates not in translation of every protein but in translation of those that are translated on membrane-bound polysomes.

Third, the majority of the bacterioopsin mRNA cosedimented with membrane-bound polysomes and was released from the membranes after puromycin treatment. After incubation with puromycin, the bacterioopsin mRNA shifted from the region of membrane-bound polysomes to the region of free polysomes, indicating that binding to the membrane is caused by the nascent polypeptide chain. This observation is consistent with a SRP-coupled translation, which implicates a cotranslational translocation event.

In mammalian cells, a crucial step in a SRP-mediated translocation mechanism is recognition of the signal sequence by the 54-kDa domain of the particle. Proteins similar to the mammalian 54-kDa protein have been found in yeast and *E. coli*, and both contain a methionine-rich domain, which has been proposed to recognize the signal sequence as it emerges from the ribosome (31). Eukaryotic and prokaryotic signal sequences are also similar and appear to follow certain rules despite the high variability of the primary sequences. Typical signal sequences possess a short hydrophilic region containing positively charged amino acids at the N terminus, followed by a region of at least eight hydrophobic amino acids (23).

However, the 13-amino acid presequence of bacteriorhodopsin is shorter than most signal sequences, does not contain the hydrophobic core, and contains two negatively charged glutamic acids instead of the positively charged amino acids. Since the halobacterial glycoprotein CGS, which is secreted extracellularly, possesses a typical signal sequence (32), the unusual bacterioopsin precursor sequence may not be characteristic for halobacterial proteins in general but may represent a specialized signal sequence characteristic of a halobacterial integral membrane protein. This hypothesis is supported by comparison to other halobacterial integral membrane proteins. In addition to bacteriorhodopsin, five other halobacterial integral membrane pro-

FIG. 5. Comparison of the N-terminal sequences of six halobacterial integral membrane proteins and the halobacterial glycoprotein. BR, bacteriorhodopsin; AR, archaerhodopsin; AR2, archaerhodopsin 2; SR, sensory rhodopsin; HR, halorhodopsin; pHR, halorhodopsin from *H. pharaonis*; CGS, halobacterial glycoprotein.

teins, which are also retinal binding proteins, have been characterized: halorhodopsin (HR) from *H. halobium* (33), halorhodopsin (pHR) from *Halobacterium pharaonis* (34), sensory rhodopsin (SR) from *Halobacterium halobium* (35), archaerhodopsin (AR) from *Halobacterium* sp. *aus-1* (36), and archaerhodopsin 2 (AR2) from *Halobacterium* sp. *aus-2* (37). All six proteins share sequence similarities in the N-terminal region (Fig. 5). A region of three to six hydrophobic amino acids is flanked at the N terminus by a negatively charged amino acid (i.e., glutamate or aspartate) and at the C terminus by threonine and alanine for bacteriorhodopsin, AR, and SR; by glutamine and alanine for AR2 and pHR; or by glutamine and serine for HR. Whether this motif acts as a signal for targeting the proteins to the membranes or for correct folding in the membrane remains to be determined. In light of the differences between the glycoprotein and the bacterioopsin presequences, two different translocation mechanisms may exist.

Note. After this manuscript was submitted for review, a paper by Ramirez and Matheson (38) was published on the gene coding for the α subunit of the SRP receptor in the archaebacterium *Sulfolobus solfataricus*.

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