The Mitochondrial Ribosome of *Xenopus laevis*

Ronald F. Swanson* and Igor B. Dawid

DEPARTMENT OF EMBRYOLOGY, CARNEGIE INSTITUTION OF WASHINGTON,
BALTIMORE, MARYLAND

Abstract. A mitochondrial ribosome with an approximate sedimentation coefficient of 60 S has been isolated from the ovary of the South African clawed toad, *Xenopus laevis*. This ribosome is active in a submitochondrial protein-synthesizing system. It contains two species of RNA, designated as "21S" and 13S RNA, which have sequences complementary to mitochondrial DNA and share no detectable sequence homology with cytoplasmic ribosomal RNA.

Introduction. Mitochondria possess partial autonomy in the cell in that they contain DNA and have the ability to synthesize DNA, RNA, and proteins.[1] An understanding of the role of mitochondrial DNA in the biogenesis of mitochondria must come through a determination of the nature of RNA and protein synthesis in these particles.

The mechanism of mitochondrial protein synthesis is as yet poorly understood. Unique ribonucleoprotein particles have been isolated from mitochondria of *Neurospora;*[2] these particles have been shown to be active in polypeptide synthesis.[3] Protein synthesis *in vitro* with submitochondrial extracts from animals has not been demonstrated. However, animal mitochondria are known to contain two high molecular weight RNA's which are coded for by mitochondrial DNA,[4,5] as well as special tRNA's[6] including *N*-formyl methionyl tRNA.[7]

This report describes the isolation and characterization of mitochondrial ribosomes from ovaries of *Xenopus laevis*, the South African clawed toad. These ribosomes contain the two high molecular weight mitochondrial-specific RNA's.

Methods. Preparation of submitochondrial fractions: All operations were performed at 0° to 5°C. Whole ovaries of *X. laevis* were homogenized in 5 vol of 0.125 M KCl, 7 mM $MgCl_2$, 0.03 M Tris·HCl (pH 8) which was mixed before use with $^1/_9$ vol of 0.06 M mercaptoethanol. Mitochondria were prepared by differential centrifugation as described previously[8] and washed three times. The mitochondria were then suspended in a hypotonic medium (0.3 ml/gm ovary) consisting of 0.04 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris·HCl (pH 7.5) which was mixed before use with $^1/_9$ vol of 0.06 M mercaptoethanol. The suspension was sonicated three times for 15 sec using the microtip attachment of the Branson Sonifier at its maximum power setting. Debris was removed by centrifugation for 20 min at 13,000 rpm; the resultant supernate is referred to as *mitochondrial extract.*† Mitochondrial particles were concentrated by high-speed centrifugation or by precipitation at pH 5. This latter method involves addition of sodium acetate buffer (pH 5) to 0.05 M and polyvinylsulfate to 2 μg/ml. The precipitate was collected by centrifugation and extracted with hypotonic medium containing 0.05 M Tris (pH 8.0).

Mitochondria from *X. laevis* ovaries are derived mainly from large oocytes, a source previously useful in studies of mitochondrial DNA[9] and RNA.[4] The absence of rough

endoplasmic reticulum in these oocytes facilitates preparation of mitochondria that are relatively free of cytoplasmic ribosomes.

Results. Requirements for submitochondrial polypeptide synthesis: The extract obtained from ovarian mitochondria was active in polypeptide synthesis. The requirements for this system are shown in Table 1. The reaction has an

TABLE 1. *Requirements for incorporation of ³H-phenylalanine into trichloroacetic acid-insoluble form by a submitochondrial extract.*

Omissions	Radioactivity incorporated (cpm)
1. None	13,200
2. ATP	110
3. GTP	6,900
4. Pyruvate kinase, phosphoenol pyruvate	40
5. Magnesium ions	40
6. Polyuridylic acid	680
7. Soluble-RNA	3,050

The complete system contained 50-μl aliquots of mitochondrial extract (see *Methods*), 1.5 mM mercaptoethanol, 0.01 *M* Tris HCl (pH 8), 0.8 mM ATP, 20 μg pyruvate kinase, 5 mM phosphoenol pyruvate, 0.04 mM GTP, 0.04 *M* KCl, 14 mM MgCl$_2$, 40 μg poly U, 65 μg yeast soluble RNA and 10 μM ³H-phenylalanine (1.46 mCi/μmole), in a final volume of 0.2 ml. Aliquots of 0.1 ml were removed at the beginning and end of a 40-min incubation period performed at 30°C. The aliquots were added to 1 ml of 5% trichloroacetic acid and heated at 70°C for 30 min, washed once by centrifugation, and suspended in 1 ml of 5% trichloroacetic acid. Precipitates were then collected on glass-fiber filters and washed with 5 ml of 5% trichloroacetic acid. The filters were dried and radioactivity was determined by liquid scintillation counting. All values were corrected for zero-time blank values which ranged from 10 to 40 cpm.

absolute requirement for magnesium ions, ATP, and an ATP-regenerating system. The function of yeast soluble RNA is unknown, but it may act as transfer RNA or as a ribonuclease inhibitor. The requirement for soluble RNA may explain previous failures[10] to obtain an active submitochondrial system. The addition of polyuridylic acid (poly U) strongly stimulated ³H-phenylalanine incorporation, but significant activity is present without poly U. If activity in the absence of poly U was due to endogenous messenger RNA it probably depended on the presence of a pool of amino acids in the mitochondrial extract. Preliminary experiments suggest the presence of such a pool in ovarian mitochondria. The rate of amino acid incorporation by the mitochondrial system was estimated at 1.2 mμmoles ³H-phenylalanine incorporated in 40 min/mg of RNA in the extract, under conditions where enzyme concentration is rate-limiting. Although this value is close to that of cytoplasmic ribosomes in other poly U-stimulated animal systems,[11] it represents a minimum value since the size of the phenylalanine pool in the extract is unknown.

All components required for polypeptide synthesis could be precipitated at pH 5; a neutral extract of the precipitate contained about half of the activity present in the original mitochondrial extract.

Particulate and soluble fractions were prepared from mitochondrial extract by high-speed centrifugation and combinations of these fractions were tested for their ability to carry out poly U-stimulated incorporation of ³H-phenylalanine. Table 2 shows that the supernate alone had no activity; the pellet fraction alone had low activity, which was removed by further purification (see

TABLE 2.   *³H-phenylalanine incorporation by fractions of the mitochondrial extract.*

Preparation	Radioactivity incorporated (cpm)
Mitochondrial extract, 80 μl	9,800
High-speed supernate of extract, 80 μl	0
High-speed pellet of extract, 120 μl	800
High-speed supernate, 80 μl, and pellet, 120 μl	10,900

Mitochondrial extract was fractionated into high-speed pellet and supernate (see *Methods*). The pellet was dissolved in a volume of hypotonic medium equal to the volume of extract from which it was derived. Each fraction was assayed for ability to support poly U-stimulated incorporation of ³H-phenylalanine into trichloroacetic acid-insoluble product during a 40-min incubation. The reaction conditions were identical to those described in the legend of Table 1.

TABLE 3.   *³H-phenylalanine incorporation by submitochondrial particles.*

Preparation	Radioactivity incorporated (cpm)
60S particles	2830
43S particles	770
32S particles	30
High-speed supernate alone	0

Submitochondrial particles were separated by centrifugation through a sucrose gradient (similar to Fig. 1). Appropriate fractions were pooled; the particles were pelleted and resuspended in a volume equal to that of the high-speed supernate from which they were derived. Aliquots of 50 μl were assayed for incorporation of ³H-phenylalanine as described in the legend to Table 1, using 100 μl freshly prepared high-speed supernate. In these experiments incorporation was entirely dependent on the addition of the high-speed supernatant fraction.

Table 3). About three fourths of the original activity of the mitochondrial extract was restored by recombining the supernatant and pellet fractions.

Isolation of the mitochondrial ribosome: The high-speed pellet was fractionated by sucrose gradient centrifugation (Fig. 1). Four types of particles were separated. A small amount of rapidly sedimenting particles traveled at the
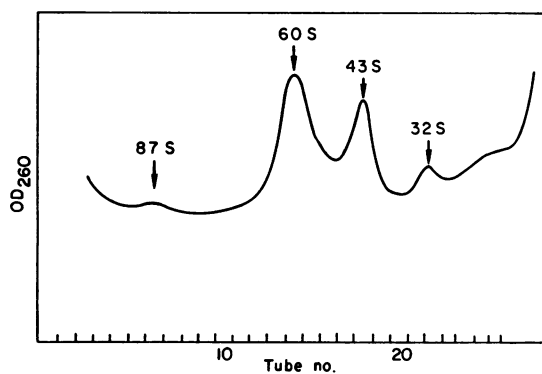

FIG. 1.—Sucrose gradient centrifugation of mitochondrial particles. High-speed pellet was obtained from mitochondrial extract (see Table 1) and resuspended in hypotonic medium (0.05 ml/gm ovary). The concentrated solution of mitochondrial particles was layered onto a 15–30% sucrose gradient made with hypotonic medium. After centrifugation at 25,000 rpm for 18 hr at 5°C in the SW 25.3 rotor of the Beckman centrifuge, the optical density was recorded at 260 nm by pumping the gradient through a flow-through cell of the Gilford spectrophotometer. Sedimentation coefficients were estimated relative to the *X. laevis* cytoplasmic (87 S) ribosome.

same rate as cytoplasmic ribosomes (about 87 S). The presence of 87S ribosomes is probably due to contamination of the mitochondria by cytoplasmic material. The other particles had sedimentation coefficients of about 60, 43, and 32 S.‡ Fractions containing each of the latter particles were pooled and the particles recovered by centrifugation at 65,000 rpm for 2 hr. In some experiments these particles were assayed for ability to support poly U-stimulated phenylalanine incorporation; in other experiments their RNA was extracted for further analysis.

About 80% of the polypeptide-synthesizing activity recovered from the gradient was associated with the 60S particles (Table 3). The remaining 20% of the recovered activity was present in the 43S fraction and probably represents 60S particles which contaminated the 43S region (see Fig. 1). The activity in the 60S particles accounts for 25% of the activity originally present in the mitochondrial extract; a similar loss of activity occurred when unfractionated mitochondrial extract was stored at 5°C for 24 hr, which is about the time required for the fractionation procedure.

The nature of the RNA in the mitochondrial particles was investigated by gel electrophoresis (Fig. 2). Cytoplasmic ribosomal RNA was used for com-
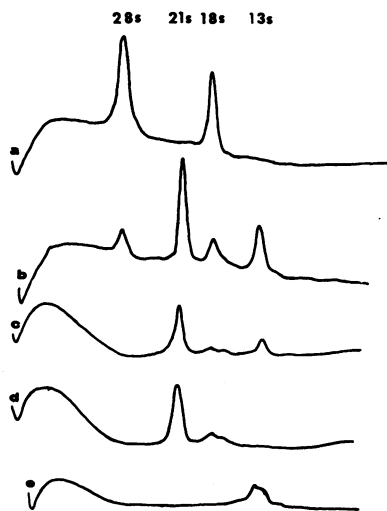

FIG. 2.—UV scans after gel electrophoresis of RNA extracted from submitochondrial particles. The particles were fractionated by sucrose gradient centrifugation (see Fig. 1). Each size class was pooled and the RNA extracted with sodium dodecyl sulfate and phenol. The RNA was analyzed by electrophoresis on 2.4% polyacrylamide gels,[16] and the gels were scanned in the ultraviolet with a Joyce Chromoscan. The direction of electrophoresis is to the right and 4S RNA has been run off the gels. Numbers at the top of the figure are relative "sedimentation coefficients." RNA from (a) cytoplasmic ribosomes, (b) total mitochondrial extract, (c) 60S particles, (d) 43S particles, and (e) 32S particles.

parison (Fig. 2a). It can be seen that a total mitochondrial extract contains four types of high molecular weight RNA (Fig. 2b). In addition, 4S RNA is present but was run off the gels. The results of previous studies[4] indicated that the 28S and 18S RNA found in such preparations originate from cytoplasmic ribosomes which contaminate the mitochondria, while the "21S" and 13S RNA's[§] are true mitochondrial components; these RNA species were shown by hybridization experiments to be homologous to mitochondrial DNA. The key question was therefore the nature of the RNA in the active submitochondrial particles. Figure 2c shows that the 60S particles contained both "21S" and 13S RNA's. The "21S" RNA was also found in 43S particles (Fig. 2d), while the 13S RNA was present in 32S particles (Fig. 2e). A likely interpretation

of these results is that the 60S particles are mitochondrial ribosomes which are composed of 43S and 32S subunits.

The mitochondrial origin of the RNA from 60S and 43S particles was confirmed by the results of hybridization experiments. RNA isolated from these particles was labeled *in vitro* by methylation with ^{3}H-dimethylsulfate[12] and then hybridized to mitochondrial DNA isolated from *X. laevis* ovarian mitochondria (Table 4). DNA from this source has been shown previously to be pure mito-

TABLE 4.   *Hybridization of RNA from mitochondrial particles with mitochondrial DNA.*

Source of ^{3}H-RNA	^{3}H-RNA (μg)	Source of unlabeled RNA	Unlabeled RNA (μg)	Mito-chondrial DNA	Nuclear DNA	*E. coli* DNA
				—^{3}H-RNA (cpm) Hybridized to:—		
From 60S particles	3.3	...	None	425	25	21
Same	3.3	Cytoplasmic ribosomal	45	445	10	23
Same	3.3	Mitochondrial*	40	76	14	17
From 43S particles	3.3	...	None	612	18	18

Mitochondrial particles were fractionated by sucrose gradient centrifugation (Fig. 1), and the RNA was extracted as described in the legend of Fig. 2. The RNA was labeled chemically by methylation with ^{3}H-dimethylsulfate.[12] The specific activities were 3300 cpm/μg for RNA from 60S particles, and 4750 cpm/μg for RNA from 43s particles. Hybridization was performed by the method of Gillespie and Spiegelman.[18] Each vessel contained one filter each with 5 μg mitochondrial DNA (prepared as described previously[9]), 5 μg nuclear DNA, and 5 μg *E. coli* DNA; and the indicated RNA in 0.5 ml 0.6 M NaCl, 0.06 M Na citrate. Incubation was for 16 hr at 65°C.

* The mitochondrial RNA used for competition was a high molecular weight fraction of mitochondrial RNA prepared by filtration through Sephadex G-100. It contained mainly "21S" and 13S RNA.[4]

chondrial DNA as judged by its circularity, renaturation behavior, and other physical properties.[9] RNA from both the 60S and 43S particles hybridized well with mitochondrial DNA; cytoplasmic ribosomal RNA did not compete in this hybridization. Hybridization of RNA from 32S particles was not tested. Comparison of Table 4 and Figure 2 shows that the mixture of "21S" and 13S RNA, as well as "21S" RNA itself, hybridize with mitochondrial DNA. These results confirm more extensive experiments presented elsewhere.[4]

The absence of cytoplasmic ribosomal RNA from the mitochondrial particles was demonstrated in a competition experiment (Fig. 3). Labeled cytoplasmic ribosomal RNA was hybridized to nuclear DNA enriched in sequences complementary to it, and RNA from mitochondrial particles was tested for its ability to compete in this hybridization. Figure 3 shows that unlabeled cytoplasmic ribosomal RNA competed very effectively; the addition of 1 μg of this RNA reduced hybridization to about 20% of the control. In contrast, 40 to 50 μg of RNA from mitochondrial particles reduced the hybridization by about 50%. From Figure 3 one can estimate that 0.2 to 0.3 μg cytoplasmic ribosomal RNA should lead to a competition level of 50%. The concentration of cytoplasmic ribosomal RNA in the RNA from the purified mitochondrial 60S and 43S particles is therefore below 1%. The absence of cytoplasmic ribosomal RNA from these particles was indicated by gel electrophoresis (Figs. 2c and d), but the hybridization-competition experiment is a more sensitive test and does not depend on the isolation of RNA in an undegraded form.
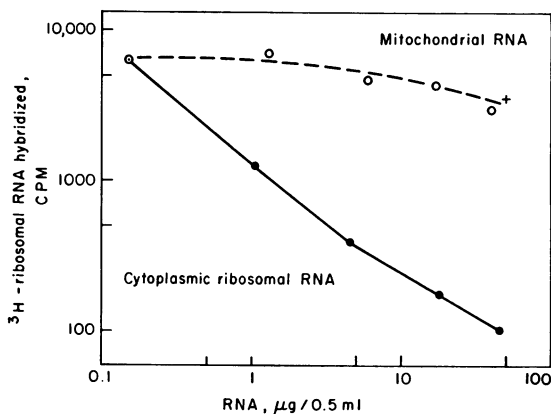
FIG. 3.—Competition by RNA from mitochondrial particles in the hybridization of cytoplasmic ribosomal RNA with nuclear DNA. The hybridization mixture contained 0.15 μg cytoplasmic ribosomal ^{3}H-RNA from *X. laevis* kidney cells (300,000 cpm/μg),[17] and unlabeled RNA as indicated. Two DNA filters were included in each hybridization vessel. A control filter contained 5 μg *E. coli* DNA, while the second filter contained 12 μg *X. laevis* nuclear DNA partially enriched for sequences homologous to cytoplasmic ribosomal RNA.[17] Hybridization was performed at 70°C as described in the footnote to Table 4. The circled dot indicates ^{3}H-RNA only, no competitor. ●—●, unlabeled cytoplasmic ribosomal RNA added; O- - -O, unlabeled RNA from 60S mitochondrial particles added (see Fig. 1 and 2); +, unlabeled RNA from 43S mitochondrial particles added.

Discussion. We wish to summarize the evidence which leads us to regard the 60S particle described in this paper as the mitochondrial ribosome. The 60S particle is the predominant particle isolated from mitochondria. This particle contains two special RNA components, termed "21S" and 13S RNA.[§] These two RNA species are unique to mitochondria, differ in base composition from cytoplasmic ribosomal RNA,[4, 5] and hybridize with mitochondrial DNA; cytoplasmic ribosomal RNA does not compete in this hybridization (Table 4 and ref. 4). The 60S particle is active in polypeptide synthesis and accounts for the bulk of polypeptide-synthesizing activity recovered from fractionated mitochondrial extract. The active particle is clearly not the large subunit of the cytoplasmic ribosome nor a degraded portion of it since (1) there is no detectable breakdown of cytoplasmic ribosomes into subunits in the buffer used, which contained 10 mM $MgCl_2$, and (2) the 60S mitochondrial particles contain no cytoplasmic ribosomal RNA as tested by gel electrophoresis (Fig. 2c) or hybridization-competition (Fig. 3). The results presented in Table 4 together with those in Figures 2 and 3 leave no room for any doubt about the mitochondrial origin of the protein-synthesizing activity associated with 60S particles. *We concluded, therefore, that the 60S particle is the mitochondrial ribosome.* It is likely that this ribosome is composed of two subunits sedimenting at about 43 and 32 S, which contain "21S" and 13S RNA, respectively. Positive identification of the subunit structure must await reconstitution experiments.

Although accurate determination of particle weight from sedimentation coefficient alone is not possible, it appears likely that the 60S mitochondrial ribosome

is the smallest ribosome so far described. Ribosomes from *Neurospora* mitochondria sediment at 73–81 S and contain 17–19S and 25S RNA.[2] A 55S particle has been isolated by O'Brien and Kalf[13] from rat liver mitochondria; polypeptide-synthesizing capacity was not detected in this particle, and the nature of its RNA was not described, which makes comparison with the mitochondrial ribosome described here difficult.

The characteristic "21S" and 13S RNA have been found in ovaries[4] and other tissues of *X. laevis*, in ovaries of *Rana pipiens*,[14] and in HeLa cells.[5] It seems likely that these tissues and cells also contain 60S mitochondrial ribosomes.

Mitochondrial DNA of all vertebrates and invertebrates studied so far consists of circular structures having a contour length between 5 and 6 μm.[1] Yeast and *Neurospora* have a different kind of mitochondrial DNA[1] and their mitochondrial ribosomes and RNA's[2, 15] also differ from those of *X. laevis*.[4] Perhaps size and structure of mitochondrial DNA and ribosomes are correlated in such a way that organisms will be found to fall into a few large classes. Mitochondrial 60S ribosomes may perhaps, be found in all metazoans which contain 5 μm mitochondrial DNA circles.

Abbreviation: poly U, polyuridylic acid.

† Definitions: *Mitochondrial extract* is the supernate obtained from mitochondria by sonication and centrifugation at 13,000 rpm for 20 min. *High-speed supernate* and *high-speed pellet* are the supernate and pellet, respectively, obtained from the mitochondrial extract by centrifugation at 65,000 rpm for 2 hr or at 40,000 rpm for 4 hr.

‡ Sedimentation coefficients of cytoplasmic and mitochondrial ribosomes were measured by band sedimentation in the analytical ultracentrifuge in 50% D_2O solutions containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, and 1 to 10 mM $MgCl_2$. Mitochondrial ribosomes are unstable in 4 mM and lower $MgCl_2$ concentrations. Corrections to $S_{20,w}$ were made assuming a partial specific volume of 0.625 cm^3/gm for ribosomes. Sedimentation coefficients were also estimated for the mitochondrial ribosome and its presumed subunits from their sedimentation rate relative to 87S ribosomes in sucrose gradients.

§ The designations of sedimentation coefficients for mitochondrial RNA's are based on their electrophoretic mobilities. The larger RNA molecule, "21 S," actually sediments at about the same rate as 18S cytoplasmic ribosomal RNA under a variety of conditions.[4, 14] The smaller RNA molecule has a sedimentation coefficient of about 13 S and therefore no quotation marks are needed. The "21S" and 13S RNA's are poorly separated on sucrose gradients and may be confused with a broad zone sedimenting at an average rate of 14 to 18 S.

[1] Roodyn, D. B., and D. Wilkie, *The Biogenesis of Mitochondria* (London: Methuen & Co., 1968); Borst, P., and A. M. Kroon, *Int'l. Rev. Cytology* 26, 107 (1969).

[2] Küntzel, H., and H. Noll, *Nature*, 215, 1340 (1967); Rifkin, M. R., D. D. Wood, and D. J. L. Luck, these PROCEEDINGS, 58, 1025 (1967).

[3] Küntzel, H., *Nature*, 222, 142 (1969).

[4] Dawid, I. B., *Carnegie Institution Year Book*, 67, 418 (1968); Dawid, I. B., "The Control of Organelle Development," in *24th Symposium of the Society for Experimental Biology*, in press.

[5] Vesco, C., and S. Penman, these PROCEEDINGS, 62, 218 (1969); Zylber, E., C. Vesco, and S. Penman, *J. Mol. Biol.*, 44, 195 (1969).

[6] Buck, C. A., and M. M. K. Nass, *J. Mol. Biol.*, 41, 67 (1969); Nass, M. M. K., and C. A. Buck, these PROCEEDINGS, 62, 506 (1969).

[7] Smith, A. E., and V. A. Marker, *J. Mol. Biol.*, 38, 241 (1968); Galper, J. B., and J. E. Darnell, *Biochem. Biophys. Res. Comm.*, 34, 205 (1969).

[8] Dawid, I. B., these PROCEEDINGS, **56,** 269 (1966).

[9] Dawid, I. B., and D. R. Wolstenholme, *J. Mol. Biol.*, **28,** 233 (1967); Dawid, I. B., and D. R. Wolstenholme, *Biophys. J.*, **8,** 65 (1968).

[10] Beattie, D. S., R. E. Basford, and S. B. Koritz, *Biochemistry*, **6,** 3099 (1967); Roodyn, D. B., *Biochem. J.*, **97,** 782 (1965).

[11] Weinstein, I. B., S. M. Friedman, and M. Ochoa, Jr., *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 671.

[12] Smith, K. D., J. L. Armstrong, and B. J. McCarthy, *Biochim. Biophys. Acta*, **142,** 323 (1967).

[13] O'Brien, T. W., and G. F. Kalf, *J. Biol. Chem.*, **242,** 2172 (1967); *ibid.*, p. 2180.

[14] Chase, J. W., and I. B. Dawid, unpublished.

[15] Wintersberger, E., and G. Viehhauser, *Nature*, **220,** 699 (1968).

[16] Weinberg, R. A., U. Loening, M. Willems, and S. Penman, these PROCEEDINGS, **58,** 1088 (1967).

[17] Brown, D. D., and C. S. Weber, *J. Mol. Biol.*, **34,** 661 (1968).

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