Forst, S. A., Delgado, J., & Inouye, M. (1989) *J. Bacteriol. 171*, 2949-2955.
Hall, M. N., & Silhavy, T. J. (1981) *Annu. Rev. Genet. 15*, 91-142.
Ikeda, R. A., & Richardson, C. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3614-3618.
Inokuchi, K., Mutoh, N., Matsuyama, S., & Mizushima, S. (1982) *Nucleic Acids Res. 10*, 6957-6969.
Kochetkov, N. K., & Budovskii, E. I., Eds. (1971) *Organic Chemistry of Nucleic Acids*, Part A, pp 234-257, Plenum Press, New York.
Krinke, L., & Wulff, D. L. (1987) *Genes Dev. 1*, 1005-1013.
Krupp, G., & Söll, D. (1987) *FEBS Lett. 2*, 271-275.
Laemmli, U. K. (1970) *Nature 277*, 680-685.
Lowry, O. H., Rosebrough, N. J., Fau, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Lugtenberg, B., Peters, R., Bernheimer, H., & Berendsen, W. (1976) *Mol. Gen. Genet. 147*, 251-262.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Matsuyama, S.-I., & Mizushima, S. (1985) *J. Bacteriol. 162*, 1196-1202.
Misra, R., & Reeves, P. R. (1987) *J. Bacteriol. 169*, 4722-4730.
Mizuno, T., Chou, M.-Y., & Inouye, M. (1983) *Proc. Jpn. Acad. 59*, 335-338.
Mizuno, T., Chou, M.-Y., & Inouye, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1966-1970.
Mullis, K. B., & Faloona, F. A. (1987) *Methods Enzymol. 155*, 335-350.
Ramakrishnan, G., Ikenaka, K., & Inouye, M. (1985) *J. Bacteriol. 163*, 82-87.
Romaniuk, P. J., de Steveson, I. L., & Wong, A. H.-H. (1987) *Nucleic Acids Res. 15*, 2737-2755.
Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., & Studier, W. (1987) *Gene 56*, 125-135.
Smith, K. C. (1976) in *Photochemistry and photobiology of nucleic acids* (Wang, S. Y., Ed.) Vol. 2, pp 187-218, Academic Press, New York.
Stoflet, E. S., Koeberl, D. D., Sarkar, G., & Sommer, S. S. (1988) *Science 239*, 491-494.
Tamm, J., & Polisky, B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2257-2261.
Van der Werf, S., Bradley, J., Wimmer, E., Studier, W., & Dunn, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2330-2334.
Wilusz, J., Feig, D. I., & Schenk, T. (1988) *Mol. Cell. Biol. 8*, 4477-4483.

In Vitro Reassembly of Active Large Ribosomal Subunits of the Halophilic Archaebacterium *Haloferax mediterranei*†

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ABSTRACT: The large ribosomal subunits of the halophilic archaebacterium *Haloferax mediterranei* have been reconstituted in vitro from the dissociated RNA and protein components. Efficient reassembly of particles fully active in poly(U)-directed polyphenylalanine synthesis requires a 2-h incubation at 42 °C in the presence of no less than 2.5 M concentrations of monovalent cations and of 60 mM magnesium. K^+ and NH_4^+ ions are equally effective in promoting subunit reconstitution; however, maximal efficiency is attained when they are combined in a 1:2 molar ratio. The reassembly process requires no heat activation step, as under the appropriate ionic conditions it takes place spontaneously within the temperature range optimal for growth of *H. mediterranei* cells (40-45 °C).

Extremely halophilic archaebacteria thrive in hypersaline environments. Unlike other kinds of halotolerant microorganisms, they counterbalance the strong external osmotic pressure by raising their internal concentration of potassium ions up to near saturation. This creates an intracellular milieu of uniquely high ionic strength, to which all cellular components are obliged to adapt.

Halobacterial ribosomes have long since attracted the biologists' attention as a suitable object for studying how macromolecules and macromolecular assemblies become modified in order to function at salt concentrations that would normally be destructive. However, apart from the well-established facts that halobacterial ribosomes are unstable in low-salt buffers (Bayley & Kushner, 1964; Bayley, 1966a; Visentin et al., 1972) and that they contain mostly acidic, instead of basic, proteins (Bayley, 1966b; Strom & Visentin, 1973), there is a considerable dearth of information on the mechanism of RNA-protein interaction and ribosome folding in hypersaline conditions.

To gain insight into these latter issues, we have developed an in vitro system that allows the spontaneous reassembly, from the separate RNA and protein moieties, of synthetically active

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large ribosomal subunits of the halophilic archaebacterium *Haloferax mediterranei*, a solar saltern species growing optimally in 20% NaCl (Rodriguez-Valera et al., 1983; Torreblanca et al., 1986). Such a reconstitution system is an effective tool to study the influence of salt and other environmental parameters on the assembly and function of halophilic ribosomes. In perspective, moreover, it allows the separate manipulation of the various ribosomal components, such as the substitution of individual proteins with modified or heterologous counterparts.

This latter possibility may be of special interest in view of the fact that halophilic ribosomes are an important material for crystallographic analyses of ribosome structure (Makowski et al., 1987; Yonath et al., 1987).

Materials and Methods

Preparation of Ribosomes and Ribosomal Subunits. *H. mediterranei* (ATCC 33500) cells were grown at 40 °C in a medium containing (per liter) NaCl, 195 g; $MgCl_2 \cdot 6H_2O$, 34.6 g; $MgSO_4 \cdot 7H_2O$, 49.4 g; $CaCl_2 \cdot 2H_2O$, 0.92 g; KCl, 0.5 g; $NaHCO_3$, 0.17 g; NaBr, 0.58 g; yeast extract, 5 g; pH was adjusted to 7.2. Cells were harvested in the mid-log phase of growth (A_{660} = 0.6–0.7) and immediately frozen at −80 °C until used. For ribosome extraction, the cells were ground in a mortar with twice their weight of alumina; the paste was diluted with two volumes (i.e., 2 mL/g of wet cells) of buffer A [3.4 M KCl; 60 mM $Mg(OAc)_2$; 30 mM Tris-HCl, pH 7.6; 7 mM β-mercaptoethanol], treated with 1 μg/mL RNase-free DNase (20 min at 0 °C), and spun at 30000*g* for 30 min to remove alumina and cell debris. The upper two-thirds of the supernatant was collected and centrifuged for 3 h at 150000*g* in a Spinco Ti 50 rotor. The resulting supernatant (S150) was stored at −80 °C in small aliquots. The ribosomal pellet was resuspended in buffer A at a concentration of 800–1000 A_{260} units/mL and stored at −80 °C in small aliquots or immediately used to separate ribosomal subunits.

To obtain ribosomal subunits, aliquots (5000–6000 A_{260} units or 300–400 mg) of 70-S ribosomes in buffer A were diluted approximately 10-fold with dissociation buffer (2.7 M KCl, 0.45 M NH_4Cl, 20 mM $MgCl_2$, 20 mM Tris-HCl, pH 8, 7 mM β-mercaptoethanol). The subunits were then separated by zonal centrifugation in a Ti 15 Beckman rotor by using a linear, 6–36% (w/v), sucrose gradient made in dissociation buffer. Absorbance peaks corresponding to 30-S and 50-S subunits were pooled and subunits were collected by high-speed centrifugation. The pellets of ribosomal subunits were lastly resuspended in buffer A and stored in small aliquots at −80 °C.

Preparation of Ribosomal RNA. Ribosomal RNA was prepared from 70-S ribosomes by phenol extraction. One hundred to two hundred A_{260} units of 70-S ribosomes in 200–300 μL of buffer A were diluted with an equal volume of SCE buffer (NaCl, 150 mM; sodium citrate, 15 mM; EDTA, 0.1 mM) and extracted twice with an equal volume of water-saturated phenol. The last aqueous phase was reextracted with chloroform–isoamyl alcohol (99:1) and RNA was precipitated with two volumes of ethanol. RNA was then resuspended in TM4 buffer [10 mM Tris-HCl, pH 7.0, 4 mM $Mg(OAc)_2$] at a concentration of 150–200 A_{260} units/mL; it was dialyzed overnight against the same buffer and stored at −20 °C in small aliquots.

The RNA preparations were analyzed by denaturing electrophoresis. Discontinuous gels (0.2 × 10 × 14 cm) consisting of a 5-cm bottom layer of 8% acrylamide and a 5-cm top layer of 4% acrylamide were made in deionized formamide containing 20 mM phosphate buffer (final pH 8.5). Before loading on the gel, the RNA samples were dissolved in formamide (pH 8.5) containing 6% sucrose and heated at 90 °C for 5 min. Phosphate buffer (pH 8), 20 mM, was used as the electrode buffer; at the end of the run, the gels were washed for 30 min with the same buffer and then stained with methylene blue (0.4 g/L in 0.3 M sodium acetate and 7% acetic acid).

Preparation of 50-S Subunit Proteins (TP 50). TP 50s were extracted from 50-S subunits by acetic acid as described by Londei et al. (1986) with some modifications. After removal of RNA, the 67% acetic acid supernatant was dialyzed for 2 h against 5% acetic acid. Proteins were then lyophilized, resuspended in 6 M urea buffer [6 M urea, 400 mM KCl, 20 mM Tris-HCl, pH 7, 4 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol], and dialyzed overnight against the same buffer. Urea was removed by 3 × 45 min consecutive dialyses (400 mL each) against 400 mM KCl, 20 mM Tris-HCl, pH 7, 4 mM $Mg(OAc)_2$, and 6 mM β-mercaptoethanol. Protein samples were stored in small aliquots at −80 °C.

Reconstitution Procedure. Reconstitution mixtures contained, in a final volume of 150 μL, 1 A_{260} unit ribosomal RNA; 2–2.5 equivalent units of TP 50 (1 equivalent unit = the amount of protein corresponding to 1 A_{260} unit of 50-S subunits); 1 M $(NH_4)_2SO_4$; 1 M KCl (or variable amounts of different salts, as specified in the Results); 60 mM $Mg(OAc)_2$; 20 mM Tris-HCl, pH 8.2; and 5 mM β-mercaptoethanol. The salt solution was made up first (KCl and ammonium sulfate were added as solids) and heated briefly at 40 °C to fully dissolve the salts; it was then supplemented with RNA and finally with proteins. The mixture was incubated at 42 °C for 2 h and either sampled for activity measurements or subjected to sucrose gradient analysis.

Analysis of Reconstituted Particles. The sedimentation behavior of the reconstructed particles was determined by centrifuging the reconstitution mixtures onto sucrose density gradients (2–32% w/v) made in 3.2 KCl, 30 mM Tris-HCl, pH 8, and 60 mM $Mg(OAc)_2$. The gradients were run for 3 h at 15 °C in a Beckman SW41 rotor.

When necessary, the reconstituted subunits were purified by collecting the appropriate fractions from the sucrose gradients and centrifuging for 15 h at 40000 rpm. The pellets were resuspended in buffer A and stored at −80 °C.

The polypeptide-synthesizing activity of the reconstituted subunits was measured either by using gradient-purified particles or by direct sampling of the reconstitution mixtures. In the latter case, particle activity was calculated by assuming that the amount of reconstituted subunits was equal to that of the 23-S RNA present in the sample (about 60% of the total A_{260}). The composition of the poly(U)-directed cell-free system (Sanz et al., 1988) was as follows (in a final volume of 100 μL): 1 M KCl; 0.4 M NH_4Cl; 1.5 M $(NH_4)_2SO_4$; 30 mM $Mg(OAc)_2$; 30 mM Tris-HCl, pH 9; 2 mM ATP; 0.5 mM GTP; 5 mM creatine phosphate; 7 mM β-mercaptoethanol; 80 μg of poly(U); 100 μg of yeast tRNA; 36 μM [^{3}H]-phenylalanine [specific activity 200 (counts/min)/pmol]; 10–15 pmol of native 30-S subunits; 7–8 pmol of native or reconstituted 50-S subunits; 3–5 μL of S150. The final pH of the mixture was approximately 7.5. Incubation was for 1 h at 40 °C; hot TCA-precipitable radioactivity was determined as described by Mans and Novelli (1961).

Results

Reconstitution Procedure. Isolation of *H. mediterranei* ribosomal components essentially conformed to standard protocols (Amils et al., 1979; Nierhaus & Dohme, 1979;

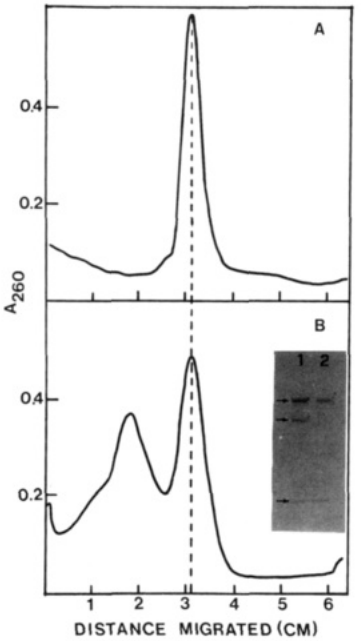

FIGURE 1: Sedimentation profiles of *H. mediterranei* native (A) and reconstituted (B) large ribosomal subunits. Inset to panel B: electrophoresis on formamide/acrylamide gels of RNA extracted from native *H. mediterranei* 70S ribosomes (lane 1) and RNA extracted from gradient-purified reconstituted subunits (lane 2). The arrows, from top to bottom, mark the positions of 23-S, 16-S and 5-S RNAs, respectively. High-salt-containing sucrose gradients and denaturing gels were prepared and run as described under Materials and Methods.

Schulze & Nierhaus, 1982; Londei et al., 1986). Both the RNA and the protein preparations could be stored for several months with no loss of activity; also, they did not require high salt for maintenance of stability or competence in reconstitution. The RNA was extracted from 70-S particles rather than from purified 50-S subunits because intact 23-S molecules capable of efficiently participating in reconstitution are obtained in higher yields from undissociated ribosomes. Upon electrophoretic analysis on denaturing gels, the RNA preparations were found to contain essentially intact 23-S, 16-S, and 5-S rRNA species (Figure 1, inset to panel B).

Optimal reconstitution of *H. mediterranei* large ribosomal subunits was found to take place following a 2-h incubation at 40 °C in the presence of 1 M KCl, 1 M $(NH_4)_2SO_4$, 30 mM Tris-HCl, pH 8.0, and 60 mM $Mg(OAc)_2$; the final pH of the mixture was about 7.5. Sedimentation analysis of mixtures of RNA and TP50 incubated under the specified conditions evidenced the presence of a discrete particle cosedimenting with native 50-S subunits (Figure 1); after purification from the gradients, such particles were found to contain 23-S and 5-S RNAs (Figure 1, inset to panel B) and to be active in polyphenylalanine synthesis when complemented with native 30-S subunits (Table I). The slower sedimenting peak appearing on the gradients of Figure 1 consists almost entirely of the 16-S RNA, which neither participates nor interferes in the reconstitution process and is usually recovered as such at the end of the incubation [see also Amils et al. (1979) and Londei et al. (1986)].

The data in Table I show that gradient-purified reconstituted subunits are very close to the native particles in their capacity for poly(U)-directed polyphenylalanine synthesis. The kinetics of phenylalanine incorporation by the native and the reconstituted subunits were also determined and found to be essentially superimposable over a 1-h incubation period; in addition, the rate of polypeptide synthesis increased linearly with the amount of reconstituted subunits added to the assay (not

Table I: Polyphenylalanine Synthesizing Activity of *Haloferax mediterranei* Reconstituted 50-S Subunits[a]

	native 50 S	reconstituted 50 S
counts min^{-1} (assay mix)$^{-1}$	10625	9987
mol of Phe/mol of 50-S subunits	6.6	6.2
% activity	100	94
% activity + 10^{-7} anisomycin	89	82
% activity + 10^{-6} anisomycin	81	70
% activity + 10^{-5} anisomycin	62	58
% activity + 10^{-4} anisomycin	21	27
% activity + 10^{-3} anisomycin	9	10

[a] Reconstituted subunits were purified from sucrose gradients (see Figure 1). Background radioactivity (all components minus 50-S subunits, approximately 1000 cpm) has been subtracted.

shown). The conformational accuracy of the reassembly products is further documented by the fact that they possess the same high sensitivity to the antibiotic anisomycin that characterizes native halobacterial ribosomes (Table I).

Influence of Various Environmental Parameters on Reconstitution. The optimal reconstitution conditions summarized above were evolved gradually by individually assessing the influence of the various environmental parameters on the reassembly process. The principal observations are reported in the following paragraphs.

(*a*) *Monovalent Cation Dependence of the Reconstitution Process.* We first investigated the possibility of obtaining subunit reassembly in the presence of an increasing concentration of four different salts: potassium chloride, ammonium chloride, ammonium sulfate, and sodium chloride. After incubation at 42 °C in the presence of 60 mM Mg^{2+} ions, the extent of reconstitution was evaluated by performing the poly(U) assay on samples directly taken from the incubation mixtures.

As the results in Figure 2a show, efficient reconstitution is observed in the presence of either potassium chloride, at concentrations above 2.5 M, or ammonium sulfate, at concentrations around 1.2 M. In both cases, a similar amount of monovalent cations (2.5–3 molar equiv) appears to be required to promote subunit reassembly. NH^+ ions, however, are maximally effective within a rather restricted concentration range, whereas reconstitution efficiency in the presence of K^+ ions increases almost linearly with cation concentration to reach a plateau level at about 3 M salt.

Subunits reconstituted in the presence of optimal amounts of KCl (3–3.2 M) or ammonium sulfate (1.2 M) attain about 80% of the activity of native halobacterial large subunits.

The other two salts tested, NaCl and ammonium chloride, proved to be completely ineffective in promoting particle reassembly (Figure 2a). The unexpected inactivity of ammonium chloride was further investigated in order to rule out the possibility of the presence of contaminants in the salt batch employed. Reconstitution experiments were repeated several times by using different brands of ultrapure, heavy-metal-free ammonium chloride; however, negative results were consistently obtained (not shown). A further potentially interesting salt, K_2SO_4, could not be reliably assayed because of its low solubility.

Next, we investigated subunit reconstitution in the presence of mixtures of two salts, all other conditions remaining invariant. As shown in Figure 2b, combining KCl and ammonium sulfate gives the best results in terms of reconstitution efficiency. The maximal polymerizing activity of the reconstructed particles (approximately 95% that of the native ones) is attained by using an equimolar mixture of the two salts; however, highly active particles are obtained at all concen-

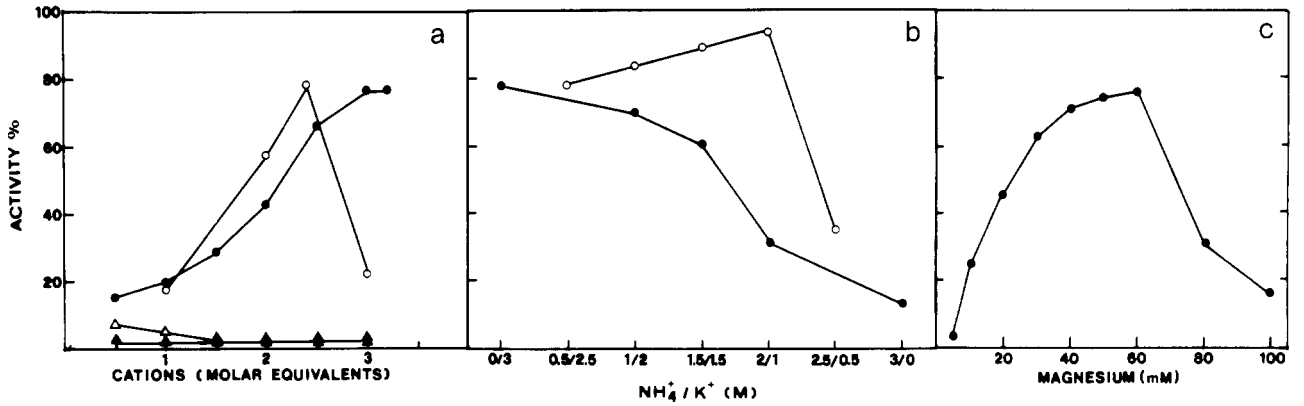

FIGURE 2: Cation dependence of the reassembly process. Panel a: polymerizing activity of the reconstituted particles as a function of increasing concentrations of the following salts: (●) KCl; (○) $(NH_4)_2SO_4$; (△) NH_4Cl; (▲) NaCl. Panel b: reconstitution in the presence of varying molar ratios of K^+ to NH_4^+ ions to a final concentration of 3 M. (○) Mixtures of $(NH_4)_2SO_4$ and KCl; (●) mixtures of NH_4Cl and KCl. Magnesium concentration was kept in all cases at 60 mM. Panel c: effect of magnesium on reconstitution, in the presence of 3.2 M KCl. In all experiments, the percentage activity of the reconstituted subunits is relative to an amount of native 50-S particles corresponding to the amount of 23-S RNA present in the aliquot of reconstitution mixture sampled for the assay.

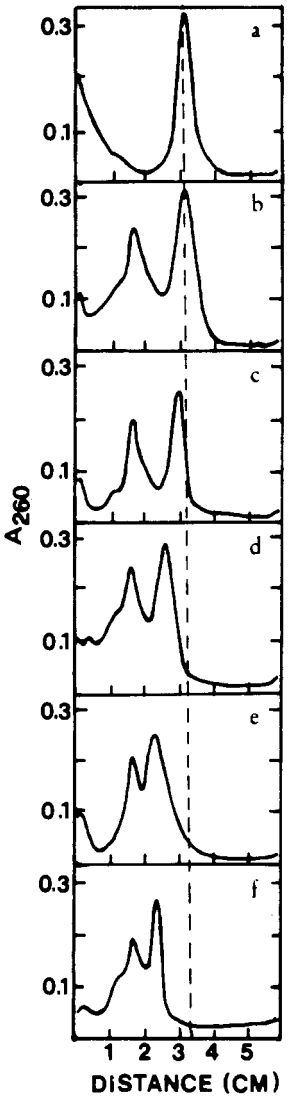

FIGURE 3: Sedimentation profiles of native 50-S subunits (a) compared to those of reconstitution mixtures containing (b) 3 M KCl, (c) 2.5 M KCl, (d) 2 M KCl, (e) 1.5 M KCl, and (f) 0.5–1 M KCl.

tration ratios tested, except for the case in which KCl falls below 1 M (Figure 2b).

However, when ammonium sulfate is substituted for by ammonium chloride in the same experiment, maximal reconstitution efficiency is observed in the absence of NH_4Cl (at 3 M KCl), and a progressive drop in particle activity accompanies the increase of the latter salt in the incubation mixture (Figure 2b).

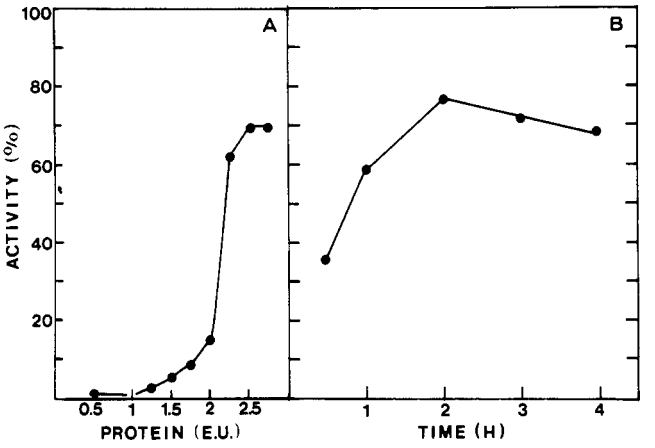

FIGURE 4: Panel A: reconstitution as a function of increasing amounts of large subunit proteins, in the presence of 3.2 M KCl and 60 mM $Mg(OAc)_2$. Protein equivalent units are defined as specified under Materials and Methods. Panel B: time course of the reassembly process, in the presence of 3.2 M KCl and 60 mM Mg^{2+}. The activity of the reconstituted particles is defined as in the legend to Figure 3.

To get some further insight in the role played by monovalent cations in the subunit reassembly process, the products of reconstitution assays performed at KCl concentrations ranging from 0.5 to 3 M (in the presence of 60 mM Mg^{2+}) were further characterized by sucrose gradient centrifugation (Figure 3). The formation of "healthy" 50-S subunits upon raising salt concentration is clearly appreciated by the progressive increase in mobility of the peak of reconstituted particles; a perfect overlapping with native large subunits is only obtained at 3 M KCl. The experiment also documents the specificity of protein binding to the 23-S RNA, as at any salt concentration the position of the 16-S peak remains invariant.

(*b*) *Effect of Divalent Cations.* The results in Figure 2c show that reassembly of *H. mediterranei* 50-S subunits can be achieved within a broad range of magnesium concentrations, most effectively between 40 and 60 mM. Beyond the latter value, a sharp decrease in the reconstitution yield is observed.

(*c*) *RNA/Protein Ratio.* As shown in Figure 4A, best reconstitution efficiency requires a 2.5 M excess of proteins over rRNA.

(*d*) *Reconstitution Kinetics.* The time course of subunit reassembly in the presence of 3.2 M KCl and 60 mM Mg-

$(OAc)_2$ is shown in Figure 4B. The synthetic activity of the reconstituted particles continues to increase during the first 2 h of incubation and declines slowly thereafter.

Discussion

We show in the present report that the large ribosomal subunits of an halophilic archaebacterium (*H. mediterranei*) can be reconstituted from the separate RNA and protein moieties under in vitro high-salt conditions. The accuracy and the efficiency of the reassembly process are demonstrated by the fact that the reconstructed subunits are strictly similar to the native ones in sedimentation behavior, ability to translate poly(U) into poly(Phe), and sensitivity to the antibiotic anisomycin.

The in vitro reassembly of the halophilic ribosomes appears to be principally regulated by the chemical nature and the concentration of the monovalent cations involved. Subunit reconstitution can be achieved in the presence of either of two salts, KCl and ammonium sulfate; however, the reaction is most efficient when they are combined in equimolar amounts. As KCl is by far the most abundant salt found in the internal environment of halobacteria, its activity in ribosome reconstitution was expected. However, it is worth noticing that the optimal KCl concentration in the reconstitution assay (3–3.2 M) far exceeds the physiological one (approximately 2 M) found in living *H. mediterranei* cells (Rodriguez-Valera et al., 1983; Sanz et al., 1988).

On the other hand, the fact that ammonium sulfate is as effective as KCl in the reconstitution assay is more difficult to explain, as ammonium ions are only present in limited amounts within halobacteria (Sanz et al., 1988). Moreover, when the two salts are used in combination, the maximal reconstitution yield is obtained with a 2-fold excess of ammonium over potassium ions. Such an interchangeability between NH_4^+ and K^+ is in agreement with the work of Sanz et al. (1988), who observed that polyphenylalanine synthesizing systems derived from various halobacterial species can function equally well when the "physiological" cation potassium is entirely replaced by ammonium.

The picture, however, is considerably complicated by the fact that when ammonium ions are supplied as NH_4Cl instead of ammonium sulfate, ribosome reconstitution cannot take place. Having excluded trivial explanations, such as contamination of the salt batch employed by heavy metals or RNases, we do not have a satisfactory interpretation for this phenomenon. It cannot be attributed to a need for SO_4^{2-} anions, as they are not present when reconstitution is performed in KCl alone; a similar reasoning holds true for a hypothetical inhibitory effect of Cl^- ions. Finally, it is very difficult to envisage some kind of incompatibility between NH_4^+ and Cl^- ions since these can be successfully mixed as ammonium sulfate and potassium chloride. Experiments in which NH_4Cl and KCl are combined show that the former, although inactive by itself, does not have an adverse effect on the reassembly process, as it can stimulate subunit reconstitution when added to suboptimal amounts of KCl to bring the overall cation concentration to 3 M. For instance, particles reconstituted in the presence of 2 M KCl alone have about 40% activity, but when 1 M NH_4Cl is added, activity goes up to 70% (cf. Figure 2a,b).

Failure to obtain reconstitution in the presence of NaCl was not surprising, as this salt is mainly present in the external environment of halobacteria; furthermore, it has long been known that Na^+ ions have a damaging effect on ribosome structure.

On the whole, the results show that optimal in vitro reconstitution of halophilic subunits requires K^+ and/or NH_4^+ ions to be present in no less than 2.5–3 M concentrations. However, the amount of monovalent cations does not have an all-or-none effect on ribosome assembly. Reconstitution assays performed at suboptimal KCl concentrations result in the formation of particles that, although sedimenting at less than 50 S, possess appreciable polymerizing activity (cf. Figures 2a and 3). Therefore, even at relatively low salt (in the presence of 60 mM magnesium), RNA and proteins can efficiently interact, forming particles containing at least the minimal set of components required for activity in polypeptide elongation. That subunits having an apparently low sedimentation coefficient display substantial polymerizing activity (see, e.g., reconstitutions performed at 2 M KCl) may be explained by surmising that they exist in a loose conformational state until placed in the high-salt environment of the poly(Phe) assay, where at least a fraction of them may fold to active 50-S particles. This interpretation is supported by earlier data (Strom et al., 1975) indicating that K^+ concentration, at high magnesium, primarily influences the folding state of halophilic ribosomes. Alternatively, it is possible that the slowly sedimenting particles reconstituted at suboptimal salt concentrations are only capable of polymerizing phenylalanine at a reduced rate.

Divalent cations such as magnesium, whose exact dosage is all-important for in vitro reconstitution of nonhalophilic ribosomes (Nierhaus & Dohme, 1974; Amils et al., 1979; Londei et al., 1986), do not appear to exhibit a pivotal role in the reassembly of *H. mediterranei* large subunits: concentrations comprised between 40 and 60 mM are almost equally effective. Probably, the optimal amount of magnesium for reconstitution (60 mM) represents a compromise between the need to ensure efficient binding of Mg^{2+} ions to the relevant ribosomal sites and the need to avoid competition with monovalent cations. Indeed, if the magnesium concentration is too high, inhibition of the reconstitution process is observed.

Another aspect of the system described in the present paper is that a more than 2-fold excess of protein over RNA is required for subunit assembly to take place, most probably because of selective loss or damage of some proteins during extraction and handling. The cooperativity of the reassembly reaction upon raising protein concentration indicates that some component essential for initiating the reconstitution process is included among the proteins present in reduced amounts.

A relevant feature that differentiates the in vitro assembly of halophilic large ribosomal subunits from that of their nonhalophilic, eubacterial counterparts (*E. coli*, *Bacillus* sp.; Nierhaus & Dohme, 1974; Fahnestock, 1979) is that fully active particles are obtained in a single incubation step at relatively low temperature. There is no apparent need to push forward the reconstitution process by performing sequential modifications of the ionic environment or by carrying out prolonged "heat-activation" steps at temperatures beyond 50 °C. Also, the reaction appears to be relatively fast, as 60% particle activity is regained within the first hour of incubation.

These observations suggest that the in vitro reassembly of halobacterial ribosomes follows a more energetically favorable pathway, probably closer to the in vivo one, than is the case for the reconstitution of the large ribosomal subunits of nonhalophilic eubacteria. It remains to be seen whether this generally applies to extremely halophilic ribosomes or whether it is a peculiarity of the *H. mediterranei* system.

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Registry No. Mg, 7439-95-4; K, 7440-09-7; NH_4^+, 14798-03-9.

REFERENCES
Amils, R., Matthews, E., & Cantor, C. (1979) *Methods Enzymol. 59*, 449–461.
Bayley, S. T. (1966a) *J. Mol. Biol. 18*, 330–338.
Bayley, S. T. (1966b) *J. Mol. Biol. 15*, 420–427.
Bayley, S. T., & Kushner, D. J. (1964) *J. Mol. Biol. 9*, 654–669.
Fahnestock, S. (1979) *Methods Enzymol. 59*, 437–443.
Londei, P., Teixido, J., Acca, M., Cammarano, P., & Amils, R. (1986) *Nucleic Acids Res. 14*, 2269–2285.
Makowski, I., Frolow, F., Saper, M. A., Wittman, H. G., & Yonath, A. (1987) *J. Mol. Biol. 193*, 819–827.
Mans, R. J., & Novelli, G. D. (1961) *Arch. Biochem. Biophys. 94*, 48–53.
Nierhaus, K. H., & Dohme, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4713–4717.
Nierhaus, K. H., & Dohme, F. (1979) *Methods Enzymol. 59*, 443–449.
Rodriguez-Valera, F., Juez, G., & Kushner, D. J. (1983) *Syst. Appl. Microbiol. 4*, 369–381.
Sanz, J. L., Marin, I., Balboa, M. A., Ureña, D., & Amils, R. (1988) *Biochemistry 27*, 8194–8199.
Schulze, H., & Nierhaus, K. H. (1982) *EMBO J. 1*, 609–613.
Strom, A. R., & Visentin, L. P. (1973) *FEBS Lett. 37*, 274–280.
Strom, A. R., Hasnain, S., Smith, N., Matheson, A. T., & Visentin, L. P. (1975) *Biochim. Biophys. Acta 383*, 325–337.
Torreblanca, M., Rodriguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M., & Kates, M. (1986) *Syst. Appl. Microbiol. 8*, 89–99.
Visentin, L. P., Chow, C., Matheson, A. T., Yaguchi, M., & Rollin, F. (1972) *Biochem. J. 130*, 103–110.
Yonath, A., Leonard, K. R., & Wittman, H. G. (1987) *Science 236*, 813–816.

Analysis of Fluorescence Energy Transfer in Duplex and Branched DNA Molecules†

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ABSTRACT: Nonradiative fluorescence energy transfer (FET) is thought to be a highly sensitive measure of distance, occurring through a dipole coupling (Forster) mechanism in which the efficiency of FET depends on the inverse sixth power of the distance between fluorophores. The current work assesses the utility of FET for measuring distances in duplex and branched DNA molecules. The apparent efficiencies of FET between donor (fluorescein) and acceptor (eosin) fluorophores attached to opposite ends of oligonucleotide duplexes of varying length were determined; the results suggest that FET is a useful qualitative indicator of distance in DNA molecules. However, the apparent FET efficiency values cannot be fit to the Forster equation without the specification of highly extended DNA-to-fluorophore tethers and motionally restricted fluorophores, conditions that are unlikely to coexist. Three other lines of evidence further suggest that factors in addition to Forster transfer contribute to apparent FET in DNA: (1) The efficiency of FET appears to depend on the base sequence in some instances. (2) Donor fluorescence changes with the extent of thermally induced DNA melting in a sequence-dependent fashion, indicating dye–DNA interactions. (3) The distances between the ends of various pairwise combinations of arms of a DNA four-way junction do not vary as much as expected from previous work. Thus, the occurrence of any nondipolar effects on energy transfer in oligonucleotide systems must be defined before distances in DNA molecules can be quantified by using FET.

The characterization of DNA structure often requires techniques for measuring distances between particular sites on DNA molecules in free solution. Such techniques can, in principle, provide information on the geometries of bent or branched DNA molecules, thus enabling the interpretation of results obtained from studies of the same molecules in constrained environments (e.g., in gels or crystals). The present report describes a preliminary assessment of the utility of fluorescence energy transfer (FET) for measuring the distances between the ends of oligonucleotide duplexes and branched structures in solution.

Our initial motivation for pursuing FET studies of oligonucleotides stemmed from our work on the structure of a synthetic DNA four-way junction (Cooper & Hagerman, 1987, 1989), in which gel electrophoretic and hydrodynamic analyses were utilized. While the limitations of the gel method (Cooper & Hagerman, 1987) were largely overcome by the determination of rotational diffusion times (Cooper & Hagerman, 1989), both methods require the attachment of reporter fragments to specified arms of the junction, followed by several purification steps. Thus, we sought a method for measuring junction interarm angles that would be performed in free solution and that would obviate the need for attachment of reporter fragments. The distance range that is considered accessible to FET (~10–90 Å) should, in principle, allow evaluation of the magnitudes of the junction interarm angles

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