OCCURRENCE OF ACTIVE 80 S RIBOSOMES IN SUBCELLULAR PARTICLES IN THE MITOCHONDRIAL FRACTION OF FETAL BOVINE LIVER

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In the course of studies on protein synthesis by mitochondria isolated from fetal calves liver, we found active[1] 80 S ribosomes associated with an unidentified subcellular particle in the mitochondrial fraction. Because these ribosomes incorporate leucine-^{3}H under conditions that label only intramitochondrial ribosomes in adult mammalian systems, we considered the possibility that they might reside within mitochondria, perhaps occurring as dimers of the mammalian 55 S mitochondrial ribo-

[1] Active under incubation conditions that support mitochondrial, but not microsomal, protein synthesis.

some. However, experiments with antibiotic inhibitors of protein synthesis show that these ribosomes are of the extramitochondrial type. Furthermore, it is shown in this report that the active 80 S ribosomes are derived from nonmitochondrial components of the mitochondrial fraction. A similar phenomenon has been described in brain mitochondrial preparations where in vitro protein synthesis occurs, under defined conditions, on active 80 S ribosomes present within particles (synaptosomes?) contained in the mitochondrial fraction (4, 6, 8, 9).

The present finding of active 80 S ribosomes in the mitochondrial fraction of fetal bovine liver is unusual and, excepting brain mitochondria, is the first reported occurrence of active 80 S ribosomes in a mammalian mitochondrial preparation. It is noteworthy that the active 80 S ribosomes have been detected in fetal, but not in adult, bovine liver. Curiously, their occurrence seems to be restricted to midterm fetuses, as no active 80 S ribosomes have been detected during the last trimester of gestation.

MATERIALS AND METHODS

Cycloheximide (CHM) was purchased from Calbiochem (Los Angeles, Calif.) and D(−)-threo-chloramphenicol (CAP) was donated by Parke, Davis & Co., Detroit, Mich. The ages of bovine fetuses obtained at a nearby slaughterhouse were estimated from measurements of forehead-rump length (13). Liver mitochondria were isolated as described previously, except that they were washed by five or six cycles of differential centrifugation at 4300 *g* (average) (10). When indicated, CAP (100 μg/ml) or CHM (100 μg/ml) were present during a 5 min preliminary incubation of the purified mitochondrial fraction. The mitochondria were then incubated with leucine-^{3}H for 5 min, as described (10), before extraction of the ribosome fraction. The incorporation time course was monitored by application of samples of the incubation mixture to filter paper discs (3). To extract the ribosome fraction, mitochondria in 1.9 ml of standard buffer (20 mM $MgCl_2$, 50 mM KCl, 50 mM NH_4Cl, 5 mM Tris, pH 7.6) were lysed by addition of 0.2 ml of 20% Triton-X-100 and centrifuged for 10 min at 60,000 *g*. The clarified lysate was layered directly onto linear gradients of 10–30% sucrose in standard buffer. Sedimentation analysis and subsequent procedures were as described previously (10).

RESULTS AND DISCUSSION

Protein synthesis in mammalian mitochondria occurs on 55 S ribosomes of the prokaryotic type which are sensitive to CAP, but not to CHM (1, 2, 5, 10–12). Because protein synthesis on extramitochondrial ribosomes is inhibited by CHM, but not by CAP, these antibiotics are often used to differentiate the two types of ribosomes in systems where both are active. Another major criterion distinguishing mitochondrial from extramitochondrial ribosomes is that the latter are generally inactive under in vitro conditions which support mitochondrial protein synthesis (10, 11).

When prepared and analyzed in standard buffer, ribosomes of fetal calf liver mitochondria exist predominantly as 55–56 S monosomes which, as in the adult system (10), are essentially the only ribosomes to become labeled when intact mitochondria are incubated in vitro with leucine-^{3}H for 5 min (Fig. 1). Even when the preparations were washed at higher centrifugal forces than were required to purify mitochondria in adult systems, relatively few 80 S extramitochondrial ribosomes are present in such preparations, and, moreover, they appear completely inactive when the mitochondrial fraction is incubated with leucine-^{3}H in the absence of exogenous tRNA, synthetases, and supernatant factors. Also, the effect of CHM and CAP on amino acid incorporation by these mitochondrial preparations conforms to the documented action of these antibiotics on mitochondria in other systems. As shown in Fig. 2 a, leucine-^{3}H incorporation by liver mitochondria of a 225-day gestation bovine fetus is essentially abolished (88% inhibition) by preincubation with CAP, and is relatively unaffected by CHM (7% inhibition).

The above results are in contrast to those obtained with mitochondrial preparations from younger fetuses. First, as shown in Fig. 2 b, not only is amino acid incorporation by these preparations less sensitive to CAP, but it is also inhib-

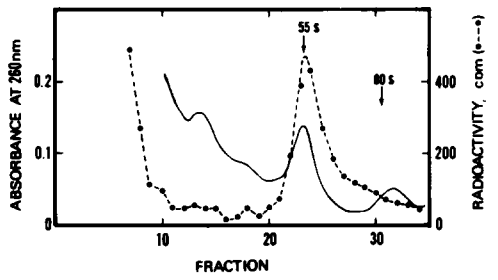

FIGURE 1 Sedimentation analysis of ribosomes isolated from the mitochondrial fraction of fetal (225 day gestation) bovine liver. Before extraction of the ribosomes, the mitochondria were incubated for 5 min with leucine-^{3}H to label active, intraorganellar ribosomes.

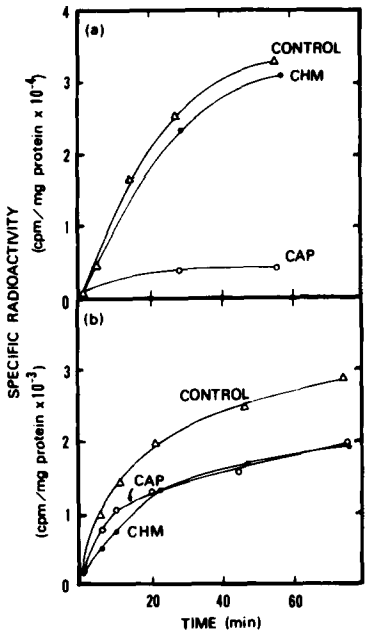

FIGURE 2 Effect of D(−)-threo-chloramphenicol (CAP) (100 μg/ml) and cycloheximide (CHM) (100 μg/ml) on leucine-^{3}H incorporation by components of the liver mitochondrial fraction of fetal bovine liver. (*a*) Fetus of 225 day gestation. Mitochondrial protein concentration during incubation was 0.5 mg/ml. (*b*) Fetuses of 162-167 day gestation. Mitochondrial protein concentration during incubation was 1.4 mg/ml.

ited by CHM. After incubation for 45 min, the inhibition of leucine-^{3}H incorporation by CHM (33%) was about the same as that by CAP (34%). Second, as shown in Fig. 3 a, these preparations contain active 80 S ribosomes. In addition to the 55 S mitochondrial ribosomes and the 29 S and 40 S peaks which are probably subunits of the 55 S ribosome (10), relatively large amounts of 80 S ribosomes are present (Fig. 3 a). It is unusual to find so many 80 S ribosomes remaining in the mitochondrial fraction which was washed six times (see Fig. 1). Furthermore, it is surprising that, like mitochondrial 55 S ribosomes, the 80 S ribosomes should be pulse labeled under the incubation conditions employed. Their activity in the absence of exogenous factors suggests that they reside within particles, such as mitochondria or some other organelle, which provide immediate access to tRNA, synthetases, and other soluble factors.

This observation raised the possibility that the active 80 S ribosomes are dimers of the 55 S mitochondrial ribosome or, alternatively, that they are 80 S monosomes present in mitochondria or other organelles contained in the liver mitochondrial fraction of younger fetuses.

That the 80 S ribosomes are dimers of mitochondrial ribosomes is most unlikely in view of their antibiotic sensitivity. Fig. 3 b shows that the labeling of the 80 S ribosomes is relatively unaffected by CAP, in contrast to that of mitochondrial 55 S ribosomes. This CAP-resistant leucine-^{3}H incorporation by 80 S ribosomes in the mitochondrial fraction explains the failure of CAP to inhibit leucine-^{3}H by this preparation more than 34% (Fig. 2 b), as opposed to the marked inhibition (Fig. 2 a) observed in preparations containing no active 80 S ribosomes (Fig. 1). Conversely, CHM inhibits leucine-^{3}H incorporation by 80 S ribosomes, and apparently stimulates labeling of the 55 S ribosomes (Fig. 3 c). This CHM effect on the labeling of 80 S ribosomes presumably is responsible for the observed partial inhibition by CHM of leucine-^{3}H incorporation by the mitochondrial

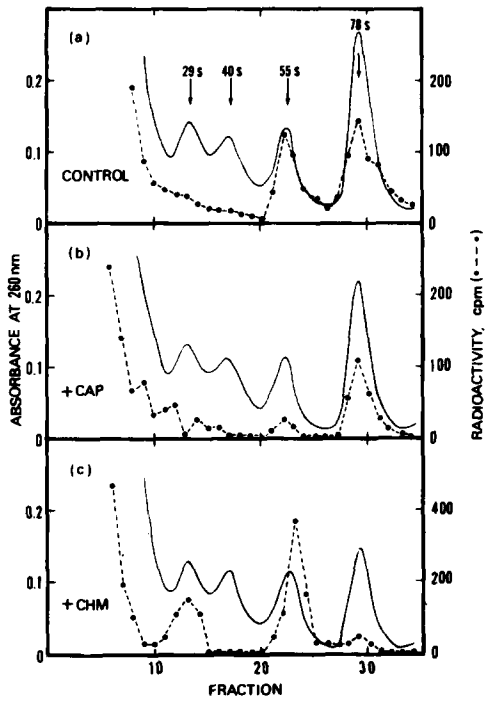

FIGURE 3 Effect of D(−)-threo-chloramphenicol (CAP) and cycloheximide (CHM) on leucine-^{3}H incorporation by ribosomes isolated from the mitochondrial fraction of fetal (162–167 day gestation) bovine liver. Samples of mitochondria were incubated 5 min with leucine-^{3}H under the conditions of Fig. 2 b before extraction and analysis of the ribosome fraction.

fraction (Fig. 2 b). One would expect CHM to inhibit most of the CAP-resistant protein synthesis in mitochondrial preparations shown to contain active nonmitochondrial ribosomes, but this effect is undoubtedly masked by the concurrent CHM-stimulation of leucine-^{3}H incorporation by mitochondrial ribosomes. Clearly, these results characterize the active 80 S ribosomes as nonmitochondrial ribosomes of the eukaryotic type.

To learn more about the origin of the active 80 S ribosomes, the mitochondrial fraction was separated into two portions on the basis of buoyant density. In this experiment, a portion of the mitochondrial fraction prepared from fetal (186 days gestation) calves liver was centrifuged over a cushion of 55% (w/v) sucrose–5 mM Tris, pH 7.5. Mitochondria generally float on such dense cushions and are recovered mainly from the supernatant fluid, while other more dense components of the mitochondrial fraction are found in the pellet. These three samples, a portion of the complete mitochondrial fraction, the recovered mitochondria, and the dense particles, were incubated separately with leucine-^{3}H as described in Materials and Methods. The results are shown in Fig. 4. Active 80 S ribosomes, present in the original mitochondrial fraction (Fig. 4 a), are not associated with mitochondria. After flotation on the sucrose cushion, mitochondria yield only 55 S ribosomes (Fig. 4 b), while particles containing active 80 S ribosomes, on the other hand, are among the more dense components of the mitochondrial fraction (Fig. 4 c).

The unidentified particles containing active 80 S ribosomes are probably not microsomes or nuclear fragments, because control experiments with microsomes and nuclei prepared in the experiment of Fig. 4 revealed no active 80 S ribosomes in these components. Because such ribosomes have been obtained only from midterm fetuses, they may be derived from membranous structures or organelles present only at this developmental stage. Alternatively, they may occur within a normal constituent of the mitochondrial fraction, but only during this period in gestation. This transient occurrence of active 80 S ribosomes presumably manifests a special role played by these ribosomes in a developmental process, perhaps in the formation or maturation of an unidentified organelle. We intend to examine preparations enriched in these particles by electron microscopy to identify these structures and elucidate their association with active 80 S ribosomes.

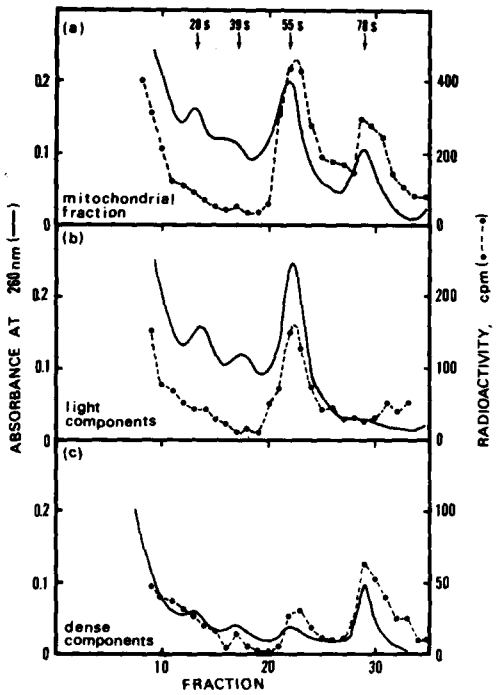

FIGURE 4 Sedimentation analysis of ribosomes isolated from the mitochondrial fraction of fetal (186 day gestation) bovine liver: (*a*) shows ribosomes obtained from 40 mg (protein) of the crude mitochondrial fraction; (*b*) shows ribosomes isolated from 40 mg (protein) of light components (mitochondria) of the mitochondrial fraction; and (*c*) shows ribosomes extracted from 17 mg (protein) of the dense components of the mitochondrial fraction. The three preparations (see text for details) were incubated separately with leucine-^{3}H. The diminished amino acid–incorporating activity of the separated components probably results from exposure to hypertonic sucrose (7) during the fractionation procedure.

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REFERENCES

1. ASHWELL, M. A., and T. S. WORK. 1970. The functional characterization of ribosomes from

rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **39**:204.

2. ATTARDI, G., and D. OJALA. 1971. Mitochondrial ribosomes in HeLa cells. *Nature (New Biology) (London).* **229**:133.
3. BOLLUM, F. J. 1968. Filter paper disk techniques for assaying radioactive macromolecules. *In* Methods in Enzymology. L. Grossman and K. Moldave, editors. Academic Press Inc., New York, XIIB:169.
4. BOSMANN, H. B., and B. A. HEMSWORTH. 1970. Intraneural mitochondria. Incorporation of amino acids and monosaccharides into macromolecules by isolated synaptosomes and synaptosomal mitochondria. *J. Biol. Chem.* **245**:363
5. BREGA, A., and C. VESCO. 1971. Ribonucleoprotein particles involved in HeLa mitochondrial protein synthesis. *Nature (New Biology) (London).* **229**:136.
6. HALDAR, D. 1971. Protein synthesis in isolated rat brain mitochondria. *Biochem. Biophys. Res. Commun.* **42**:899.
7. HALDAR, D., and K. B. FREEMAN. 1969. Importance of the osmolarity of the incubation medium on amino acid incorporation into protein by isolated rat liver mitochondria. *Biochem. J.* **111**:653.
8. HERNANDEZ, A., I. BURDETT, and T. S. WORK. 1971. Protein synthesis by brain-cortex mitochondria. Characterization of a 55 S mitochondrial ribosome as the functional unit in protein synthesis by cortex mitochondria and its distinction from a contaminant cytoplasmic protein-synthesizing system. *Biochem. J.* **124**:327.
9. MORGAN, I. G. 1970. Protein synthesis in brain mitochondrial and synaptosomal preparations. *FEBS Letters.* **10**:273.
10. O'BRIEN, T. W. 1971. The general occurrence of 55 S ribosomes in mammalian liver mitochondria. *J. Biol. Chem.* **246**:3409.
11. O'BRIEN, T. W., and G. F. KALF. 1967. Ribosomes from rat liver mitochondria. II. Partial characterization. *J. Biol. Chem.* **242**:2180.
12. PERLMAN, S., and S. PENMAN. 1970. Protein-synthesizing structures associated with mitochondria. *Nature (London).* **227**:133.
13. WINTERS, L. M., W. W. GREEN, and R. E. COMSTOCK. 1942. Prenatal development of the bovine. *In* University of Minnesota Technical Bulletin 151. University of Minnesota Press, Minneapolis, Minnesota.