Biochemistry

 Volume 19, Number 26 December 23, 1980

Distances between 3′ Ends of Ribosomal Ribonucleic Acids Reassembled into *Escherichia coli* Ribosomes†

Obed W. Odom, Jr., David J. Robbins, James Lynch, Diane Dottavio-Martin, Gisela Kramer, and Boyd Hardesty*

ABSTRACT: The three ribonucleic acids (RNAs) from *Escherichia coli* ribosomes were isolated and then labeled at their 3′ ends by oxidation with periodate followed by reaction with thiosemicarbazides of fluorescein or eosin. Ribosomal subunits reconstituted with the labeled RNAs were active for polyphenylalanine synthesis. The distances between the 3′ ends of the RNAs in 70S ribosomes were estimated by nonradiative energy transfer from fluorescein to eosin. The percentage of energy transfer was calculated from the decrease in fluorescence lifetime of fluorescein in the quenched sample compared to the unquenched sample. Fluorescence lifetime was measured in real time by using a mode-locked laser for excitation and a high-speed electrostatic photomultiplier tube for detection of fluorescence. The distances between fluorophores attached to the 3′ ends of 16S RNA and 5S RNA or 23S RNA were estimated to be about 55 and 71 Å, respectively. The corresponding distance between the 5S RNA and 23S RNA was too large to be measured reliably with the available probes but was estimated to be >65 Å. Comparison of the quantum yields of the labeled RNAs free in solution and reconstituted into ribosomal subunits suggests that the 3′ end of 16S RNA does not interact appreciably with other ribosomal components and may be in a relatively exposed position, whereas the 3′ ends of the 5S RNA and 23S RNA may be buried in the 70S ribosomal subunit.

Small angle X-ray diffraction data from the small subunit of *Escherichia coli* ribosomes indicated a structure that could be accurately represented by an ellipsoid of revolution having axes of 55 × 220 × 220 Å (Van Holde & Hill, 1974). The corresponding values for the 50S subunit were 115 × 230 × 230 Å. The distance between the centers of mass of the two subunits in 70S particles was estimated to be 93 ± 15 Å (Koch et al., 1978). Proteins and RNA[1] appear not to be uniformly distributed in either 30S or 50S subunits of *E. coli* ribosomes. Small-angle neutron scattering data indicate that the central region of both subunits is relatively rich in RNA with the ribosomal proteins distributed toward the exterior of the particles (Crichton et al., 1977; Stuhrmann et al., 1978).

The approximate location of the 3′ end of the 16S RNA in 30S subunits is indicated by several lines of evidence. Using immunoelectron microscopy, Olson & Glitz (1979) concluded that the 3′ end is located on the upper portion of the platform region of the 30S subunit. They employed antibodies against a dinitrophenol derivative covalently attached to the 3′ end of the 16S RNA. Using the same approach, Stöffler et al. (1980) found that the 3′ end of the 16S RNA is not buried and that antibody attachment sites are located between the groove and the large lobe of the 30S subunit. Shatsky et al. (1979) used the same technique with a phenyl lactoside derivative covalently linked to the 3′ end of the 16S RNA. They found the 3′ terminus to lie in the groove between the side "ledge" and the "head" of the subunit at the level of the top of the ledge. Zimmermann distinguished three domains of 16S rRNA which correspond roughly to the 5′, central, and 3′ regions of the molecule (Zimmerman, 1980). Small subunit proteins S7, S9, S13, and S19 bind to the 3′ domain. S1 appears to interact specifically with nucleotides in the region of the bulge loop involving nucleotides 10–14 from the 3′ end (Yuan et al., 1979). Immunoelectron microscopy indicates that these proteins are located in the head (Stöffler et al., 1980) or platform (Lake, 1980) region of the 30S ribosomal subunit. The same general location is indicated by immunoelectron microscopy using antibodies against N^6,N^6-dimethyladenosine (Politz & Glitz, 1977). Two successive residues of this modified base occur at positions 24 and 25 from the 3′ end of the 16S RNA.

The 3′ end of the 16S rRNA can be cross-linked to a number of ribosomal proteins (Rinke & Brimacombe, 1978) and to IF-3 (Van Duin et al., 1975), thus suggesting that this portion of the molecule may retain considerable flexibility in

† From the Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas, Austin, Texas 78712. *Received June 4, 1980.* Supported in part by Grant No. PCM78-13632 awarded by the National Science Foundation. Fluorescence lifetimes were determined at the Center for Fast Kinetics Research, which is supported by National Institutes of Health Grant RR-00886 from the Biotechnology Branch of the Research Resources and by the University of Texas.

[1] Abbreviations used: RNA, ribonucleic acid; rRNA, ribosomal RNA; tRNA, transfer RNA; poly(U), poly(uridylic acid); $Mg(OAc)_2$, magnesium acetate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease; RNase, ribonuclease; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; AMP, adenosine 5′-phosphate.

0006-2960/80/0419-5947$01.00/0

intact particles. Approximately 50 nucleotides at the 3′ end of 16S RNA are particularly sensitive to chemical modification and hydrolysis by ribonuclease, suggesting that they are in a relatively exposed position in 30S subunits (Zimmermann, 1980). Periodate-oxidized, 3′-terminal ribose of 16S RNA in 30S subunits could react with proflavine semicarbazide but was unreactive in 70S ribosomes (Schreiber et al., 1979), thus suggesting that this end of the molecule is near the surface of the 30S particle that interacts with the 50S subunits.

The topographical location of the 3′ end of 23S RNA in 50S subunits is poorly defined. L5, L18, and L25 appear to have binding sites in the 120-nucleotide sequence beginning 600 residues from the 3′ end of the molecule (Branlant et al., 1977). The same sequence may provide a point of interaction with the 5S RNA. A binding site for L1 occurs in the adjacent 5′ sequence. These and other data have led Zimmermann to place the 3′ domain of the 23S RNA near the center of the chairlike 50S subunit. 5S RNA appears to be located in this same general area (Zimmermann, 1980). A strong binding site for L25 occurs between residues 69 and 110 of 5S RNA. Guanine residues at positions 24 and 69 are strongly protected from kethoxal modification by L18 (Garrett & Noller, 1979). The 3′ end of 23S RNA is fully accessible for reaction with proflavine semicarbazide in both 50S subunits and 70S ribosomes; however, the 3′ end of 5S RNA has low reactivity suggesting that it may be buried in 50S subunits or in intact ribosomes (Schreiber et al., 1979).

Here we describe the results of experiments in which nonradiative energy transfer was used to estimate the distance between fluorophores attached to the 3′ ends of the three ribosomal RNAs. Distances are determined relative to R_0, the distance at which the energy transfer proceeds at half of its maximum rate. We have evaluated R_0 from the equation (Förster, 1959)

$$R_0 = 9.79 \times 10^3 (Q_0 n^{-4} \kappa^2 J)^{1/6}$$

where Q_0 is the quantum yield of the energy donor under the experimental conditions but in the absence of an acceptor, n is the refractive index of the medium, κ^2 is an orientation factor for dipole–dipole transfer, and J is the spectral overlap integral given by

$$J = \frac{\int_0^\infty F(\lambda)\epsilon(\lambda)\lambda^2 \, d\lambda}{\int_0^\infty F(\lambda)\lambda^{-2} \, d\lambda}$$

where $F(\lambda)$ is the relative fluorescence intensity of the donor at wavelength λ (in units of centimeters) and $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor at this wavelength.

One can experimentally determine the efficiency (E) of the energy transfer by measuring the fluorescence lifetime of the donor both in the presence and in the absence of the acceptor. The efficiency is given by

$$E = 1 - \tau/\tau_0$$

where τ_0 is the lifetime of the donor without the acceptor and τ is the lifetime in the presence of the acceptor. This efficiency can be related to the distance (r) between the donor and acceptor via

$$r = R_0(E^{-1} - 1)^{1/6}$$

Fluorescein and eosin were chosen to be the energy donor and acceptor, respectively, not only because they can be bound specifically to the 3′ terminus of the rRNAs, but also because they have a large spectral overlap integral. The reliability of the measurements is discussed.

Experimental Procedures

Materials

E. coli K12, strain A19, was a kind of gift from Drs. K. Nierhaus and H. G. Wittmann, Berlin, for early experiments. 5-Fluorescein thiosemicarbazide was synthesized in our laboratory by reacting fluorescein isothiocyanate with hydrazine. Recently, both fluorescein thiosemicarbazide and eosin thiosemicarbazide have been purchased from Molecular Probes, Inc. (Plano, TX). Casein hydrolysate, glucose, poly(ethylene glycol)-4000, phosphocreatine, and creatine phosphokinase were obtained from Sigma Chemical Co. (St. Louis, MO). Autolyzed yeast extract powder was from Yeast Products, Inc. (Clifton, NJ), and sucrose (RNase free) and urea (ultra pure) were from Becton Dickinson Immunodiagnostics (Orangeburg, NY). Poly(U) was obtained from Miles Research Products, Inc. (Elkhart, IN) and [^{14}C]phenylalanine (420 Ci/mol) from ICN Life Sciences (Irvine, CA). Phenol and dimethylformamide were redistilled before use. All other chemicals used were reagent grade.

Solutions. Solution A contained 20 mM Tris-HCl (pH 7.7), 10 mM $MgCl_2$, 1 mM dithioerythritol, and 0.1 mM EDTA. Solution B contained 50 mM Tris-HCl (pH 7.7), 10 mM $MgCl_2$, 1.0 M NH_4Cl, 1 mM dithioerythritol, and 0.1 mM EDTA. Solution C contained 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 M NH_4Cl, 1 mM dithioerythritol, and 0.1 mM EDTA. Solution D contained 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM NH_4Cl, and 6 mM β-mercaptoethanol. Solution E contained 10 mM Tris-HCl (pH 7.6), 50 mM KCl, and 1% methanol. Solution F contained 30 mM Tris-HCl (pH 7.4), 20 mM $Mg(OAc)_2$, 500 mM KCl, and 1 mM dithioerythritol. Solution G contained 20 mM Tris-HCl (pH 7.5), 4 mM $Mg(OAc)_2$, 400 mM NH_4Cl, 1 mM dithioerythritol, 0.2 mM EDTA, and 6 M urea. Solution H contained 10 mM Tris-HCl (pH 7.6), 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl, and 4 mM β-mercaptoethanol.

All solutions were made up from glass-distilled, deionized water. Contact with detergent-washed plasticware was strictly avoided to minimize contamination from fluorescent detergents, which adhere to plastic. The pH of all solutions was adjusted at room temperature.

Methods

(*1*) *Growth of E. coli. E. coli* K12, strain A19, was grown in a 250-L fermenter (New Brunswick Scientific Co., New Brunswick, NJ) containing 180 L of medium made from 1 kg of casein hydrolysate, 1 kg of yeast extract, 250 g of $K_2HPO_4 \cdot 3H_2O$, 500 g of $MgSO_4 \cdot 7H_2O$, and 500 g of glucose. The medium was inoculated from a 5-L culture containing 50 g of casein hydrolysate, 50 g of yeast extract, and 25 g of $K_2HPO_4 \cdot 3H_2O$. This culture was grown from a 25-mL overnight culture that contained the same medium plus 25 mg of glucose. *E. coli* cells were harvested in early log phase ($A_{650} \simeq 0.5$).

(*2*) *Preparation of 70S Ribosomes and Ribosomal Subunits.* The procedure described by Nierhaus & Dohme (1979) was followed with minor modifications. Frozen *E. coli* (300 g) was ground with alumina in solution A; DNase was omitted. After two low-speed centrifugations to remove alumina and cell debris, 70S ribosomes were collected by centrifugation for 4 h at 35 000 rpm in the Ti-45 rotor (Spinco, Beckman Instruments, Palo Alto, CA). The pellets were suspended in solution B, kept on ice overnight, and centrifuged for 5 h at 35 000 rpm in the Ti-45 rotor to collect the ribosomal particles. The pellets were resuspended in solution C, and the aggregates were removed by low-speed centrifugation. The ribosomal

suspension was stored frozen at −80 °C in aliquots of 20 000 A_{260} units.

One of these aliquots was loaded onto a 7.5–40% sucrose (w/v) gradient in solution C in a Ti-15 zonal rotor (Beckman Instruments, Palo Alto, CA). After centrifugation (15 h at 30 000 rpm) 30S and 50S ribosomal subunits were collected separately and precipitated with poly(ethylene glycol)-4000. The subunits were collected by centrifugation, resuspended in solution D at a concentration of 400–600 A_{260} units/mL, and stored frozen at −80 °C in small aliquots.

(*3*) *Preparation of Ribosomal RNA.* 50S ribosomal subunits were extracted with phenol as described in detail by Nierhaus & Dohme (1979). The 23S and 5S RNAs were separated by chromatography on Sephadex G-100 as described in the same reference. About 1000 A_{260} units of 23S plus 5S RNA in a total vlume of 3 mL was loaded on a 2 × 120 cm column equilibrated in solution E.

The 16S RNA was extracted from 30S ribosomal subunits with phenol and sodium dodecyl sulfate as described above for the extraction of 50S subunits. Care was taken to keep the concentration at ≤400 A_{260} units/mL.

(*4*) *Preparation of Total Proteins (TP) from Ribosomal Subunits and Subunit Reconstitution.* (*a*) *TP30 and 30S Reconstitution.* The procedures of Traub & Nomura (1968) as modified by Hardy et al. (1969) were followed for isolation of a fraction containing the total protein from the 30S subunit, TP30, and for reconstitution of 30S particles containing labeled 16S RNA. Briefly, the procedures are extraction of the total proteins with acetic acid, dialysis against 8 M urea, 1 mM dithioerythritol, and 30 mM Tris-HCl (pH 7.4), and finally passage over a Sephadex G-25 column equilibrated with solution F. The column procedure was instituted by us in lieu of dialysis. The concentration of TP30 was determined from the A_{230} reading by assuming 1 A_{230} unit equals 10 equivalent units of TP30 (Nierhaus & Dohme, 1979). The total reconstitution involves incubation of 16S RNA and TP30 for 1 h at 42 °C in 30 mM Tris-HCl (pH 7.4), 330 mM KCl, 20 mM $Mg(OAc)_2$, and 1 mM dithioerythritol. Activity of reconstituted 30S particles routinely was measured by poly(U)-dependent polyphenylalanine synthesis.

(*b*) *TP50 and Reconstitution of 50S Subunits.* The methods described the Nierhaus & Dohme (1979) were followed for isolation of unfractionated proteins from the 50S subunit, TP50, and for reconstitution of 50S subunits containing labeled 5S RNA and/or 23S RNA. Briefly, these methods involve extraction of the total proteins with acetic acid, precipitation with acetone, redissolution in solution G, and dialysis for three 1-h periods against a similar solution without urea that is adjusted to pH 6.0 with acetic acid. The pH of 6.0 is extremely important to prevent precipitation of the proteins during dialysis. The concentration of TP50 is determined from the absorbance at 230 nm, 1 A_{230} unit being equal to 10 equivalent units of TP50 (Nierhaus & Dohme, 1979). The total reconstitution involves incubation of 23S RNA, 5S RNA, and TP50, first for 20 min at 44 °C at 4 mM $Mg(OAc)_2$ and then for 90 min at 50 °C at 20 mM $Mg(OAc)_2$ in the reconstitution mixture described by Nierhaus & Dohme (1979). Assay of reconstituted 50S was by poly(U)-dependent polyphenylalanine synthesis.

(*5*) *Poly(U)-Dependent Polyphenylalanine Synthesis.* Polyphenylalanine synthesis was measured in an assay containing the following components in a total volume of 100 μL: 25 mM Tris-HCl (pH 7.5), 12–14 mM $MgCl_2$, 150 mM NH_4Cl (25 mM NH_4Cl plus 100 mM KCl when reconstituted 30S subunits were assayed), 3 mM dithioerythritol, 4 mM β-mercaptoethanol, 0.8 mM ATP, 0.05 mM GTP, 5 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 4 μg of tRNA (isolated by phenol extraction from rabbit liver and deacylated), 20 μg of poly(U), ~60 μg of *E. coli* postribosomal supernatant (concentrated and partially fractionated by precipitation with ammonium sulfate at 70% saturation), 5×10^{-5} M [^{14}C]Phe (5 Ci/mol), and 30S and 50S ribosomal subunits modified or reconstituted as indicated for the individual experiments, generally 0.8 A_{260} units of 50S ribosomal subunits with the optimal amount of 30S ribosomal subunits, usually 0.45–0.5 A_{260} units. The reaction mixture was incubated for 30 min at 37 °C. Then 100 μL of 1 N NaOH was added and the incubation continued for 5 min at 37 °C. Protein was precipitated with 5% trichloroacetic acid and collected on glass fiber filters (Schleicher & Schuell, No. 30), and its radioactivity was determined by liquid scintillation in 10 mL of a counting fluid containing 5 g of 2,5-diphenyloxazole/L of toluene. Under these conditions polyphenylalanine containing ~2100 pmol of phenylalanine was synthesized.

(*6*) *Oxidation and Labeling of Ribosomal RNAs.* (*a*) *5S RNA.* 5S RNA at a concentration of 50 A_{260}/mL or less was oxidized at its 3′ end by incubation with 0.09 M sodium periodate in 0.1 M sodium acetate, pH 5.0, for 90 min at room temperature in the dark. At the end of the incubation KCl was added to a final concentration of 0.2 M, the solution was allowed to stand on ice for 10 min, and the precipitate of potassium periodate was removed by centrifugation for 5 min at 10000*g*. The supernatant was passed over a Sephadex G-25 column equilibrated with 0.1 M sodium acetate, pH 5.0, to remove remaining periodate. The combined fractions from the Sephadex G-25 column were labeled by incubation in the dark for 2 h at room temperature with either 1 mM eosin thiosemicarbazide or 2 mM fluorescein thiosemicarbazide. Appropriate amounts of these labels were added from 0.1 M stock solutions in dimethylformamide. At the end of the incubation the samples were extracted 3 times with equal volumes of 70% phenol to remove most of the unbound label. The phenol was stored frozen after redistillation, and water was added just before use. After the phenol extraction the samples were made 0.1 M in KCl and precipitated with 2 volumes of 95% ethanol at −20 °C. The ethanol precipitation was repeated twice, after which the samples were taken up in the desired buffer.

(*b*) *16S and 23S RNA.* 16S or 23S RNA at concentrations of no more than 200 or 400 A_{260} units/mL, respectively, were oxidized by incubating with 0.09 M sodium periodate in 0.1 M sodium phosphate, pH 7.0, for 2 h at 0 °C in the dark. KCl then was added to a final concentration of 0.2 M, and the precipitate of potassium periodate was removed as described for 5S RNA. The supernatant then was passed over a Sephadex G-25 column equilibrated with 0.05M sodium phosphate, pH 7.0, to remove remaining periodate. The samples from the Sephadex G-25 column, in 0.05 M sodium phosphate, pH 7.0, are labeled as follows. 16S RNA is incubated for 1 h in the dark at room temperature with 1 mM eosin thiosemicarbazide or 2 mM fluorescein thiosemicarbazide. 23S RNA is incubated 2 h at 0 °C with 1 mM eosin thiosemicarbazide or 2 mM fluorescein thiosemicarbazide. The samples are then treated as described for 5S RNA, except that, just prior to ethanol precipitation, 2 volumes of 0.1 M KCl are added. This dilution is necessary to prevent precipitation of sodium phosphate by the ethanol.

The thiosemicarbazide adducts tend to degrade above pH 8 and at elevated temperatures (Dulbecco & Smith, 1960). Samples of labeled RNA and ribosomal subunits were rig-

Table I: Spectral Properties of Ribosomal RNAs Labeled at Their 3′ End with Fluorescein or Eosin

rRNA	A_{max} (Å)		ϵ (A_{max}) ($\times 10^{-3}$)		max emission (λ)		Q_0	
	free RNA	in subunits	free RNA	in subunits	free RNA	in subunits	free RNA	in ribosomes
fluorescein								
5S	494	494	69	66	520	520	0.55	0.29
16S	492	497	69	65	518	520	0.55	0.52
23S	492	494	69	66	518	520	0.49	0.23
eosin								
5S	520	525	91	91	544	544	0.048	0.052
16S	522	526	91	91	542	542	ND[a]	0.072
23S	522	525	91	91	541	542	0.069	ND[a]

[a] ND = not determined.

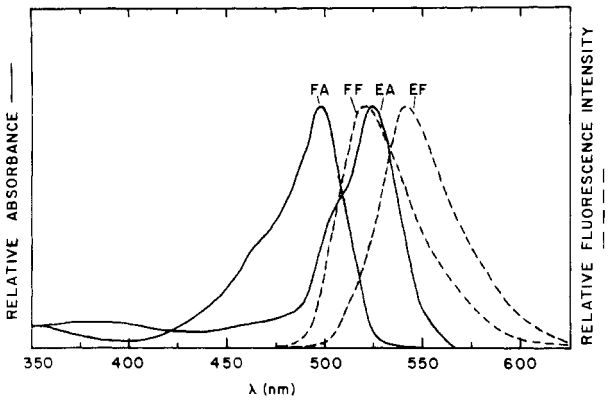

FIGURE 1: Absorbance and fluorescence spectra of fluorescein- and eosin-labeled 16S RNA in 30S ribosomal subunits. All spectra were scaled to the same height. Quantitative relationships may be calculated from the ϵ and Q_0 values given in Table I. FA and FF indicate the absorbance and emission spectra, respectively, of fluorescein-labeled 16S RNA reconstituted into 30S ribosomal subunits; EA and EF are the absorbance and emission spectra, respectively, of 30S subunits containing eosin 16S RNA.

Table II: R_0 and Limits from Fluorophore Orientation

location of fluorophore				
fluorescein	eosin	R_0[a]	polarization (acceptor)[b]	half-height limits of $Q(r'/r)$[c]
5S	16S	49	0.41	0.90–1.03
16S	5S	54	0.36	0.91–1.06
23S	16S	47	0.41	0.90–1.03
16S	23S	54	0.40	0.90–1.03
5S	23S	49	0.40	0.90–1.03
23S	5S	47	0.36	0.91–1.06

[a] R_0 is the distance for half-maximum energy transfer assuming $\kappa^2 = {}^2/_3$. [b] Excitation (acceptor) = 490 nm, and emission = 535 nm; excitation (donor) = 353 nm, and emission = 520 nm. This yields values of <0.05 in all cases. [c] As determined by the full width at half-maximum height of the function $Q(r'/r)$. These values were estimated from polarization data by the method of Haas et al. (1978). r' is the distance between the donor and acceptor pair calculated from R_0 for which $\kappa^2 = {}^2/_3$. r is the actual distance between the donor and acceptor pair. $Q(r'/r)$ is the apparent distribution of distances between donor and acceptor pairs estimated as described by Haas et al. (1978).

orously maintained under cold, dark conditions except as required for specific experimental procedures.

(7) *Determination of Factors Involved in Estimation of R_0.* Absorption spectra were taken on a Cary 15 recording spectrophotometer. Fluorescence spectra were taken with the steady-state photon-counting fluorometer [described by Odom et al. (1978)]. Absorbance and emission spectra for fluorescein- and eosin-labeled 16S RNA that has been reconstituted into 30S ribosomal subunits is shown in Figure 1. Note that the intensities were adjusted to produce spectra of approximately the same peak height.

The data in Table I compare the absorbance and emission maxima between free ribosomal RNA labeled with either fluorescein or eosin thiosemicarbazide and the same RNA reconstituted into ribosomal subunits. Only slight shifts to longer wavelengths are observed in the latter cases. Also indicated in Table I are the molar extinction coefficients, ϵ, at the absorbance maxima. These were obtained in the following way. A given amount of either fluorescein or eosin thiosemicarbazide was weighed into 0.2 M NaOH, and thus a standard solution of known concentration was obtained. Also, both of the thiosemicarbazides were reacted with periodate-oxidized AMP as a model compound. The spectrum of a given sample of labeled RNA or RNA reconstituted into subunits was taken at pH 7.6. The sample then was made 0.2 M in NaOH by adding $^1/_{50}$ volume of a 10 M NaOH solution, and after 1 h at 37 °C its spectrum was recorded. This spectrum was then compared to the standard solution described above with the results recorded in Table I.

The quantum yield, Q_0, defined as the ratio of photons emitted by a species to photons absorbed, is found by comparison to a standard whose quantum yield is known (Freifelder, 1976)

$$Q_x = \frac{I_x Q_s A_s}{I_s A_x}$$

where x represents the unknown species, s represents the standard, I is the integrated amount of fluorescence observed, and A is the absorbance at the excitation wavelength. For the data indicated in Table I, freshly prepared disodium fluorescein in 0.10 M NaOH, Q = 0.92 (Weber & Teale, 1957), was used as standard.

A value must be found for the orientation parameter, κ^2, which can range from 0 to 4, in order to compute R_0 from these spectral data. Although κ^2 cannot be experimentally determined, limits on its value can be estimated from fluorescence polarization data (Haas et al., 1978). However, if the donor and acceptor are free to tumble rapidly on the time scale of the fluorescence, κ^2 will average to a value of $^2/_3$ (Förster, 1949). This value is assumed in the initial computation of R_0. Haas et al. (1978) have tabulated limits on the ratio r/r' (where r is the actual distance between the donor and the acceptor and r' is the calculated distance based on a value of $^2/_3$ for κ^2) as a function of the polarization of both the donor and acceptor.

The fluorescence polarization data given in Table II were obtained in the following way. The fluorophore was excited with vertically polarized light in a steady-state photon-counting fluorometer [described by Odom et al. (1978)], and the fluorescence intensity was measured as a function of its polarization angle. The polarization, P, (Förster, 1951) is given by

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ is the intensity of fluorescence polarized parallel to the excitation light and $I_{\perp}$ is the intensity polarized perpendicular to the excitation. For simulation of the conditions existing during the energy transfer experiments, fluorescein was excited at 353 nm (the chosen laser line) whereas eosin was excited at 490 nm (within the fluorescein emission band). It was noted that the polarization of the fluorescein increased when it was excited nearer its absorption maximum.

(*8*) *Measurement of Fluorescent Lifetimes.* A Nd:YAG mode-locked laser (NG50, Quantel International, Sunnyvale, CA) equipped with a Pockels cell is used to provide a light pulse of ~30 ps full width at half of its maximum intensity. The primary frequency of 1060 nm is passed through a temperature-tuned frequency-doubling crystal and then through an angle-tuned crystal in which third harmonic photons are generated (Hänsch, 1977) to give excitation pulses of ~3 mJ at a frequency of 353 nm. Fluorescence is measured by an all electrostatic high-speed photomultiplier tube (Model VPM-152M, Varian Instruments, Palo Alto, CA) equipped with seven stages of gain (total gain of $\sim 10^5$), converted to digital signals by a Tektronix R7912 digitizer, and analyzed with an on-line computer (PDP-11T34, Digital Equipment Corp., Maynard, MA).

Deconvolution of the fluorescence decay from the observed signal was carried out by means of a least-squares program similar to that described by Knight & Selinger (1971). A one- or two-exponential function is convoluted with the impulse response curve in order to generate a model for the observed signal. The parameters of the trial function are then varied so as to minimize the standard deviation of the model from the observed signal.

Thus, a measurement of energy transfer between donor and acceptor probes in ribosomes involves the following steps. (1) The impulse response of the measuring system is obtained by observation of laser light scattered from distilled water in a quartz cuvette. (2) The fluorescence signal from the donor fluorophore in the absence of an acceptor is determined. A series of optical filters is used to exclude both scattered laser light and any fluorescence from the acceptor which may occur during step 3. For the case of fluorescein to eosin, a 398-nm cut-on filter (KV 408, Schott Optical Glass, Inc., Duryea, PA) with a transmission of 10^{-3}% at 353 nm was used to exclude laser light, while a 10-nm band-pass filter with 50% transmission at 500 nm and 10^{-4}% transmission on either side of the peak transmission (Ditric Optics, Inc., Marlboro, MA) was used to exclude any eosin fluorescence. (3) The fluorescence from the donor in the presence of the acceptor was measured under the same conditions as were used in step 2. In all cases, the signals from 10 laser shots were combined to provide an average response curve for analysis by the computer.

Results

Activity of Labeled Ribosomal RNAs. The three *E. coli* ribosomal RNAs were isolated from separated ribosomal subunits by a procedure involving phenol as described under Methods. Labeling was accomplished by reaction of the isolated rRNA species with either fluorescein thiosemicarbazide or eosin thiosemicarbazide following periodate oxidation of the 3′-terminal ribose. The labeling reaction was carried out to give a fluorophore/rRNA ratio of about 1:1 as determined from their respective extinction coefficients. The corresponding ratios were less than 0.05:1 for aliquots of the same rRNA samples carried through an identical procedure except that they were not subjected to periodate oxidation. The preparations used for the experiments reported below gave a single fluorescent band corresponding to undegraded rRNA as judged by polyacrylamide gel electrophoresis in formamide (data not shown). Preparations for which a higher fluorophore/rRNA ratio was observed or for which more than one component was detected by gel electrophoresis were discarded. These results indicate that the primary product formed during the labeling reaction results in attachment of a single fluorophore residue to the 3′ end of the rRNAs used in these studies. The labeling ratios reported here are equivalent to those found by Dulbecco & Smith (1960) for the reaction of thiosemicarbazide with periodate-oxidized RNAs and are consistent with the formation of a morpholine derivative similar to the product proposed by Hansske et al. (1974) for the reaction of hydrazides with periodate-oxidized nucleotides. Labeling ratios of greater than unity have been interpreted to indicate reaction of each of the two aldehyde groups formed by periodate oxidation of the ribose with a separate reactive group thus yielding two fluorophore residues per RNA chain (Reines & Cantor, 1974).

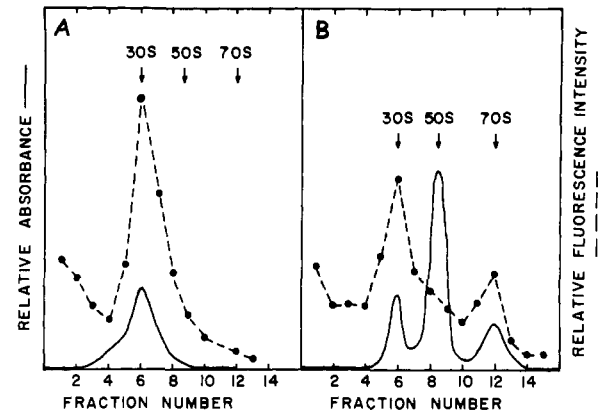

FIGURE 2: Fluorescein-labeled 16S RNA in 30S and 70S ribosomal particles. An aliquot of 30S ribosomal subunits (0.9 A_{260} units) reconstituted with fluorescein-labeled 16S RNA was incubated without (A) or with (B) 1.8 A_{260} units of 50S ribosomal subunits for 10 min at 37 °C in solution H and then loaded on a 10–40% (w/v) linear sucrose gradient in solution H. Centrifugation was for 18 h at 4 °C at 20000 rpm (SW-41 rotor, Spinco). After centrifugation the gradients were fractionated with automatic scanning of the absorbance at 254 nm (solid line). Then the fluorescence of each fraction was determined [excitation at 470 nm, emission at 515 nm (broken line); peak fraction in panel A, $\sim 16 \times 10^3$ cpm; peak fractions in panel B, about 12×10^3 and 6×10^3 cpm].

Labeled 16S RNA may be efficiently reconstituted into 30S subunits. Absorbance measurements of the reconstituted particles indicate a <10% loss of the label during the reconstitution. These reconstituted 30S particles can reassociate with unmodified 50S subunits to form 70S ribosomes. Data leading to this conclusion are shown in Figure 2 which depicts a typical sucrose gradient profile of an aliquot of a sample containing fluorescein-labeled 16S RNA in 30S subunits used for an energy-transfer experiment. Similar results were obtained with either fluorescein- or eosin-labeled 16S RNA. Under the conditions used, about one-third of the labeled 30S subunits combine with unlabeled 50S subunits to form stable 70S ribosomes. A very similar profile is obtained with 30S subunits that have been reassociated with unlabeled 16S RNA. The proportion of ribosomes in which energy transfer has occurred, as judged by the weighting factor of each of the two fluorescence lifetimes from the quenched and unquenched species, consistently has been larger than the proportion of 30S subunits that combine to form 70S ribosomes, as judged by sucrose gradient analysis. The basis for this phenomenon is

Table III: Activity in Polyphenylalanine Synthesis of 30S Ribosomal Subunits Reconstituted with 3'-Modified 16S RNA[a]

RNA	[^{14}C]Phe incorporated (pmol)	relative act. (%)
unmodified 16S	1008	100
eosin 16S	990	98
fluorescein 16S	961	95

[a] 30S ribosomal subunits were reconstituted as described under Methods. An aliquot of the reconstitution mixture containing 0.6 A_{260} units of 30S was assayed in duplicate in the poly(U)-dependent polyphenylalanine assay with 0.9 A_{260} units of 50S subunits. Unmodified 30S subunits were assayed concurrently as controls. The 30S particles reconstituted from unlabeled 16S routinely gave 60–70% of the activity of these unmodified 30S subunits.

unclear. It may reflect the formation of loose 30S–50S complexes that dissociate during sucrose gradient centrifugation.

Results similar to those shown in Figure 2 also have been obtained with 50S subunits reconstituted with either 5S RNA or 23S RNA labeled with either eosin or fluorescein as described above (data not shown). Here again, there is a <10% loss of the label during reconstitution. These results indicate that even the relatively large eosin moiety attached to the 3' ends of the ribosomal RNA does not appreciably interfere with reassociation of the ribosomal subunits. Cantor and his coworkers (Schreiber et al., 1979) found that 30S particles reconstituted from periodate-oxidized 16S RNA that had been reacted with proflavine semicarbazide would not form stable 70S ribosomes. The basis for this apparent discrepancy is not clear.

Peptide synthesis using 30S subunits reconstituted with labeled 16S RNA is shown in Table III. The results indicate that attachment of fluorescein or eosin to the 3' end or the labeling procedure itself does not appreciably reduce the activity of the 16S RNA for peptide synthesis. For these experiments 30S subunits reconstituted with eosin or fluorescein 3' labeled 16S RNA are compared in their activity to promote poly(U)-directed synthesis of polyphenylalanine with 30S subunits reconstituted with unlabeled 16S RNA. Polyphenylalanine synthesis was carried out with native 50S subunits. Typically, 30S ribosomes reconstituted with unlabeled 16S RNA exhibited about 60–70% of the activity of 30S subunits that had not been reconstituted but incubated under the conditions by which 30S subunits are reconstituted.

Typical results for 50S subunits reconstituted with either 5S RNA or 23S RNA labeled with either fluorescein or eosin and native 30S subunits are shown in Table IV. Polyphenylalanine synthesis at near control levels is seen with all combinations of fluorescein- or eosin-labeled 5S RNA and with fluorescein-labeled 23S RNA. However, 23S RNA labeled with eosin appears to have reduced activity. This may be due to the relatively large size and mass of eosin. The lowest activity (21% of the unlabeled controls) is observed with 50S subunits containing fluorescein-labeled 5S RNA and eosin-labeled 23S RNA. However, it appears that even in this worst situation peptide synthesis can take place on labeled ribosomes, since the degree of labeling approaches 100%. Polyphenylalanine synthesis with eosin-labeled 23S RNA in 50S subunits and fluorescein- or eosin-labeled 16S RNA in 30S subunits was approximately as active as only eosin 23S RNA in 50S subunits (data not shown).

Distances between 3' Ends of 16S, 5S, and 23S RNA. The rRNAs labeled at their 3' end with either fluorescein or eosin were reconstituted into the respective ribosomal subunits, and then their fluorescence properties were analyzed as described

Table IV: Polyphenylalanine Synthesis with Ribosomes Having 50S Subunits Reconstituted with 3'-Labeled 23S or 5S RNA[a]

23S RNA	5S RNA	[^{14}C]Phe incorporated (pmol)	relative act. (%)
unmodified	unmodified	1580	100
fluorescein	unmodified	1501	95
eosin	unmodified	513	32
unmodified	fluorescein	1481	94
unmodified	eosin	1452	92
fluorescein	eosin	1178	75
eosin	fluorescein	330	21

[a] 50S subunits were reconstituted from TP50 and the RNA indicated above as described under Methods. An aliquot, ~0.74 A_{260} units, was assayed in duplicate with 0.45 A_{260} units of 30S ribosomal subunits for activity in poly(U)-dependent polyphenylalanine synthesis. A blank obtained with 30S subunits alone (60 pmol) was subtracted. Omission of 5S RNA from the reconstitution mixture resulted in reduction of 50S activity to ~5%. Unmodified 50S subunits were assayed concurrently as controls, compared to which unlabeled reconstituted 50S particles exhibited 40–60% activity.

Table V: Determination of Distances between the 3' Ends of Ribosomal RNAs in Ribosomal Subunits or Ribosomes

3' label on RNA		E		limits of r[a]
fluorescein	eosin	(% quenching)	r' (Å)	(Å)
5S	16S	42	52	50–58
16S	5S	46	55	52–60
23S	16S	<15	>62	
16S	23S	16	71	69–79
5S	23S	<15	>65	
23S	5S	<15	>63	

[a] Calculated from the limits given in Table II.

in detail under Methods. Generally the sample used for determination of energy transfer contained ~1 nmol of ribosomol subunits in 0.5 mL of solution H. Data are obtained as a computer-drawn curve of fluorescence intensity vs. time on which the actual data points and the plot of the exponential function derived by the computer to fit the data are shown. Numerical values are given for lifetimes and zero time intensities. First, the fluorescence lifetime from ribosomes labeled only with the donor is determined. For a typical experiment in which energy transfer from fluorescein-labeled 5S RNA to eosin-labeled 16S RNA was to be determined, fluorescein 5S RNA was found to have a lifetime of 2.94 ns. This value for the fluorescence lifetime of the unquenched species then was used as one component of the double-exponential function fitted to the decay curve from the sample in which fluorescein fluorescence was quenched by energy transfer to eosin, i.e., in the sample containing both fluorescein-labeled 5S RNA and eosin-labeled 16S RNA. By this means a fluorescence lifetime of 1.72 ns was determined for the fluorescein in ribosomes also labeled with eosin. This is a 42% reduction in the lifetime of the unquenched species. With an R_0 value of 49 Å for this donor–acceptor pair (Table II), 42% quenching corresponds to distance of 52 Å. This calculation assumes a value of $^2/_3$ for κ^2. The distance of 52 Å has probable distribution limits of plus 5.8 Å and minus 1.5 Å estimated by the method of Haas et al. (1978) for the uncertainty of the value of the orientation factor, κ (see Table II and related text).

Data analyzed as described above for energy transfer between the 3' ends of the three ribosomal RNAs are summarized in Table V. Each value represents the average of at least two independent experiments using different RNA prepara-

Table VI: Fluorescence Intensities Determined on a Steady-State Photon-Counting Fluorometer for Energy Transfer from Fluorescein-Labeled 5S RNA to Eosin-Labeled 16S RNA

fluorophore	fluorescence at 500 nm (cpm × 10^3)	
	353 nm[a]	480 nm[a]
none	0.4	0.6
fluorescein 5S	25.4	234
eosin 16S	3.1	4.8
fluorescein 5S + eosin 16S	19.6	152

[a] Excitation wavelength.

tions. Time-resolved fluorescence for energy transfer between the 3′ ends of 5S RNA and 23S RNA indicates a component with a relatively long fluorescent lifetime. Typically, this long-lived fluorescence has corresponded to a component with a fluorescence lifetime indicating about 10% quenching. However, this degree of quenching is too low to resolve dependably. Quenching of less than 15% has been indicated in Table V. This corresponds to a distance of >65 Å. Energy transfer of 16% from the 3′ end of 16S RNA to the 3′ end of 23S RNA is at the lower limit for reliable determination. This energy transfer corresponds to a distance of 71 Å. Energy transfer in the reverse direction from 23S RNA to 16S RNA is smaller and is indicated as <15%. Note that the quantum yield of fluorescein on both 5S RNA and 23S RNA is considerably reduced compared to its quantum yield on 16S RNA (Table I). This is the primary factor in the lower R_0 values for energy transfer from 16S RNA (Table II). The quantum yield for the fluorescein-labeled RNAs in free solution is ~0.5. The quantum yield for the fluorescein thiosemicarbazide reagent was determined to be 0.56. These results suggest that the 3′ end of 16S RNA in ribosomes may be in an exposed position in which it does not interact significantly with other ribosomal components. In contrast, the quantum yield for fluorescein on the 5S and 23S RNA is reduced in ribosomes compared with that for fluorescein on free rRNA, suggesting that the ends of these RNAs may be in positions in which the immediate environment of the attached probe is altered by other ribosomal components. Fluorescence intensities for the experiment in which energy transfer from fluorescein-labeled 5S RNA to eosin-labeled 16S RNA were measured are shown in Table VI. The reduction in fluorescein fluorescence by using excitation light at 353 nm, the maximum of the laser light, is ~34%, which can be compared with the value of 42% calculated from the time-resolved fluorescence as described above. The difference appears to reflect 50S subunits containing fluorescein-labeled 5S RNA that are not joined with a 30S subunit containing eosin-labeled 16S RNA. This phenomenon of incomplete association also has been observed in the sucrose gradient profile shown in Figure 2.

Discussion

The probes attached to the 3′ ends of the three ribosomal RNAs may be visualized as forming a triangle in 70S ribosomes in which the base is formed between the 5S RNA and the 23S RNA. The longest side, ~71 Å, is formed between the 16S RNA and the 23S RNA. The shorter side between the 5S RNA and 16S RNA is ~54 Å (average of 5S to 16S and 16S to 5S distances). The length of the base of the triangle between the 5S RNA and 23S RNA is too long to be measured reliably with the probes used in this study. It appears to be more than ~65 Å.

The quantum yield of fluorescence on the 3′ end of 16S RNA is consistent with a ribosomal model in which the 3′ terminus lies in an exposed position on or near the platform or neck of the 30S subunit. Evidence favoring this location is considered in the introduction. The distances between the 3′ ends of the RNAs in the two subunits are considerably shorter than the distance between the centers of mass of the two ribosomal subunits, 93 ± 15 Å, as determined by neutron scattering (Koch et al., 1978). The data presented here do not favor a model of 70S ribosomes in which there is a physical interaction of the 3′ region of the 16S RNA with the 3′ region of either the 23S RNA or 5S RNA.

The reliability of the distances reported here is of primary concern for further determination of ribosome structure. As briefly explained under Methods, energy transfer is calculated directly from the decrease in the fluorescence lifetime of an energy donor in a donor–acceptor pair, thereby eliminating the requirement for a separate quantitative determination of the concentration of these pairs. Such a determination is required in making energy-transfer calculations from changes in fluorescence intensity. This fundamental difference constitutes a major advantage in the use of fluorescent lifetimes over steady-state techniques. In our experience generally it is difficult or impossible to accurately evaluate the concentration of the donor–acceptor pairs in ribosomal systems of the types employed here. Problems arise from incomplete labeling, nonspecific binding of the dyes, loss of the dyes due to photo or enzymatic degradation during the course of the experiment, and incomplete binding of components such as association of 30S and 50S subunits in some of the experiments reported here.

Another source of potential error involves evaluation of the factors required to calculate R_0, the distance at which energy transfer occurs at half of its maximum rate between the donor–acceptor pair. These factors have been evaluated under the conditions of the experiments as described above. There appears to be no source of major error in these determinations. Furthermore, it should be noted that R_0 is relatively insensitive to small variations in any of the values considered above in that they enter the calculation as a function raised to the $^1/_6$ power. The limits due to the relative orientation of the fluorophores have been established by using the procedure of Haas et al. (1978) as described under Methods. These limits are relatively narrow for the results presented here.

We believe sources of error other than determination of fluorescence lifetimes or R_0 are apt to be more serious. The probes themselves are relatively large and are attached by side chains of significant length. These side chains may be quite flexible. Fluorescence from the probes shows a considerable degree of depolarization. Thus, the position of the probes with respect to the 3′ end of the RNA is not known precisely. Also, it is entirely possible that the probes themselves cause a significant perturbation of the structure. Despite these limitations, we believe that the results described here demonstrate the potential utility of fluorescence lifetime measurements for determination of ribosome structure.

Acknowledgments

We gratefully acknowledge the excellent technical expertise and assistance of G. Zardeneta, R. Evans, M. Rodgers, and K. Pham. We also thank M. A. Henderson for preparation of the typescript and F. Hoffman for the photography and artwork he provided for the figures.

References

Branlant, C., Sri-Widada, J., Krol, A., & Ebel, J.-P. (1977) *Nucleic Acids Res. 4*, 4323–4345.

Crichton, R. R., Engelman, D. M., Haas, J., Koch, M. H. J., Moore, P. B., Parfait, R., & Stuhrmann, H. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5547–5550.

Dulbecco, R., & Smith, J. D. (1960) *Biochim. Biophys. Acta 39*, 358–361.

Förster, T. (1949) *Z. Naturforsch., A 4*, 321–327.

Förster, T. (1951) *Fluoreszenz organischer Verbindungen*, p 312, Vandenhoeck and Ruprecht, Göttingen, Germany.

Förster, T. (1959) *Discuss. Faraday Soc. 27*, 1–17.

Freifelder, D. (1976) *Physical Biochemistry: Applications to Biochemistry and Molecular Biology*, pp 410–441, W. H. Freeman, San Francisco.

Garrett, R. A., & Noller, H. F. (1979) *J. Mol. Biol. 132*, 637–648.

Haas, E., Katchalski-Katzir, E., & Steinberg, I. Z. (1978) *Biochemistry 17*, 5064–5070.

Hänsch, T. W. (1977) in *Dye Lasers* (Schäfer, F. P., Ed.) pp 228–235, Springer-Verlag, Berlin, Heidelberg, and New York.

Hansske, F., Sprinzl, M., & Cramer, F. (1974) *Bioorg. Chem.* 3, 367–376.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897–2905.

Knight, A. E. W., & Selinger, B. K. (1971) *Spectrochim. Acta, Part A 27A*, 1223–1234.

Koch, M. H. J., Parfait, R. Haas, J., Crichton, R. R., & Stuhrmann, H. B. (1978) *Biophys. Struct. Mech. 4*, 251–262.

Lake, J. A. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 207–236, University Park Press, Baltimore.

Nierhaus, K., & Dohme, F. (1979) *Methods Enzymol. 59*, 443–449.

Odom, O. W., Craig, B. B., & Hardesty, B. A. (1978) *Biopolymers 17*, 2909–2931.

Olson, H. M., & Glitz, D. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3769–3773.

Politz, S. M., & Glitz, D. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1468–1472.

Reines, S. A., & Cantor, C. R. (1974) *Nucleic Acids Res. 1*, 767–786.

Rinke, J., & Brimacombe, R. (1978) *Mol. Biol. Rep. 4*, 153–156.

Schreiber, J. P., Hsiung, N., & Cantor, C. R. (1979) *Nucleic Acids Res. 6*, 181–193.

Shatsky, I. N., Mochalova, L. V., Kojouharova, M. S., Bogdanov, A. A., & Vasiliev, V. D. (1979) *J. Mol. Biol. 133*, 501–515.

Stöffler, G. Bald, R., Kastner, B. Lührmann, R., Stöffler-Meilicke, M. Tischendorf, G., & Tesche, B. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 171–205, University Park Press, Baltimore.

Stuhrmann, H. B., Koch, M. H. J., Parfait, R., Haas, J., Ibel, K., & Crichton, R. R. (1978) *J. Mol. Biol. 119*, 203–212.

Traub, P., & Nomura, M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 777–784.

Van Duin, J., Kurland, C. G., Dondon, J., & Grunberg-Manago, M. (1975) *FEBS Lett. 59*, 287–290.

Van Holde, K. E., & Hill, W. E. (1974) *Ribosomes*, [*Meet.*], *1973*, 53–91.

Weber, G., & Teale, F. W. J. (1957) *Trans. Faraday Soc. 53*, 646–655.

Yuan, R. C., Steitz, J. A., Moore, P. B., & Crothers, D. M. (1979) *Nucleic Acids Res. 7*, 2399–2418.

Zimmermann, R. A. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 135–169, University Park Press, Baltimore.