The Origins of Mitochondrial Ribonucleic Acids in *Tetrahymena pyriformis**

Y. Suyama

ABSTRACT: Mitochondria contain two classes of ribonucleic acid (RNA), one soluble RNA (sRNA) and the other RNA referred to as pRNA which can be extracted from the pellet sedimentable after centrifugation of a mitochondrial lysate at 105,000*g* for 90–120 min. The origins of these RNAs were examined by DNA–RNA hybridization studies. The pRNA shows specific hybridization with mitochondrial deoxyribonucleic acid (DNA) up to 6.8% (RNA/DNA × 100), while sRNA does not hybridize to a significant extent. It is concluded that a large portion of mitochondrial sRNA is not transcribed from mitochondrial DNA.

Recent investigations suggest that mitochondria of all organisms contain deoxyribonucleic acid (DNA). Several properties of mitochondria indicate that this DNA is essential for the synthesis of some mitochondrial proteins. Such phenomena as the extrachromosomal inheritance of defects in mitochondria (Ephrussi, 1952; see Wagner and Mitchell, 1955), as well as defects in mitochondrial DNA (Mounolou *et al.*, 1966), the semiautonomous nature of mitochondrial replication (Luck, 1963), the presence of ribonucleic acid (RNA) (Roodyn *et al.*, 1961; Truman and Korner, 1962), as well as DNA-dependent RNA polymerase activity (Kalf, 1964; Luck and Reich, 1964; Wintersberger, 1966), and protein-synthesizing activity in isolated mitochondria (Roodyn *et al.*, 1961, 1962; Truman and Korner, 1962; Kalf and Grece, 1964) support this hypothesis. Furthermore, several lines of investigation suggest that mitochondrial DNA specifies structural protein as well as contractile protein of mitochondria (Roodyn *et al.*, 1962; Kalf and Grece, 1964; Woodward and Munkres, 1966).

To clarify the functional role of mitochondrial DNA in protein synthesis, it is essential to investigate whether RNA found in mitochondria originates from this DNA as template. Work in our laboratory has shown (Y. Suyama and J. Eyer, manuscript in preparation) that isolated mitochondria of *Tetrahymena* synthesize messenger-like RNA, which is specifically produced by mitochondrial DNA as demonstrated by DNA–RNA hybridization studies. Mitochondria also contain active transfer RNA (tRNA) or soluble RNA (sRNA) (Barnett and Brown, 1967; Y. Suyama and J. Eyer, manuscript in preparation) as well as structures similar to ribosomes (Rendi, 1959; Nass *et al.*, 1965; Pollard *et al.*, 1966). Molecular hybridization studies on these structural RNAs would indicate whether or not they are transcribed from mitochondrial DNA. This paper presents evidence that RNA from ribosome-like material from *Tetrahymena* mitochondria hybridizes with mitochondrial DNA, while sRNA does not, to a significant extent.

Materials and Methods

Culture. An axenic culture of a strain of *Tetrahymena pyriformis* previously designated ST (Suyama, 1966) was used throughout these studies. Stock cultures were maintained in culture tubes containing about 5 ml of PP medium (2% proteose–peptone and 0.1% yeast extract). For obtaining experimental cells, a 2-l. erlenmeyer flask containing 300 ml of PP medium was inoculated with 0.5 ml of stock culture and maintained at 28 ± 0.5° for a period of 66–68 hr. In some experiments cultures were obtained from a culture flask containing 500 ml of P medium (1% proteose–peptone) after an incubation period of 90 hr. Both culture methods yield about 1–1.3-ml of packed cells/culture flask in a late exponential or early stationary state of culture growth.

^{32}P Incorporation. [^{32}P]Orthophosphate (Squibb & Sons) was added to a 42-hr-old culture and cells were allowed to grow for an additional 24 hr for PP culture and 48 hr for P culture. A 2.5-mc/PP culture or 0.625-mc/P culture flask was added under sterile conditions. RNA prepared from these cells had an initial specific activity of 1 or 0.5 × 10^4 cpm/μg, respectively, for PP or P culture.

Preparation of Mitochondria. Cells after collecting by an International oil testing centrifuge were resuspended in four times their packed volume of the raffinose medium (0.2 M raffinose, 1 mM potassium phosphate buffer (pH 6.2), and 0.25% bovine serum albumin) and homogenized with a cream homogenizer. Mitochondria were prepared as previously described (Suyama, 1966). Mitochondria washed three times were used throughout these studies.

Isolation of the Bulk Mitochondrial RNA. Mitochondria obtained from about 6 ml of cells were lysed in 2 ml

* From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania. *Received April 10, 1967.* This research was supported by a Contract (AT(304)-3588) from the Atomic Energy Commission.

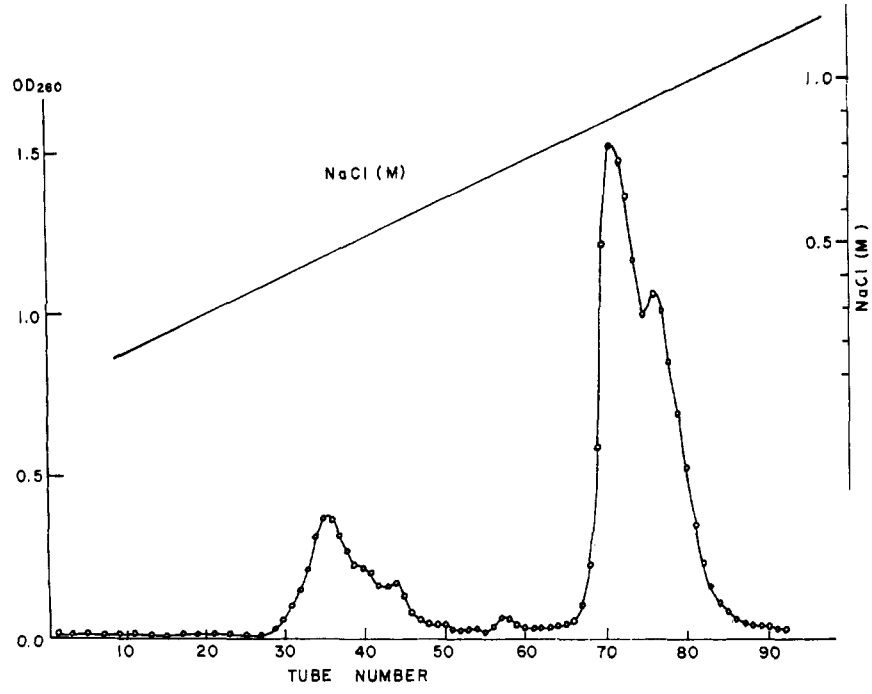

FIGURE 1: Elution pattern of postmitochondrial RNA of *Tetrahymena* on MAK column. RNA (2 mg) was applied to the column, and RNA was eluted by a linear salt gradient from 0.2 to 1.35 N NaCl. NaCl concentration was determined by measuring the refractive index of every tenth fraction.

of 0.01 M acetate buffer (pH 5.1) containing 2% SDS.[1] RNA was isolated by a cold phenol method as follows. The lysate was mixed with an equal volume of water-saturated phenol (Merck & Co.) and the mixture was shaken in the cold for 20 min. This mixture was then centrifuged and the resulting supernatant was treated with phenol again. After centrifugation, the supernatant was mixed with twice its volume of cold ethanol and kept in a freezer for at least 3 hr to ensure the complete precipitation of RNA. The precipitate was redissolved in saline–phosphate buffer (0.1 N NaCl–0.05 M sodium phosphate buffer, pH 6.7) and phenol was extracted with ether.

RNA thus isolated shows sedimentation values of 18, 14, and 4 S in a ratio of approximately 1:1:1. Additions such as Bentonite (Frankel-Conrat *et al.*, 1961), Macaloid (Stanley and Bock, 1965), Mg^{2+}, and changes in isolation medium (10^{-2} M Tris-HCl, pH 7.2) and condition (60° phenol treatment such as used by Scherrer and Darnell (1962)) do not change the sedimentation pattern of this RNA greatly.

Isolation of Postmitochondrial RNA. Cells suspended in four times their packed volume of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.01 M $MgCl_2$ and 0.5% Macaloid were forced through a cream homogenizer. The homogenate was spun for 15 min at 20,000*g*. To the supernatant, $^1/_{20}$ its volume of 10% SDS and an equal volume of water-saturated phenol were added. A flask containing this mixture was immersed in a 60° water bath and shaken vigorously for a period of 3 min as described (Scherrer and Darnell, 1962). The mixture was then cooled rapidly by immersing the flask in an ice bath. The cooled mixture was then centrifuged for 2 min at 2000*g* in the cold. The supernatant was carefully pipetted out and the phenol treatment was repeated twice on this material. RNA was precipitated from solution with one-ninth its volume of 1.8 N NaCl plus two times its volume of cold ethanol. The precipitate was collected and dissolved in saline–phosphate buffer. Residual phenol was removed by ether extraction and ether was expelled by bubbling filtered air through the solution. The final solution was usually turbid, presumably due to glycogen contamination, most of which could be removed by a centrifugation of 1 hr at 39,000*g*.

In order to obtain competitor sRNA and ribosomal RNA (rRNA) used for hybridization studies (see Results), the above RNA solution was treated with DNase (20 μg/ml, 5 mM Mg^{2+}) and chromatographed on methylated albumin-coated Kieselguhr (MAK) column (Sueoka and Cheng, 1962). A typical elution profile of postmitochondrial RNA is presented in Figure 1. The first peak eluting at about 0.5 N NaCl is largely sRNA species, and the second and third peaks eluting close together at higher salt concentrations represent two rRNA species. In *Escherichia coli*, 16S and 23S rRNA can be separated by MAK column chromatography into two peaks, the first eluting peak representing 16 S and

[1] Abbreviations used: SDS, sodium dodecyl sulfate; MAK, methylated albumin-coated Kieselguhr; DOC, sodium deoxycholate.

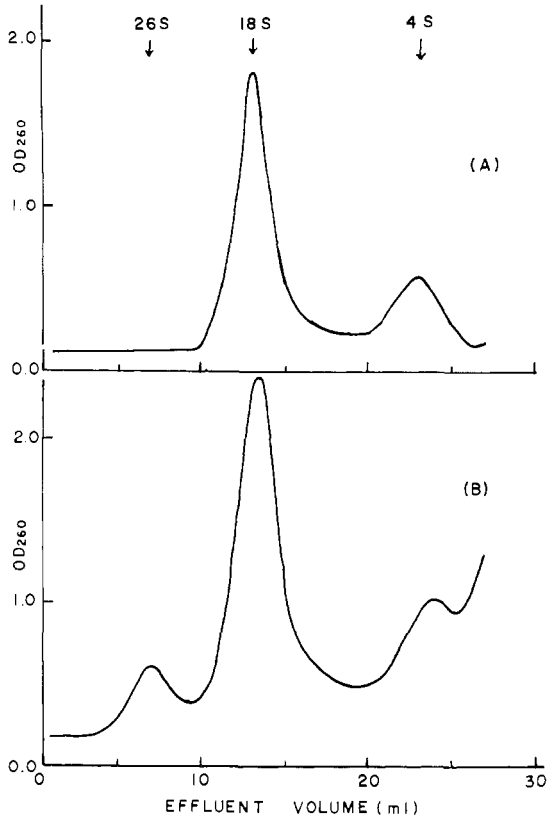

FIGURE 2: Sucrose gradient centrifugation of purified postmitochondrial RNA (A) and postmitochondrial extract lysed with 1% SDS (B). A linear sucrose gradient from 5 to 20% made in 0.01 M Tris-HCl (pH 7.4), 10^{-2} M Na_2EDTA, 0.015 M NaCl, and 1% SDS was used. Centrifugation was made in a Spinco Model L centrifuge at a temperature setting of 15° at an average speed of 25,000 rpm for 15 hr. The continuous OD_{260} profiles were obtained by monitoring the contents through a flow cell (path length/volume = 10 mm/0.10 ml). Postmitochondrial lysate (1.5 ml) and 9.3 OD_{260} of purified postmitochondrial RNA were layered over 28-ml sucrose gradients.

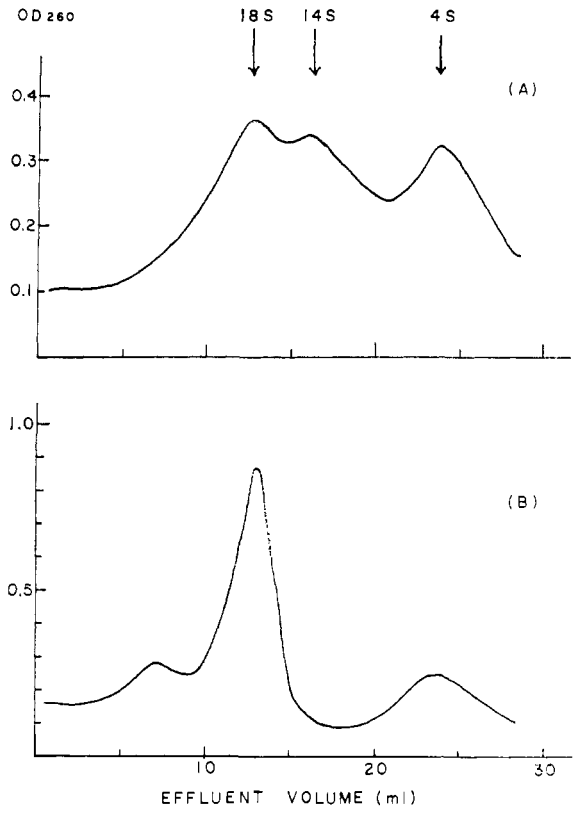

FIGURE 3: Sucrose gradient centrifugation analysis on bulk mitochondrial RNA (A) purified from washed mitochondria and whole-cell RNA (B) run as control. A sample (1.5 ml) was layered over a 28-ml volume linear 5–20% sucrose gradient in 0.01 M Tris-HCl (pH 7.4), 0.015 N NaCl, 0.01 M EDTA, and 1% SDS, and tubes were centrifuged for 15 hr at an average speed of 25,000 rpm.

the late peak 23 S, with RNA in the late peak twice as much as that in the first peak in quantity (Sueoka and Cheng, 1962). On the other hand, in *Tetrahymena* the situation is not quite the same. Both rRNAs resolved by MAK column chromatography show a sedimentation value of 18 S as determined by sedimentation velocity analysis in the presence of 10^{-2} M Mg^{2+}. These sRNA and 18S rRNA fractions isolated by MAK column chromatography were used for hybridization studies.

Various methods of isolation of RNA from *Tetrahymena* cells have so far failed to produce a typical rRNA pattern such as 28 (23 S) and 18 S (16 S) in a ratio of 2:1 in quantity. However, the presence of about 10% of 26S RNA can be demonstrated in RNA extracted from postmitochondrial or whole-cell extracts by phenol extraction in the cold or by direct sucrose gradient sedimentation analysis (Figure 2). Again, the use of Bentonite or dextran sulfate (1 mg/ml) does not modify this rRNA pattern. Ages of culture as well as times (1–30 min) allowed for collecting cells before preceding to RNA isolation steps do not alter the picture. From these observations, I maintain the opinion that a large portion of ribosomes in *Tetrahymena* cells raised in the present condition are accessible to nuclease attack *in vivo*, so that 28S rRNA is already cut at a specific point as postulated to occur *in vitro* during isolation of ribosomes (see Peterman, 1964).

Isolation of DNA. Tetrahymena mitochondrial DNA (ρ 1.686 g/ml) was purified as described previously (Suyama, 1966). *Tetrahymena* nuclear DNA (ρ 1.692 g/ml) was purified as follows. A cell suspension made in 20 times its packed volume of saline-EDTA (0.15 M NaCl–0.1 M EDTA, pH 8.0) was crushed in a cream homogenizer and the homogenate was centrifuged at 10,000*g* for 10 min to remove the bulk cellular debris and mitochondria. The supernatant was treated with saline-EDTA-saturated phenol in the cold for 20

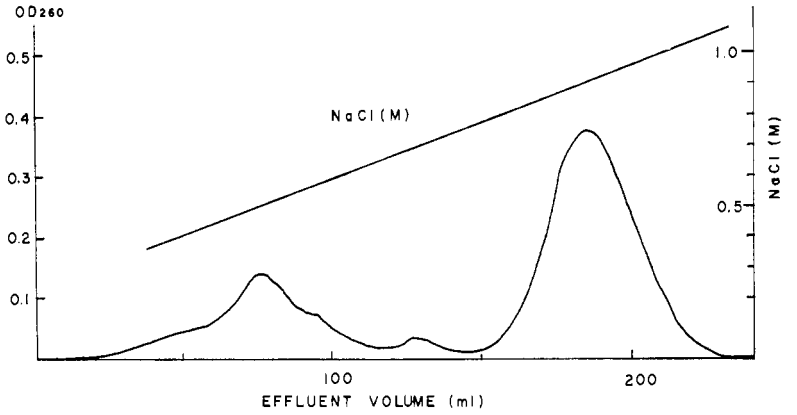

FIGURE 4: MAK column chromatography of bulk mitochondrial RNA isolated from washed mitochondria as described (see Methods). RNA was eluted with a linear salt gradient made with 130 ml each of 0.3 and 0.9 N NaCl–sodium phosphate buffer (0.05 M, pH 6.5). The continuous OD_{260} profile was obtained by monitoring the effluent through a flow cell (path length/volume = 10 mm/0.50 ml) in a Beckman DB spectrophotometer with an attached recorder.

min by shaking. The emulsified mixture was centrifuged and the supernatant was extracted. DNA was precipitated by two times its volume of cold ethanol and subsequent centrifugation. The DNA redissolved in 1 times SSC (0.15 N NaCl–0.015 M sodium citrate) was treated with ribonuclease (RNase) (20 μg/ml for 20 min at room temperature) and again treated with phenol to remove RNase. DNA was then dialyzed against 1 times SSC. *Bacillus subtilis* DNA was isolated by the methods of Saito and Miura (1963). All DNAs were repurified by MAK column chromatography, dialyzed against 0.01 times SSC, and stored in a freezer until used.

Tetrahymena mitochondrial DNA was free of nuclear DNA and *vice versa*, as determined by CsCl density gradient equilibrium centrifugation. Detection of any cross contamination below 3% of the total DNA is difficult by this method, because of the closeness of the densities of these DNAs. Although the actual contamination might be less than the limit of detection, even 3% contamination of mitochondrial DNA with nuclear would not greatly hamper our experimental analysis for the present studies. Therefore, no further effort was taken to improve the method of detection of contaminating DNA or to more thoroughly eliminate any contamination.

DNA–RNA Hybridization. DNA–RNA hybridization technique of immobilized DNA on membrane filters (Gillespie and Spiegelman, 1965) was employed. A hybridization temperature of 64 or 60° and 2 or 6 times SSC were used. DNAs in 0.01 times SSC were denatured at pH 12.5 and after neutralization heated for 10 min in a 98° water bath and quickly cooled in an ice-water bath. Denaturation of DNAs was ascertained by CsCl density gradient equilibrium centrifugation. DNAs on membrane filters (BacT, B6, Schleicher & Schuell) were quantitated by monitoring the optical density at 260 mμ of the DNA solution as well as of filtrate. Over 73% of DNA was retained on the filter.

Concentrations. Protein determinations were made by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. The RNA determination was made by the orcinol assay of Dische (1955) using D-ribose as the standard. For estimating the radioactive RNA, 24 OD_{260} was assumed to correspond to 1 mg of RNA/ml. DNA was estimated from absorbance at 260 mμ assuming 1 OD_{260} = 50 μg/ml for native DNA and 1 OD_{260} = 36 μg/ml for denatured DNA.

Results

Distribution of Nucleic Acids in Different Fractions of Mitochondrial Lysate. A lysate of mitochondria made in Tris–DOC medium (10^{-2} M Tris-HCl (pH 7.2), 3×10^{-3} M $MgCl_2$, and 0.5% deoxycholate) was centrifuged to remove unlysed material and some debris. This pellet contains no nucleic acid. The supernatant was further centrifuged at 105,000*g* for 2 hr. Distributions of RNA, DNA, and protein are presented in Table I. Almost 100% of DNA was solubilized, while about 75–80% of the total RNA remained in the pellet after 105,000*g* centrifugation. A lower centrifugation speed (34,000*g* for 30 min) of the lysate sediments only 6.8% of the total sedimentable RNA. Sodium dodecyl sulfate at a concentration of 0.5% completely solubilizes these pellet materials.

Characterization of Mitochondrial RNA. RNA isolated by the cold phenol method (see Methods) was analyzed by sucrose gradient centrifugation analysis (Figure 3). The heavy peak corresponds to 18 S, the middle 14 S, and the lightest 4 S. MAK column fractionation was carried out on the same RNA preparation (Figure 4). The first peak, eluting at about 0.5 N NaCl, corresponds to the 4S peak of the sucrose gradient and contains tRNA, since it was shown to be chargeable with leucine (Y. Suyama and J. Eyer, manuscript in preparation). The second, minor peak represents DNA.

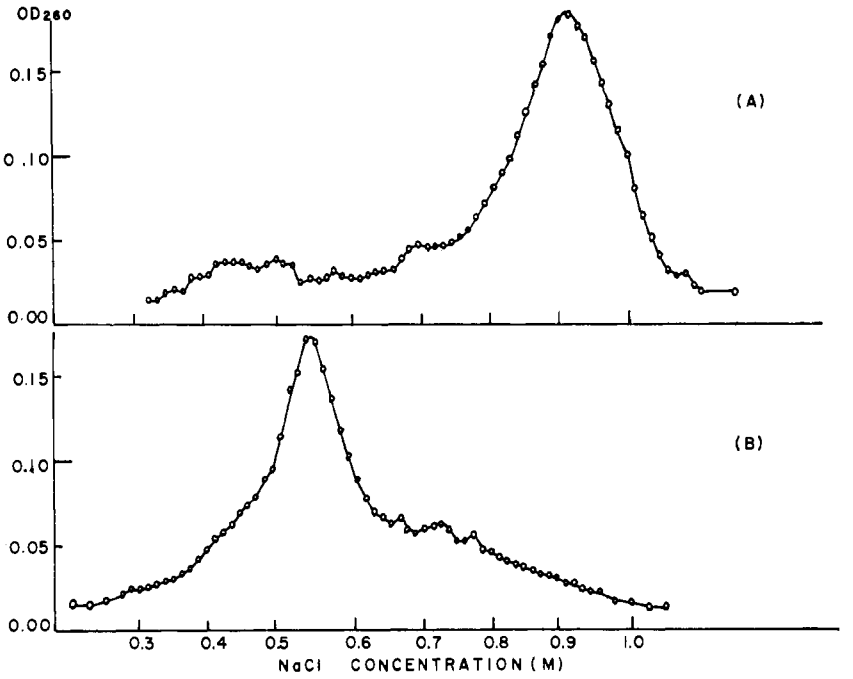

FIGURE 5: MAK column chromatography of RNA isolated from a 105,000*g* pellet (A) and the supernatant (B) obtained from mitochondria lysed with 0.5% DOC in 3×10^{-3} M $MgCl_2$–0.01 M Tris-HCl (pH 7.4) buffer.

The third peak, eluting at about 0.9 N NaCl, is in the same position as rRNA in a postmitochondrial RNA preparation, and is the same as RNA which can be extracted from the 105,000*g* sedimenting material of a mitochondrial lysate. This conclusion was obtained from separate MAK column fractionations of RNAs isolated from the 105,000*g* supernatant and pellet fractions (see Table I). The supernatant RNA was eluted from a column at low salt concentration, while the pellet RNA was eluted at higher salt concentrations (Figure 5). The pellet RNA, whose sedimentation values were determined to be 18 and 14 S, behaves on MAK column in a manner similar to rRNA from higher organisms (Ritossa *et al.*, 1966). There is no clear separation into two peaks as seen in chromatographic elution profiles of *Tetrahymena* postmitochondrial rRNA as well as those of *E. coli* rRNA.

TABLE I: Distribution of Nucleic Acids in Different Fractions of Mitochondrial DOC Lysate after Centrifugation for 2 hr at 105,000*g*.[a]

	I (mg)			II (mg)	
Fractions	Protein	RNA	DNA	Protein	RNA
105,000*g* supernatant		0.42	0.16	259	2.0
105,000*g* pellet		1.89	0.02	124	4.0
Total	130	2.3	0.16	383	6.0

[a] Mitochondria were lysed in Tris–DOC buffer. The lysate was spun at 10,000*g* for 10 min to remove debris. The supernatant was further centrifuged at 105,000*g* for 2 hr. Protein was determined by the method of Lowry *et al.* (1951). RNA and DNA contents were determined by the orcinol method of Dische (1955) and indole method of Keck (1956) after the lysates were treated as described (Volkin and Cohn, 1954).

The high protein to RNA ratio of the 105,000*g* pellet material of mitochondrial lysate and unusual sedimentation values (18 and 14 S) of RNA extractable from this material make it difficult to compare this RNA to rRNA isolated from generally accepted ribosomal particles of bacteria as well as of higher organisms. However, MAK column chromatography separates two classes of RNAs, one, sRNA, and the other, RNA bound to the 105,000*g* pellet. The latter RNA will be referred to as mitochondrial pellet RNA (pRNA) in this paper for simplifying the identification of this particular class of RNA. The origins of these RNAs were then examined by DNA–RNA hybridization studies.

Hybridization of Mitochondrial RNA and DNA. A MAK column chromatography elution pattern of bulk ^{32}P-labeled RNA isolated from mitochondria is presented in Figure 6. The figure indicates also those areas from which different classes of RNAs were separately pooled, reisolated, and used in the following hybridization studies. Fractions 1 and 2 represent sRNA and 3

TABLE II: Hybridization of Mitochondrial RNA with Mitochondrial, Nuclear, and *B. subtilis* DNA.[a]

DNA Immobilized on Membrane	[^{32}P]RNA (μg)	Cpm Bound	Sp Act.[b] (cpm)	% (RNA/DNA)
Mitochondrial	No. 1 (21)	608	512	0.51
Nuclear	No. 1 (21)	146	49	0.049
B. subtilis	No. 1 (21)	96		
Mitochondrial	No. 2 (15)	1192	1049	1.0
Nuclear	No. 2 (15)	270	127	0.13
B. subtilis	No. 2 (15)	143		
Mitochondrial	No. 3 (23)	4978	3332	3.3
Nuclear	No. 3 (23)	2359	714	0.71
B. subtilis	No. 3 (23)	1645		
Mitochondrial	No. 4 (23)	4546	3711	3.7
Nuclear	No. 4 (23)	981	146	0.15
B. subtilis	No. 4 (23)	835		

[a] Immobilized DNA membrane filters were prepared by the methods of Gillespie and Spiegelman (1965). Each DNA (20 μg) was loaded on a membrane filter. A vial containing 5 ml of [^{32}P]RNA (5000 cpm/μg) in 2 times SSC as indicated in the table was incubated at 64° for a period of 10 hr. RNAs numbered 1–4 indicate RNA as prepared from MAK column chromatography (see Figure 6). [b] Counts per minute bound on *B. subtilis* DNA membranes were subtracted.

TABLE III: Competition Experiments between Mitochondrial [^{32}P]RNA and Postmitochondrial Nonradioactive RNA for Hybridization with Mitochondrial RNA.[a]

DNA Immobilized on Membrane	[^{32}P]RNA (μg)	Postmitochondrial RNA (μg)	Sp Act.[b]	% (RNA/DNA)
Mitochondrial	No. 2 (4.5)	sRNA (15)	352	0.35
Nuclear	No. 2 (4.5)	sRNA (15)	34	0.035
Mitochondrial	No. 2 (4.5)	None	283	0.28
Nuclear	No. 2 (4.5)	None	35	0.035
Mitochondrial	No. 3 (6.4)	rRNA (31.5)	1545	1.5
Nuclear	No. 3 (6.4)	rRNA (31.5)	305	0.31
Mitochondrial	No. 3 (6.4)	None	1566	1.6
Nuclear	No. 3 (6.4)	None	193	0.19

[a] Immobilized membrane filters were prepared by the methods of Gillespie and Spiegelman (1965). Each DNA (20 μg) was loaded on a membrane filter. A vial containing 2 ml of [^{32}P]RNA (5000 cpm/μg) and postmitochondrial nonradioactive RNA as indicated was incubated at 64° for 7.5 hr. For RNAs numbered 2 and 3, see Table II and Figure 6. [b] Counts per minute bound on *B. subtilis* DNA membranes were subtracted.

and 4 pRNA, but separations into these fractions are arbitrary.

The results of the hybridization experiments are shown in Table II. Nuclear DNA and *B. subtilis* DNA were used as controls in these experiments. The results show that all the fractions of mitochondrial RNA exhibited higher percentages of hybridization with mitochondrial DNA than nuclear DNA. Relatively high noise level encountered in this experiment, which presumably resulted from insufficient washing of MAK column (Gillespie and Spiegelman, 1965), makes it difficult to evaluate the count associated with nuclear DNA membrane filters. At this time, because of this high noise level as well as relatively low specific activity of these RNAs, investigation on the hybridization with nuclear DNA was not pursued further. However, as it will be shown in a later section, the pellet RNA hybridized specifically with mitochondrial DNA.

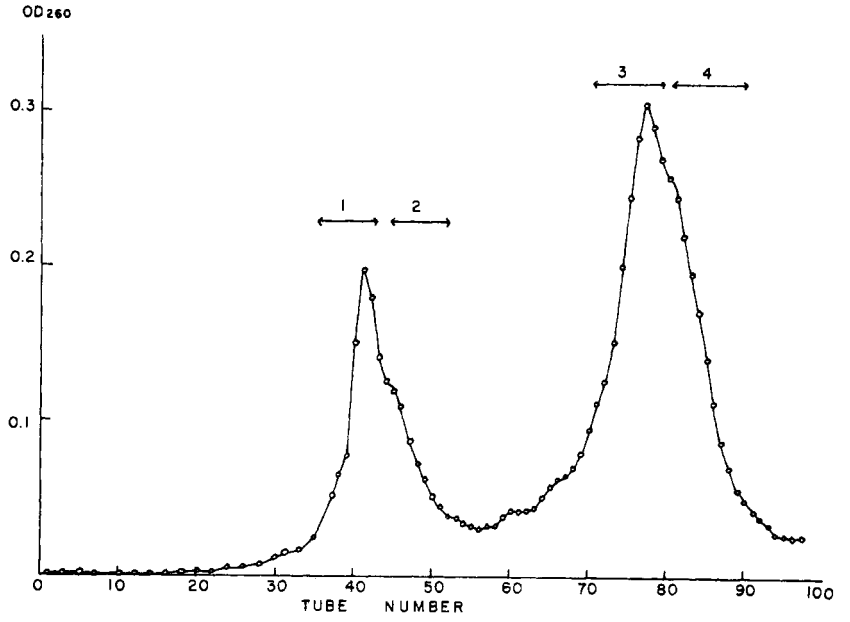

FIGURE 6: Elution pattern of mitochondrial ^{32}P-labeled RNA isolated from cells grown in ^{32}P–P medium. RNA was eluted by a linear salt gradient from 0.1 to 1.35 N NaCl. Underlined areas 1–4 were reisolated and used for hybridization studies after dialyzing against 2 times SSC (see Tables II and III).

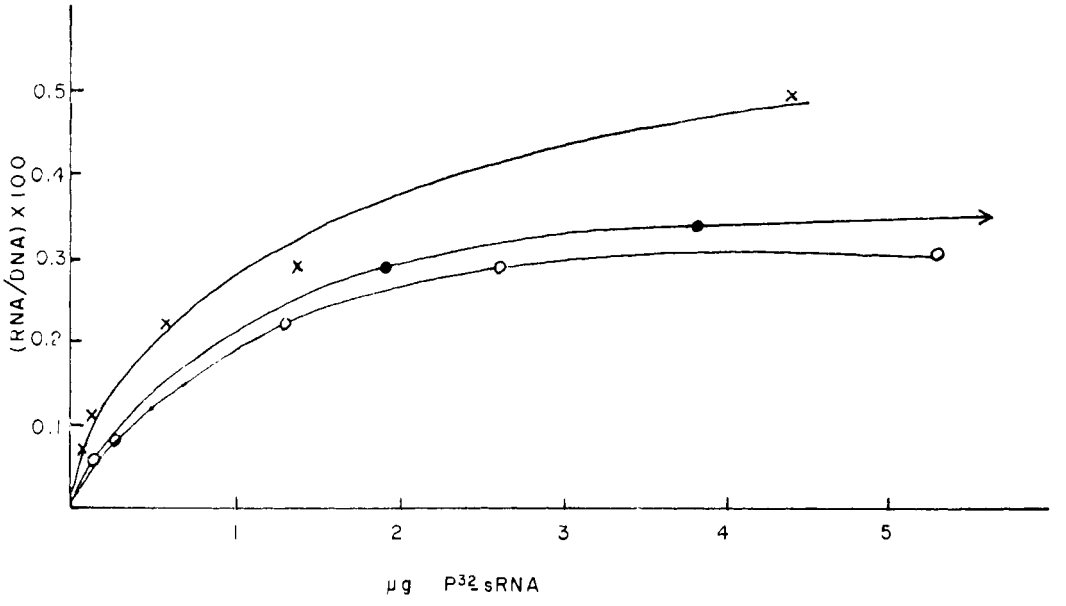

FIGURE 7: Hybridization of mitochondrial DNA with three independently isolated sRNA preparations. (×—×) [^{32}P]-sRNA isolated from 105,000*g* supernatant of mitochondrial lysate (7900 cpm/μg RNA). (●—● and ○—○) sRNA isolated directly from mitochondria by hot (60°) and cold phenol methods, respectively. Specific activities are 2500 and 4500 cpm/μg of RNA, respectively. An arrow indicates that the hybridization continues up to 38 μg of sRNA and 0.7% (RNA/DNA × 100).

In order to demonstrate that the hybridization of mitochondrial RNA with the mitochondrial DNA is a specific reaction, a competition experiment was carried out as follows. If the hybridization occurring between mitochondrial sRNA and pRNA and mitochondrial DNA is due to contaminating cytoplasmic (or post-mitochondrial) components, an appropriate amount of nonradioactive cytoplasmic RNA added to the hy-

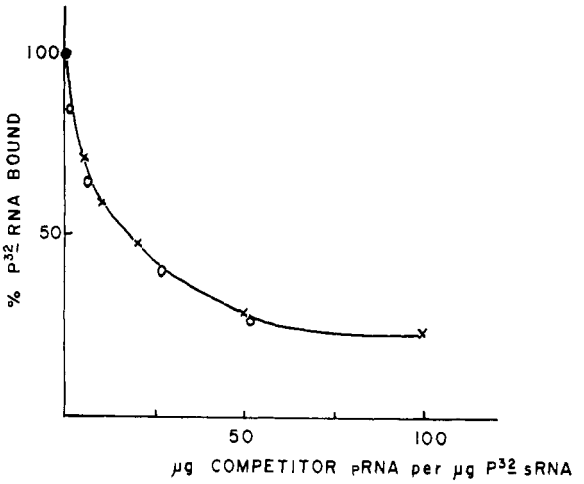

FIGURE 8: Competitive displacement of [^{32}P]sRNA by nonradioactive pRNA. (×) DNA membrane filter (10 μg) and 2.6 μg of [^{32}P]sRNA in 3 ml of 6 times SSC were incubated at 60° for 11 hr. (○) DNA membrane filter (10 μg) and 1.4 μg of [^{32}P]sRNA in 3 ml of 6 times SSC were incubated at 60° for 20 hr. These [^{32}P]RNA preparations were the same as those used in Figure 7 with corresponding markings.

bridization mixture should competitively displace radioactive RNA and thus apparent inhibition of hybridization should result. The results of such experiments show that binding of mitochondrial sRNA and pRNA to mitochondrial DNA was not inhibited by the corresponding postmitochondrial components, suggesting that these are specific products of mitochondrial DNA (Table III).

sRNA Hybridization. The mitochondrial sRNA fraction, separately isolated after repeated deoxyribonuclease (DNase) treatment and MAK column chromatography (DNA contamination level is below 0.5% as determined by 0.3 N KOH hydrolysis for 3 hr at 37°), hybridizes increasingly with increasing concentrations of sRNA. The results of three independent experiments run with three independently isolated sRNA preparations are presented in Figure 7.

It should be pointed out that DNA–RNA hybridization experiments carried out in other laboratories (Gillespie and Spiegelman, 1965; Ritossa *et al.*, 1966) demonstrated that a few micrograms of rRNA or sRNA are sufficient to establish a saturation plateau of about 0.5% RNA/DNA. Therefore, in the present studies, 38 μg of sRNA used in one experiment should have certainly brought a saturation plateau for sRNA at the level of at least 2–3% RNA/DNA. Contrary to this expectation, the observed hydridization was only 0.7% RNA/DNA and a saturation plateau was not reached.

This rather inefficient hybridization might be explained if this sRNA preparation contains a small amount of contaminating material which continues to hybridize after sRNA itself reached a plateau. As it has been shown to occur in other systems (Ritossa *et al.*, 1966), the present sRNA fraction was also shown to be contaminated with degraded rRNA (here pRNA). When varying concentrations of unlabeled mitochondrial pRNA were added to the hybridization vials, ^{32}P count bound on membrane filters diminished exponentially (Figure 8). Almost 80% of the total hybridization was inhibited at the ratio of cold mitochondrial pRNA to [^{32}P]sRNA of 100. This result means that, if a saturation for sRNA was reached at 38 μg of the sRNA preparation, at least 80% or more of the 0.7% hybridization value would be accounted for by contaminating pRNA, and therefore the remaining 20% of 0.7%, 0.14% is considered to be the maximum meaningful hybridization of sRNA species of mitochondria. As will be discussed in a later section, this value is too low for a significant number of sRNA species to be transcribed from mitochondrial DNA.

But the possibility cannot be ruled out, however, that a very small portion of the sRNA preparation represents sRNA transcribed from mitochondrial DNA and a major portion of it nonhybridizable sRNA transcribed from nuclear DNA. In order to elucidate this point, it is necessary to carry out a hybridization experiment with a very large quantity of [^{32}P]sRNA combined with nonlabeled pRNA as competitor. The relatively small amount of sRNA recoverable from mitochondria has made such an experiment impractical at present. However, it is possible to obtain an estimate on the limit of detection of the amount of sRNA transcribed from mitochondrial DNA, if such RNA exists. As will be shown in the following section, approximately 3% hybridization (RNA/DNA × 100) was attained when 5 μg of [^{32}P]pRNA was used in a hybridization vial, while only 0.3% hybridization was obtained with the sRNA preparation at 5 μg in a vial. This means that at most 10% pRNA contamination in the sRNA preparation would account for the hybridization pattern obtained with this sRNA preparation. If we assume that the hybridization efficiency of sRNA is not too different from that of pRNA, at least 20% of this hybridizable material, 2% of the total, might represent the maximum amount of hybridizable sRNA that could be present in the sRNA preparation. Since about 25% of the total mitochondrial RNA is sRNA (Table I), only 0.5% of the total mitochondrial RNA could be sRNA made by mitochondrial DNA.

pRNA Hybridization. The major problem in the hybridization between pRNA and DNA is that the pRNA fraction obtained by MAK column chromatography might be contaminated with messenger RNA (mRNA), although previous studies (Y. Suyama and J. Eyer, manuscript in preparation) have demonstrated that pRNA does not compete with RNA synthesized *in vitro* in isolated mitochondria, which is probably mRNA. In order to improve the purity of pRNA further, RNA was extracted from the carefully rinsed pellet obtained after centrifugation of mitochondrial lysate for 90 min at 105,000*g* as described. Hybridization experiments were performed on this RNA after further purification with DNase treatment followed by MAK

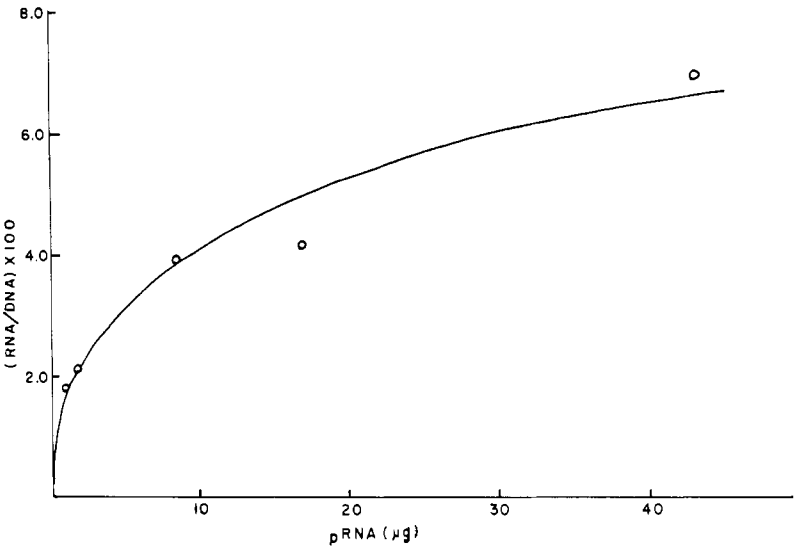

FIGURE 9: Hybridization of mitochondrial DNA with 105,000*g* pellet [^{32}P]pRNA obtained from mitochondrial lysate. DNA membrane filter (4.5 μg) and varying amounts of [^{32}P]pRNA in 5 ml of 2 times SSC were incubated for 22 hr at 64°. Specific activity of this RNA was 7900 cpm/μg.

TABLE IV: Hybridization of 105,000*g* Pellet RNA with Mitochondrial, Nuclear, and *B. subtilis* DNA.[a]

DNA Immobilized on Membrane	Cpm of [^{32}P]RNA (μg)	Cpm Bound	Sp Act.[b] (μg of RNA)	% DNA Bound
Mitochondrial	335,750 (42.5)	2,660	2,440 (0.31)	6.8
	134,300 (17)	1,600	1,454 (0.18)	4.1
Nuclear	335,750 (42.5)	145		
	134,300 (17)	75		
B. subtilis	335,750 (42.5)	220		
	134,300 (17)	146		

[a] Each DNA (4.5 μg) was loaded on a membrane filter. Each hybridization vial contained the indicated amount of RNA in 5 ml of 2 times SSC. Hybridization temperature at 64° and a 22-hr incubation period were employed. Specific activity of pRNA = 7900 cpm/μg of RNA. [b] Counts per minute bound on *B. subtilis* DNA membranes were subtracted.

column chromatography (alkaline-resistant ^{32}P count of this RNA was below 0.04%). This RNA shows specific hybridization with mitochondrial DNA (Table IV), and binding of this pRNA to DNA increased with increasing concentration of pRNA. The maximum level of hybridization so far attained was 6.8% RNA/DNA (Figure 9).

Although there is no critical test to prove that this pRNA is indeed free of other RNA such as mRNA, two lines of investigation pursued so far might be pertinent. First, mitochondrial pRNA collected from late stationary culture of *Tetrahymena* cells, which are presumably less active in mRNA synthesis, exhibits similarly extensive hybridization. Secondly, pRNA purified from the pellet sedimented at 105,000*g* from the mitochondrial lysate to which 1.5 μg of RNase/ml was added before centrifugation, showed a linear increase of hybridization up to 2.2% RNA/DNA at 3.3 μg of pRNA/vial with a membrane filter containing 10 μg of DNA. Although some degradation of pRNA was noted with this RNase treatment, the hybridization value with the remaining pRNA is comparable to that obtained with the pellet RNA without the treatment (see Figure 9). Furthermore, it can be shown that the same RNase treatment solubilizes the RNA component (mRNA) which is synthesized with mitochondria *in vitro*. Therefore, these results appear to support the notion that the pRNA which is hybridizable to such a high degree

TABLE V: Nucleic Acids Contents of *Tetrahymena* Mitochondria and *E. coli* Cells.

Nucleic Acid	*E. coli*[a]		Mitochondria[b]	
	mg/100 mg of Protein	Approx No./Cell	mg/100 mg of Protein	Approx No./ Mitochondrion
DNA	4.5		0.12	39[c]
RNA	27		1.5	
rRNA (16 S + 23 S)	22	3×10^4	1.1	2×10^{3}[d]
sRNA	5	4×10^5	0.0075[e]	1×10^3
			0.37[f]	5×10^4

[a] Data modified after Watson (1965). [b] Mitochondrial protein (1 mg) corresponds to 2×10^9 mitochondria. [c] A molecular weight of mitochondrial DNA is assumed to be 10×10^6 Daltons. [d] A molecular weight of pRNA is assumed to be 2×10^6 Daltons. [e] An estimate of sRNA transcribed from mitochondrial DNA as based on 0.5% of the total RNA (see text). [f] A total sRNA which corresponds to about 25% of the total mitochondrial RNA (Table I).

represents a stable and unique structural RNA similar to rRNA commonly found in other organisms.

Discussion

The present studies demonstrate that mitochondria of *Tetrahymena* cells contain a RNA species highly hybridizable with mitochondrial DNA. Since this RNA is a major RNA component found in the pellet obtained by centrifugation of mitochondrial lysate at 105,000*g* for 90–120 min and since it does not compete with messenger-like RNA synthesized *in vitro*, it appears to be bound to a structure analogous to the ribosome found in every organism. The apparently high protein to RNA ratio of this particle (see Table I), unlike the regular ribosomes, might be explained by the presence of certain submitochondrial particles such as EP (Fernández-Morán *et al.*, 1964) in this fraction.

However, this RNA with sedimentation properties of 18 and 14 S is not comparable to RNA species generally isolatable from ribosomes of bacteria or any other organisms. But it would not be surprising if these unusual RNAs result from degradation during isolation of mitochondria, since *Tetrahymena* is known to have highly active nucleases. The possibility cannot be ruled out, however, that the rRNAs of mitochondria are not like those of whole organisms, since similar RNA species (19 and 15 S) are reported to occur in *Euglena* chloroplast ribosomes (Brawerman and Eisenstadt, 1964).

In spite of the above difficulties, if one accepts that these 18S and 14S RNAs are rRNA, the hybridization efficiency is extremely high compared to values reported in other organisms (Gillespie and Spiegelman, 1965; Ritossa *et al.*, 1966; McConkey and Hopkins, 1964). In *E. coli* and *Drosophila*, about 0.3% hybridization was reported for rRNA, while in the present system 6.8% (or a higher value) is obtained. However, this is not surprising, since DNA of mitochondria is reported to be very small. For example, it was reported (Kroon *et al.*, 1966; Nass, 1966; Sinclair and Stevens, 1966) that several mammalian mitochondria all contain approximately 10 million molecular weight DNA. We have previously reported that mitochondrial DNA of *Tetrahymena* has a sedimentation value of 41 S (Suyama, 1966) and this value is comparable to the same molecular weight range reported in other organisms if a supercoiled ring structure is assumed (Kroon *et al.*, 1966).

If this DNA is the only DNA species within the mitochondrion, which then directs the synthesis of rRNA of 18 and 28 S whose molecular weights would be roughly 0.6 and 1.4×10^6 (Peterman, 1964), a hybridization up to 20% (RNA/DNA $\times$ 100) would be expected. Conversely, the 6.8% value obtained here would give a value of 30×10^6 mol wt DNA. However, uncertainties about the molecular weight of mitochondrial pRNA and the maximum hybridization efficiency in the present case make it impossible to obtain an accurate estimate on the molecular weight of DNA, but the estimated value, 30×10^6 mol wt computed as above, should be an absolute maximum for the molecular weight of the species of mitochondrial DNA in *Tetrahymena*, if the pRNA region on the DNA is not redundant. Variations in the foregoing assumptions, such as molecular weights of pRNA smaller than 2×10^6 and hybridization efficiency higher than 6.8%, would result in a smaller molecular weight estimate for mitochondrial DNA.

Now, we turn our attention to sRNA of mitochondria. It has been reported that mitochondria of *Neurospora* (Barnett and Brown, 1967) contain at least 18 different tRNAs. *Tetrahymena* mitochondria contain leucyl-tRNA which appears to be different from the corresponding cytoplasmic tRNA. The present studies however demonstrate that although the tRNA of mitochondria hybridizes with mitochondrial DNA this hybridization is largely due to contaminating pRNA. From the reasoning above, it appears that only 0.14% or less hybridization between sRNA and DNA is significant. Since the molecular weight of a tRNA molecule is 25,000 Daltons (Cantoni, 1966), 0.14% DNA binding means that just one or two tRNA species can be transcribed from a 30×10^6 mol wt DNA species. This

clearly shows that mitochondria are largely deficient in their own tRNA. Furthermore, since the 0.14% DNA binding represents the maximum limit, it is possible that mitochondrial DNA does not code for any tRNA.

From the present experimental data, however, it is not possible to eliminate the alternate possibility that mitochondria contain a small amount of a variety of tRNA species transcribed from mitochondrial DNA. It can be estimated that roughly 0.5% of the total mitochondrial RNA could be sRNA made by mitochondrial DNA. This quantity is below the 1% limit of detection for synthesis experiments, and helps to explain why sRNA synthesis could not be demonstrated with mitochondria *in vitro* (Y. Suyama and J. Eyer, manuscript in preparation).

Table V presents a summary of nucleic acid contents in mitochondria in comparison to those in *E. coli* cells. Mitochondria contain comparatively small amounts of nucleic acids. Particularly, the estimated content of sRNA transcribed from mitochondrial DNA is very low as compared to the estimate for *E. coli* cells. Whether or not this sRNA indeed exists and serves as an adapter for protein synthesis in mitochondria can not be determined at this time.

However, in view of the existence of a relatively large amount of sRNA in mitochondria, it is conceivable that a specific sRNA transport mechanism is operating in association with mitochondria. Although the present studies do not establish any specific hybridization between this sRNA and nuclear DNA, a logical hypothesis to explain the present situation would be that mitochondrial sRNA is largely transcribed from nuclear DNA and transported into mitochondria.

Acknowledgment

The author thanks Mr. Joe Eyer for his excellent technical assistance and also for critical reading of the manuscript.

References

Barnett, W. E., and Brown, D. H. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 452.

Brawerman, G., and Eisenstadt, J. M. (1964), *J. Mol. Biol. 10*, 403.

Cantoni, G. L. (1966), *in* Molecular Architecture in Cell Physiology, Hayashi, T., and Szent-Gyorgyi, A. G., Ed., Englewood Cliffs, N. J., Prentice-Hall, p 147.

Dische, Z. (1955), *in* the Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, p 285.

Ephrussi, B. (1952), Nucleo-cytoplasmic Relations in Microorganisms, Oxford, England.

Fernández-Morán, H., Oda, T., Blair, P. V., and Green, D. E. (1964), *J. Cell Biol. 22*, 63.

Frankel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.

Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.

Kalf, G. F. (1964), *Biochemistry 3*, 1702.

Kalf, G. F., and Grece, M. A. (1964), *Biochem. Biophys. Res. Commun. 17*, 674.

Keck, K. (1956), *Arch. Biochem. Biophys. 63*, 446.

Kroon, A. M., Borst, P., van Bruggen, E. F. J., and Ruttenberg, G. J. C. M. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1836.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. T. (1951), *J. Biol. Chem. 193*, 265.

Luck, D. J. L. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 233.

Luck, D. J. L., and Reich, E. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 931.

McConkey, E. H., and Hopkins, J. W. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1197.

Mounolou, J., Jakob, H., and Slonimski, P. (1966), *Biochem. Biophys. Res. Commun. 24*, 218.

Nass, M. M. K. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1215.

Nass, M. M. K., Nass, S., and Afzelius, B. A. (1965), *Exptl. Cell Res. 37*, 516.

Peterman, M. L. (1964), The Physical and Chemical Properties of Ribosomes, New York, N. Y., Elsevier.

Pollard, C. J., Stemler, A., and Blaydes, D. F. (1966), *Plant Physiol. 41*, 1323.

Rendi, R. (1959), *Exptl. Cell Res. 17*, 585.

Ritossa, F. M., Atwood, K. C., and Spiegelman, S. (1966), *Genetics 54*, 663.

Roodyn, D. B., Reis, P. J., and Work, T. S. (1961), *Biochem. J. 80*, 9.

Roodyn, D. B., Suttie, J. W., and Work, T. S. (1962), *Biochem. J. 83*, 29.

Saito, H., and Miura, K. (1963), *Biochim. Biophys. Acta 72*, 619.

Scherrer, K., and Darnell, J. E. (1962), *Biochem. Biophys. Res. Commun. 7*, 486.

Sinclair, J. H., and Stevens, B. J. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 508.

Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 7*, 1302.

Sueoka, N., and Cheng, T. (1962), *J. Mol. Biol. 4*, 161.

Suyama, Y. (1966), *Biochemistry 5*, 2214.

Truman, D. E. S., and Korner, A. (1962), *Biochem. J. 83*, 588.

Volkin, E., and Cohn, W. E. (1954), *Methods Biochem. Anal. 1*, 287.

Wagner, R. P., and Mitchell, H. K. (1955), *in* Genetics and Metabolism, New York, N. Y., Wiley, p 313.

Watson, J. D. (1965), Molecular Biology of the Gene, New York, N. Y., Benjamin, p 85.

Wintersberger, E. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliaviello, E., and Slater, E. C., Ed., New York, N. Y., Elsevier, p 439.

Woodward, D., and Munkres, K. D. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 872.