5S RNA–Protein Complex from an Extreme Halophile, *Halobacterium cutirubrum*. Comparative Studies on Reconstituted Complexes†

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ABSTRACT: Partially and fully reconstituted complexes of the 5S RNA and two 5S RNA binding proteins (HL13 and HL19) from an extreme halophile, *Halobacterium cutirubrum*, were examined using ethidium bromide binding, circular dichroism spectra, and limited nuclease digestion. The binding of 5S RNA to HL13 was studied by means of the quenching of tryptophan flurorescence; a value of 10^8 M^{-1} was estimated for the association constant in 3.4 M potassium chloride. Circular dichroism spectra in the 240–320-nm region indicated that only HL13 elicited a conformational change in the 5S RNA. A study of the CD spectra in the 200–240-nm region also indicated that this protein underwent some conformational change when bound. The addition of HL13 chased ethidium bromide bound to the 5S RNA. The only effect observed on binding HL19 was an enhanced association constant for HL13 as inferred from a change in shape of the ethidium bromide chasing curve. Addition of HL13 also gave rise to a partial protection of the 5S RNA against T_1 ribonuclease digestion; in contrast, it brought about an enhanced susceptibility to pancreatic ribonuclease digestion. Addition of HL19 had no effect in either case. These results demonstrate that protein HL13 is alone responsible for the corresponding circular dichroism and nuclease digestion observation on the native *H. cutirubrum* 5S RNA–protein complex and 5S RNA previously reported [Nazar, R. N., Willick, G. E., & Matheson, A. T. (1979) *J. Biol. Chem. 254*, 1506–1512]. Furthermore, despite the limited sequence homology, the results demonstrate a remarkable structural homology, within their respective 5S RNA complexes, between protein HL13 from *H. cutirubrum* and protein EL18 (plus possibly EL25) from *Escherichia coli*. Similarly, HL19 appears to have a similar role as EL5 in the *E. coli* complex.

Because they are easily isolated pure and in large yields, 5S RNA–protein complexes from procaryotic or eucaryotic ribosomes are useful simple systems for studying ribosomal RNA–protein interactions. In procaryotes the complex contains two to three proteins (Monier, 1974; Erdmann, 1976) with a combined molecular weight of about 40 000, whereas eucaryotic complexes contain a single protein of a similar molecular weight (Blobel, 1971; Mazelis & Petermann, 1973).

The complex from *Escherichia coli* is the most extensively studied. Depending on the method of preparation, this complex contains up to three proteins (Horne & Erdmann, 1972; Yu & Wittmann, 1973), but the 5S RNA interacts strongly with only two, EL18 and EL25 (Gray et al., 1973). The third protein, EL5, interacts weakly with the RNA (Yu & Wittmann, 1973) but assembles with EL18 and EL25 to form a stable quaternary complex (Erdmann, 1976). Ethidium bromide (EthBr) binding (Feunteun et al., 1975) and circular dichroism (CD) (Bear et al., 1977; Fox & Wong, 1978) have been used to characterize the interaction of the 5S RNA with the individual binding proteins. In addition, partial ribonuclease digestion studies have provided information about the nucleotide sequence which are involved in protein binding (Erdmann, 1976; Monier, 1974).

Recently, we have isolated a 5S RNA–protein complex from an extreme halophile *Halobacterium cutirubrum* (Smith et al., 1978). This complex contains two highly acidic proteins, HL13 and HL19, with molecular weights of 18 700 and 18 000, respectively. It requires a high salt concentration for optimum stability (Willick et al., 1978) and binds less EthBr than the RNA, and the RNA undergoes a characteristic conformational change with respect to the free state (Nazar et al., 1979). Partial ribonuclease digestion studies have identified sequences in the 3′ half of the 5S RNA molecule which are involved in protein binding. All of these results showed surprising similarities to the results obtained with *E. coli*, despite the marked contrast between the components of the two complexes. Since comparison of these two complexes should provide valuable information on the critical features of the RNA–protein interactions which have been retained during evolution, we have extended our studies to include the reconstituted complexes from the individual components.

Materials and Methods

(*a*) *5S RNA and 5S RNA Binding Proteins.* Ribosomal 50S subunits from *H. cutirubrum* were isolated as previously described (Ström et al., 1975). The subunits (2-g amounts) were suspended in 400 mL of buffer A (50 mM KCl, 100 mM $MgAc_2$, 10 mM Tris-HCl, 6 mM β-mercaptoethanol) and dialyzed against 6 L of buffer A overnight. The suspension was centrifuged for 24 h at 165000*g* in a Spinco Ti 50 rotor (Ström et al., 1975), and the supernatant was dialyzed against a total of 18 L (3 × 6 L) of Tris–urea buffer (6 M urea, 10 mM Tris-HCl, 9 mM methylamine, 0.1 mM dithiothreitol, pH 6.0). The dialysate was then titrated to pH 8.0 by the addition of solid Tris and applied directly to a DEAE-cellulose column (DE-52 Whatman) (1.5 × 90 cm) previously equilibrated with pH 8.0 Tris–urea buffer. The proteins were eluted with a linear 0–0.3 M KCl gradient with a flow rate of 20 mL/h, and the proteins were detected by scanning the column fractions (10 mL) at 230 nm (Figure 1). The locations of the proteins were analyzed by polyacrylamide gel electrophoresis. The appropriate fractions were pooled, dialyzed extensively against water, and freeze-dried. The freeze-dried proteins were stored at −20 °C. The identities of the proteins (HL13 and HL19) were confirmed by their amino acid compositions and mobilities on two-dimensional gels, as compared to the proteins originally isolated from the native complex (Smith et al., 1978).

After the elution of the ribosomal proteins, the 5S RNA, which is also extracted in buffer A, was eluted by the addition of 0.5 M KCl to the column (Figure 1).

(*b*) *Reconstitution of Complexes.* Complexes were formed by mixing appropriate concentrations of 5S RNA and HL13

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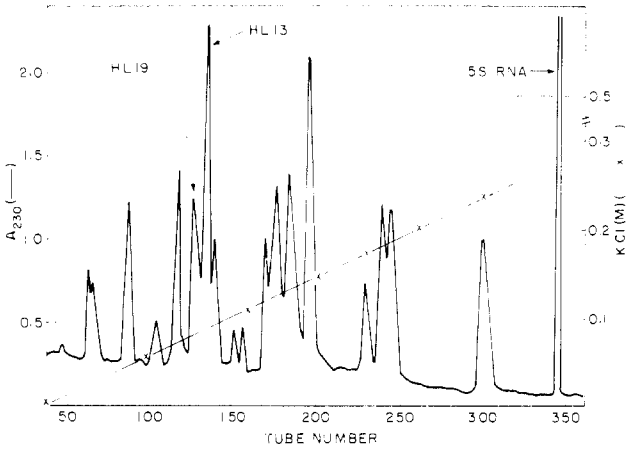

FIGURE 1: Isolation of *H. cutirubrum* 5S RNA and 5S RNA binding proteins. The 150000*g* supernatant from buffer A extracted subunits was applied to a 1.5 × 90 cm column of DEAE-cellulose equilibrated with Tris–urea buffer (pH 8.0) and eluted with a linear 0–0.3 M gradient of KCl (3.2 L). Fractions (10 mL) were collected. The positions, as identified by polyacrylamide gel electrophoresis, of elution of HL13 and HL19 are shown. The 5S RNA was eluted at the end by stepping the gradient to 0.5 M KCl.

or HL19 which had been predialyzed against high salt buffer (3.4 M KCl, 0.003 M $MgAc_2$, 0.01 M Tris-HCl, 0.01 M β-mercaptoethanol, pH 7.6) and were allowed to stand at 20 °C for at least 15 min before experimental observations. No time-dependent changes were observed.

(*c*) *Partial RNase Digestions.* Nucleotide sequences involved in protein binding were probed by limited pancreatic or T_1 ribonuclease digestion as previously described (Nazar et al., 1979).

(*d*) *Fluorescence Measurements.* The fluorescence enhancement of EthBr on binding to free or complexed 5S RNA was measured in a Hitachi Perkin-Elmer spectrophotofluorometer thermostated at 21 °C. Excitation and emission wavelengths were 500 and 605 nm, respectively. Binding of protein HL13 to 5S RNA was measured from the quenching of the tryptophan fluorescence at 340 nm as RNA was added. The excitation wavelength was 300 nm. The quenching was corrected for absorbance of the RNA by using the equation of Hélène et al. (1971).

(*e*) *Circular Dichroism (CD) Spectra.* The spectra were obtained on a Cary 61 circular dichrograph at 27 °C. Protein concentrations were estimated both from Rayleigh fringe displacements in the analytical ultracentrifuge (Millar et al., 1969) and from amino acid analyses. Values for the extinction coefficients at 280 nm for HL13 and HL19 were thus estimated at 0.83 and 0.28 mL/(mg cm), respectively. RNA concentrations were determined by using a value of 23.4 cm^{-1} $(mg/mL)^{-1}$ for the extinction coefficient at 260 nm. A value of 320 was used as the mean residue weight for the RNA and 110 for each of the two proteins. The spectrum of proteins complexed to 5S RNA was calculated from the difference between complexed and free 5S RNA as previously described (Nazar et al., 1979). Spectral changes between 5S RNA and 5S RNA–protein complexes in the 240–320-nm spectral region were measured by using a tandem mixing cell with a 0.90-cm total path length (Hellma Cells, Inc.) as described by Fox & Wong (1978).

Results

(*a*) *Ethidium Bromide Binding Studies.* Recently, we reported that the 5S RNA–protein complex of *H. cutirubrum* binds less EthBr than the free 5S RNA (Nazar et al., 1979). In order to establish whether one or both of the binding proteins were responsible for this effect, an EthBr chasing experiment, similar to that of Feunteun et al. (1975) with the *E. coli* 5S RNA–protein complex, was carried out. Figure 2 shows that only HL13 itself chases EthBr, although the chasing curve was altered when protein HL19 was also present. Protein HL19 itself had no effect (result not shown). The results are very similar to those observed by Feunteun et al. (1975) with the *E. coli* system. In that case, of the three 5S RNA binding proteins (EL18, EL5, and EL25), only EL18 itself chases EthBr, although either EL5 or EL25 altered the shape of the chase curve. This suggests that HL13 is equivalent to EL18 in the complex structure. In a previous study, some sequence homology was observed in the N-terminal ends of these two molecules (Smith et al., 1978).

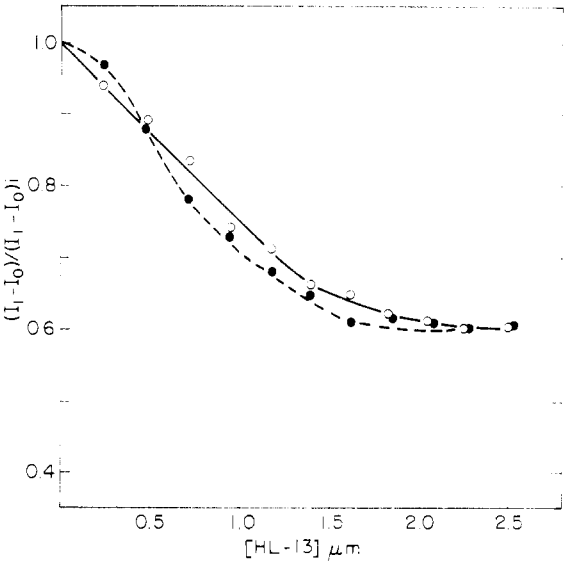

FIGURE 2: HL13 induced chase of ethidium bromide bound to *H. cutirubrum* 5S RNA. HL13 was added to a solution of 5S RNA (0.5 μM) in high salt buffer containing 1 μM EthBr (○) and with an addition of 1.5 μM HL19 (●). Excitation wavelength = 500 nm; emission wavelength = 605 nm. The temperature was 21 °C.

(*b*) *Binding of HL13 to 5S RNA.* HL13 contains 2 mol of tryptophan per mol of protein (Smith et al., 1978). Consequently, it was possible to follow the binding of 5S RNA to this protein by means of a small (10%) quenching of the tryptophan fluorescence that was observed (Figure 3). On the basis of an equivalency of 1 mol of HL13 per mol of 5S RNA, an association constant of 1 (±0.5) × 10^8 M^{-1} was estimated. This can be compared to the values of 10^7 (Feuteun et al., 1975) and 10^8 M^{-1} (Spierer et al., 1978) estimated for the binding of EL18 to *E. coli* 5S RNA. Due to the absence of tryptophan in HL19, it was not possible to use this method to measure its binding to the RNA.

(*c*) *Conformational Changes in 5S RNA on Complex Formation.* CD spectra in the region of 250–320 nm were measured to assess possible conformational changes in the 5S RNA as each protein was bound. In this spectral region, the contribution of protein is negligible compared to that of RNA. Only HL13 showed any effect (Figure 4); there was an increase in the ellipticity at 268 nm of about 5%, coupled with a red shift of 1 nm. There was also a slight red shift of the 300-nm minimum. Addition of HL19, either directly to the 5S RNA or after the addition of HL13, had no further effect. These results are qualitatively the same as those previously observed with the native complex and free 5S RNA (Nazar et al., 1979), although not quite as large. These results can be compared with those of Bear et al. (1977) on 5S RNA–protein complexes from *E. coli*. EL18 had the largest effect

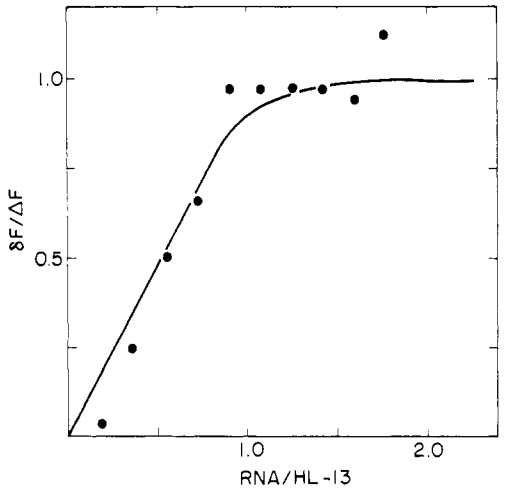

FIGURE 3: Binding of HL13 to *H. cutirubrum* 5S RNA in high salt buffer. The curve shows the quenching of protein fluorescence on addition of 5S RNA. Excitation wavelength = 300 nm; emission wavelength = 340 nm. The concentration of HL13 was 0.29 μM. The curve shown used a value for K_a of 10^8 M^{-1}.

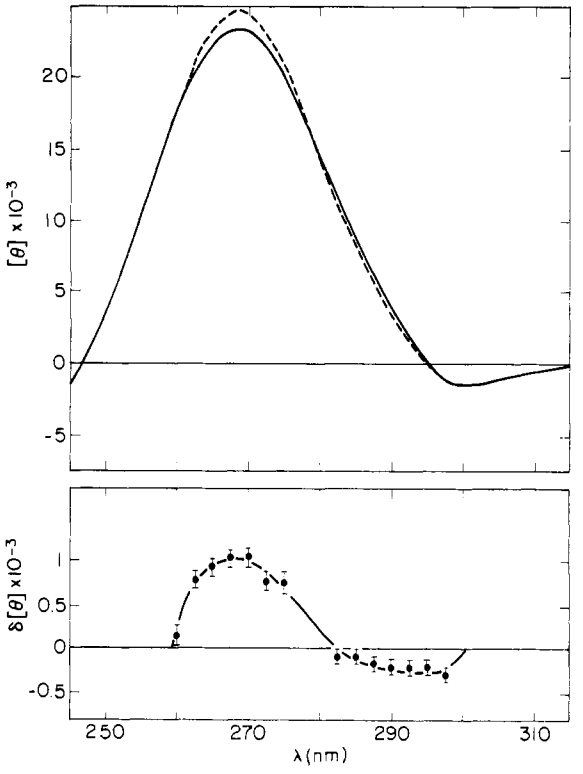

FIGURE 4: Near-UV circular dichroism spectra of *H. cutirubrum* 5S RNA (—) and the reconstituted HL13–5S RNA complex (- - -) in high salt buffer. The difference spectrum (lower) was calculated from the two spectra.

and gave rise to a change in the spectrum similar to that observed here with HL13. EL25 brought about a small decrease in the ellipticity of the CD maximum, whereas EL5 had no effect.

(*d*) *Conformational Changes in Proteins on Complex Formation.* Secondary structural estimations from the difference spectra in the 200–240-nm region between the native complex and free 5S RNA suggested that a conformational change occurred in the bound proteins as compared to the free proteins (Nazar et al., 1979). To determine whether one or both of the binding proteins were involved in this change, we performed the same experiment with the reconstituted complexes. Figure 5 (top) shows the spectrum derived from

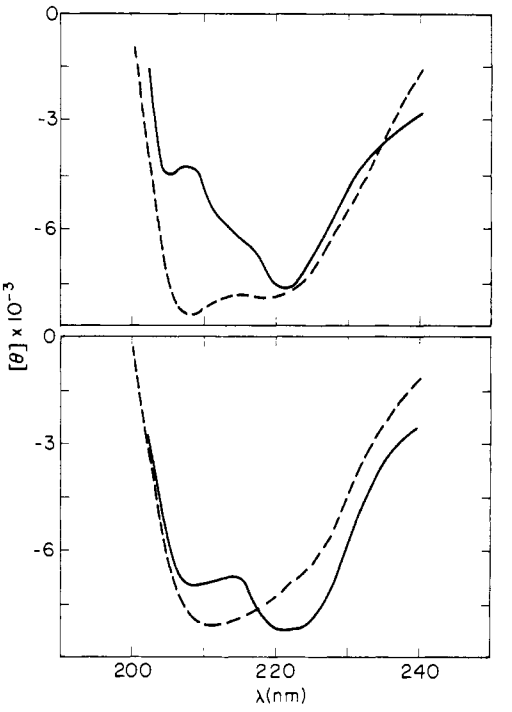

FIGURE 5: Far-UV circular dichroism spectra of free and complexed *H. cutirubrum* 5S RNA binding proteins. The protein spectra of the complex were estimated from the difference between complex and free 5S RNA as described under Materials and Methods. All experiments were carried out in high salt buffer: (upper figure) free HL13 (- - -) vs. HL13 in the reconstituted HL13–5S RNA complex (—); (lower figure) free HL19 (- - -) vs. HL19 in the reconstituted HL19–5S RNA complex (—).

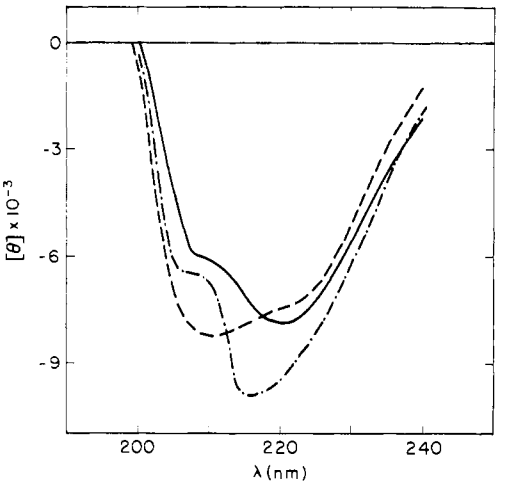

FIGURE 6: Far-UV circular dichroism spectrum of *H. cutirubrum* 5S RNA binding proteins in the fully reconstituted complex (—). The complex was re-formed on a 1:1:1 molar basis. Also included for comparison is the corresponding spectrum previously derived from the native complex (Nazar et al., 1979) (–·–) and that of a 1:1 molar mixture of HL13 and HL19 (- - -). All experiments were conducted in high salt buffer.

the 5S RNA–HL13 complex; the corresponding data for the 5S RNA–HL19 complex are shown in Figure 5 (bottom). The high solvent absorption below 240 nm results in a much lower signal to noise ratio than in the region above this wavelength. This ratio is of the order of 5:1; hence, only the spectral difference observed with HL13, as opposed to HL19, can be considered with reasonable certainty to be significant. Figure 6 shows the difference curve for the completely reconstituted complex (5S RNA–HL13–HL19) and, for comparative purposes, the same curve derived from the native RNP.

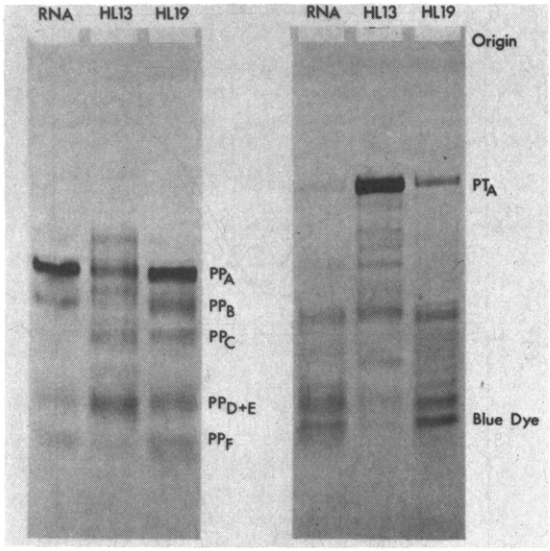

FIGURE 7: Limited pancreatic (left) and T_1 (right) ribonuclease digests of free 5S RNA or partially reconstituted 5S RNA–protein complexes from *H. cutirubrum*. Samples (one A_{260} unit each) containing threefold excesses of protein were digested in high salt buffer at 37 °C with 1 μg of enzyme for 1 h and extracted with $NaDodSO_4$–phenol, and the RNA fragments were fractionated on 8% polyacrylamide gel slabs at pH 8.3. Gels were stained with methylene blue, and fragments were labeled to correspond to an earlier study (Nazar et al., 1979) on the native 5S RNA–protein complex.

Qualitatively, these two curves agree well, although there is some difference in the magnitude of the two curves. The average spectra of HL13 and H19 (Willick et al., 1978) are shown on the same figure. The free proteins have a ratio of the ellipticity values at 220 and 210 nm of less than 1, whereas the complexed proteins have values for this ratio of 1.3–1.5. Comparison of Figures 5 and 6 indicates that the observed spectral change results mainly from a conformational change in HL13 on complex formation. Neither the data from the reconstituted complexes nor our methods for analyzing the CD spectra in terms of conformational components are sufficiently precise to suggest exactly what the conformational change was. Linear least-squares analysis of the curves (Willick & Zuker, 1977), using the reference spectra of Chen et al. (1974), gave a value of 26 ± 3% for the α-helix content in all cases, with little or no β structure inferred. However, the data from the native RNP had inferred a small increase, from 25 to 30%, in the α-helical content (Nazar et al., 1979).

(*e*) *Partial Nuclease Digestion Studies on Reconstituted Complexes.* Previously, comparative studies showed striking differences between the native complex and free 5S RNA after limited ribonuclease digestion (Nazar et al., 1979). The results indicated that conformational changes had occurred and suggested that specific regions of the 3′-nucleotide sequence were involved in protein binding. To evaluate the relative contribution of the individual proteins to these observations, we repeated the studies using partially reconstituted complexes. With both pancreatic and T_1 ribonuclease (Figure 7), HL13 alone was able to fully mimic the previous observations and HL19 had virtually no effect on the digestions. As was reported with the native complex (Nazar et al., 1979), in the presence of HL13 a large portion (fragment PT_A) was preferentially protected from T_1 ribonuclease digestion. In contrast, after limited pancreatic ribonuclease digestion, two regions (residues 65–68 and 89–92) of fragment PP_A were preferentially cleaved to yield fragments PP_{C-F} (Nazar et al., 1979). Our previous studies had indicated that the *H. cutirubrum* 5S RNA binding proteins had no effect on the RNase digestion of chick 5S RNA. Consequently, the effects of HL13 observed in this study were entirely the result of the binding of the protein to the 5S RNA molecule. Therefore, as indicated by the physical studies, *H. cutirubrum* interacts primarily with HL13, with a concomitant conformational change in its secondary (and probably tertiary) structure.

Discussion

Before discussing the present results, it is useful to review some of the previous data on the *H. cutirubrum* 5S RNA binding proteins and their relationship to the *E. coli* 5S RNA binding proteins. The *E. coli* proteins EL18, EL25, and EL5 have molecular weights of 12770 (Brosius et al., 1975), 10695 (Bitar & Wittmann-Liebold, 1975), and 20172 (Chen & Ehrke, 1976), respectively. In contrast, the two proteins, HL13 and HL19, of the *H. cutirubrum* complex have molecular weights of 18700 and 18000, respectively (Smith et al., 1978). Partial sequence data at the amino termini (Smith et al., 1978) suggest homologies between EL18 and HL13 and EL5 and HL19. At this point, the corresponding protein to EL25 has been unidentified. However, in view of the fact that HL13 has a molecular weight approaching the sum of EL18 and EL25, the possibility of HL13 having homology to both proteins exists. Further protein sequence analyses are being undertaken to test this hypothesis.

The work reported here strongly supports a structural homology between EL18 and HL13. The chasing of EthBr from *H. cutirubrum* 5S RNA as HL13 is bound and the apparent enhancement of the binding of HL13 on addition of HL19 (Figure 2) are very similar to the effect of EL18 on 5S RNA bound EthBr in *E. coli* (Feunteun et al., 1975). Both EL25 and EL5 enhanced the binding of EL18 in this case. Our CD results showed that HL13 is the major contributor to conformational changes brought about in the 5S RNA on addition of the binding proteins. In *E. coli*, it has been shown that E18 brings about the largest conformational change (Bear et al., 1977). EL25 has been reported to bring about either a small change (Bear et al., 1977) or no change (Spierer et al., 1978) in the RNA as it was bound. EL5 brought about no change (Bear et al., 1977; Spierer et al., 1978), and in this negative sense EL5 and HL19 are similar.

The nuclease digestion studies reported here indicate that HL13 is responsible for all of the protection against T_1 RNase digestion or increased reactivity to pancreatic RNase digestion previously reported for the native complex (Nazar et al., 1979). In this respect, HL13 is similar in the *H. cutirubrum* complex to EL18 plus EL25 in the *E. coli* complex (Gray et al., 1973). EL5 gave no protection, again a point of similarity to HL19.

Although the mechanism by which HL13 and HL19 bind to the *H. cutirubrum* 5S RNA is unclear, our results suggest several fundamental features. Sedimentation equilibrium results have implied an overall equilibrium constant of about 10^{12} M^{-2} (Willick et al., 1978), whereas in this work the measured binding constant of HL13 was 10^8 M^{-1}. This would imply a binding constant for HL19 to the 5S RNA–HL13 complex of about 10^4 M^{-1}, but a relatively small error in the overall constant gives rise to a large error in this estimate. We have no direct evidence for independent HL19 binding, although it could be inferred from the cooperative shape of the EthBr-chased curves when HL19 was present (Feunteun et al., 1975). We have previously shown that there is no direct interaction between HL13 and HL19 under either low or high salt conditions (Smith et al., 1978). Consequently, binding of HL13 and HL19 as a dimer can be ruled out. In comparison, the situation in *E. coli* has recently become more clear. A direct measurement of the binding of protein EL18 to *E.*

coli 5S RNA gave a binding constant of 2.3×10^8 M^{-1} (Spierer et al., 1978), very similar to the value of 10^8 M^{-1} measured here for HL13 binding to *H. cutirubrum* 5S RNA. They also measured a value of 10^5 M^{-1} for the binding of EL5 (Spierer et al., 1978), and the association of EL5 was further shown to be cooperative with the binding of EL18 (Spierer & Zimmermann, 1978). Spierer & Zimmermann (1978) suggested that the secondary and tertiary structural changes observed in the 5S RNA on binding EL18 would increase the binding of EL5 and account for the cooperativity. Our work suggests that structural changes occur in 5S RNA as HL13 is bound and furthermore that there appear to be secondary structure changes in the HL13 as well. Thus, we postulate a type of induced fit for binding of the HL13 to the 5S RNA. This would suggest the possibility of a similar cooperativity as observed in *E. coli* in the binding of HL19 to the HL13–5S RNA complex. Thus far, we have not been able to directly measure the equilibria involving HL19 and firmly establish this point.

Finally, an important result of these comparisons is the demonstration of an apparent enormous retention of structural roles in the evolutionary sequence relating *E. coli* to *H. cutirubrum* despite the large differences between the primary sequences of their 5S RNAs and binding proteins. The *H. cutirubrum* complex has been shown to require high salt concentrations for optimum stability, and this requirement is apparently due to a requirement of high salt concentrations for the correct secondary structure of the binding proteins (Willick et al., 1978). The chemical stabilization of protein–nucleic acid interaction is likely a complicated combination of several processes. However, a mechanism such as counterion release, invoked to explain some nucleic acid–protein interactions (de Haseth et al., 1977), can be ruled out in this case. Thus, the comparing and contrasting of complexes of this type from an extreme halophile with those of procaryotes or eucaryotes existing under less extreme conditions could prove valuable in sorting out these complex processes.

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