MITOCHONDRIAL PROTEIN SYNTHESIS: RESISTANCE TO EMETINE AND RESPONSE TO RNA SYNTHESIS INHIBITORS

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Mitochondrial protein synthesis is not inhibited by concentrations of emetine that completely suppress cytoplasmic protein synthesis. The emetine resistant, chloramphenicol sensitive structures sediment in a broad band about 95S and in a sharp band at 55S after brief treatment with RNAse. The activity of the structures has a half-life of 3 hours in cordycepin or actinomycin D and of less than 30 minutes in ethidium bromide.

A mitochondrial specific protein synthesizing structure from mammalian tissue sedimenting at 55S was first reported by O'Brien & Kalf (1). Swanson & Dawid (2) later showed a 55S structure in mitochondria of Xenopus Laevis oocytes which dissociates into subunits and incorporates phenylalanine in the presence of polyuridylic acid. In a previous report (3), we showed that a mitochondrial specific protein synthesizing structure could be identified by selectively inhibiting HeLa cells and labeling in vivo.

In HeLa cells the polyribosomes bound to endoplasmic reticulum cannot be separated physically from the mitochondria. The mitochondrial specific protein synthetic structures could be identified in HeLa cells only after membrane-bound ribosomal synthesis was suppressed because the synthetic activity of the membrane-bound ribosomes is far greater than that of the mitochondrial structures. Poliovirus infection and cycloheximide combined were found to effectively suppress protein synthesis on membrane-bound ribosomes. Using these inhibitors, we described several characteristics of the mitochondrial protein synthetic structures. They are not inhibited when the cell is treated with

cycloheximide or infected with poliovirus. The activity is inhibited by chloramphenicol and by ethidium bromide, both agents that have no effect on cytoplasmic ribosomes. The buoyant density in cesium chloride of gluteraldehyde fixed mitochondrial protein synthetic structures is 1.40gm/cc compared to 1.54g/cc for (4) cytoplasmic ribosomes.

The mitochondrial protein synthetic structures sediment as a broad peak with a nominal velocity of about 95S. When the structures are treated briefly with pancreatic ribonuclease they are converted to a sharp band sedimenting at 55S. When divalent ions are removed with EDTA, the structures are converted to one sedimenting at about 35S which, unlike cytoplasmic ribosomes, still retains its nascent polypeptide chain (3).

The combined use of polio infection and cycloheximide to suppress ribosome directed synthesis was tedious. Cycloheximide alone is not an adequate inhibitor of bulk ribosomal protein synthesis even at the highest concentrations used. The drug, emetine, however is a more potent inhibitor of ribosomes and provides the means for a simple measurement of mitochondrial protein synthesis. Using this assay, the effect of inhibiting mitochondrial RNA synthesis on mitochondrial protein synthesis has been examined.

MATERIALS AND METHODS

HeLa cells were grown in suspension culture as previously described. Before labeling, cells were concentrated to 8 X 10^6 cells/ml in leucine-free Eagle's medium. Either emetine or cycloheximide was added and 5 minutes later the cells were pulse labeled with ^{3}H-leucine. The cells were harvested and homogenized in hypotonic buffer and a crude mitochondrial fraction prepared as previously described (3). The mitochondrial pellet was resuspended in HRSB (0.1M NaCl 0.01M $MgCl_2$, 0.01M Tris pH 7.4) and solublized by the addition of sodium deoxycholate and Brij 58 to

a final concentration of one per cent. The solublized mitochondria were layered on a 15-40% sucrose gradient made with HRSB and centrifuged at 40,000 rpm for 150 minutes in the SW40 rotor of the Beckman ultracentrifuge.

Actinomycin D was the generous gift of Dr. William McCormick of Merck Sharp & Dohme. Cordycepin was purchased from Sigma. Ethidium bromide was obtained from Calbiochem.

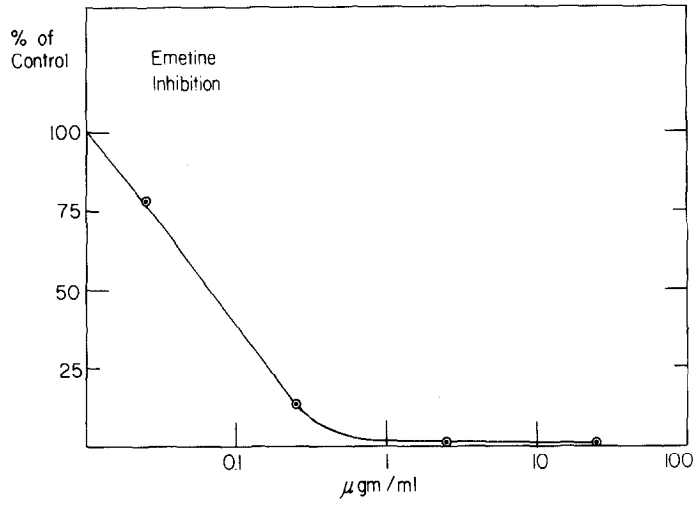

Figure 1: Effect of emetine on total cell protein synthesis. 2 X 10^6 cells were concentrated 5-fold and incubated for 5 minutes in the indicated concentrations of emetine. The cells were pulsed for 10 minutes with 1μc of ^{14}C-leucine (316Mc/mM). The reaction was stopped with a 5-fold excess of cold Earle's saline and the cells assayed for TCA precipitable radioactivity.

RESULTS AND DISCUSSION

Emetine has been shown to be an extremely potent inhibitor of ribosome directed protein synthesis (5). Figure 1 shows the response of HeLa cell protein synthesis to different concentrations of the drug. 4μg/ml of emetine is sufficient to inhibit total amino acid incorporation by more than 99%. In subsequent experiments a concentration of 80μg/ml of emetine is used. This reduces the protein synthesis by ribosomes contaminating the mitochondrial preparations to a negligible amount.

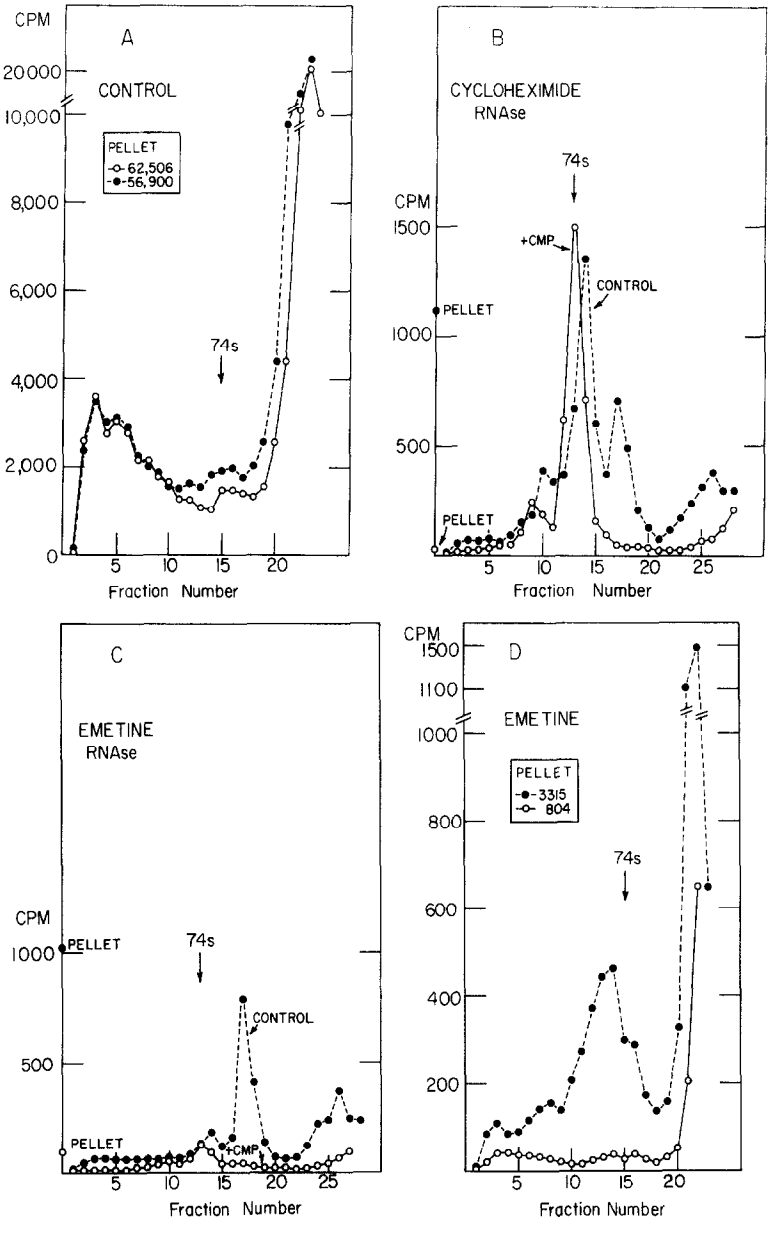

Figure 2: Effect of chloramphenicol on control, cycloheximide-treated and emetine-treated cells.
(a) 8 X 10^7 cells were resuspended in 20 ml leucine-free medium. 100µg/ml of chloramphenicol was added to one-half. Five minutes later, each sample was pulsed for 3 minutes with 100µc ^{3}H-leucine (53.9C/mM). Mitochondria were isolated, treated with detergent and analyzed by sucrose density centrifugation as described in Materials and Methods.
(b) 4 X 10^7 cells were concentrated 20-fold in leucine-free medium, treated with 600µg/ml cycloheximide and divided

into two samples. Chloramphenicol (100µg/ml) was added to one sample. Five minutes later, each sample was pulsed for 10 minutes with 50µc ^{3}H-leucine and treated as described in materials and methods except that the samples were treated with one microgram/ml of pancreatic RNAse for 5 minutes at 0°C prior to centrifugation.
(c) As in 2b, except that the cycloheximide was replaced by emetine at a concentration of 80µg/ml.
(d) As in 2c, except that 8 X 10^{7} cells were used and RNAse was omitted.

The necessity for using a potent inhibitor of ribosome directed protein synthesis is illustrated in Figure 2a. The sedimentation profile of amino acid pulse labeled structures from a mitochondrial fraction is shown. Most of the incorporation into nascent polypeptides is either associated with polyribosomes sedimenting faster than 100S or in released proteins at the top of the gradient. Chloramphenicol, which is used to identify the mitochondrial specific protein synthetic system (6,7), has only a very small effect on structures sedimenting around 95S. Most of the radioactivity appears on contaminating polyribosomes. It should be noted that the pulse time used here had to be restricted to 3 minutes to prevent radioactive proteins released from ribosomes to the top of the gradient from overwhelming the region of interest.

The relative efficacy of cycloheximide and emetine in suppressing the amino acid incorporation by ribosomes that contaminate the mitochondrial fraction is shown in Figures 2b and 2c. The quantitative comparison of the activity of contaminating ribosomes and the mitochondrial specific structures is facilitated by degrading all structures to a uniform sedimentation velocity with RNAse prior to sucrose gradient sedimentation. Thus, all cytoplasmic polyribosomes are converted to 74S and the active mitochondrial structures to 55S.

The sedimentation pattern in Figure 2b shows that even in the presence of 600µg/ml of cycloheximide, some amino acid incorporation occurs on 74S particles while the chloramphenicol sensitive structures,

which are of interest here, are found exclusively at 55S. In contrast, 80μg/ml of emetine completely suppresses all activity of the 74S ribosomes but leaves the 55S mitochondrial specific structures apparently unaffected. Thus, emetine reduces contaminating ribosome activity to a negligible amount and permits the study of the mitochondrial specific activity alone.

Figure 2d shows the distribution in sedimentation velocity of the amino acid incorporating structures from mitochondria treated with emetine, but not with RNAse. The mitochondrial structures sediment in a broad peak with a maximum at about 95S in agreement with previous results (3).

An effective, rapid selective inhibitor of cytoplasmic protein synthesis permits experiments on mitochondrial protein synthesis which were not possible using the more elaborate procedure of virus infecting cells (3). In particular, the time course of mitochondrial response to various agents cannot be studied because in a two hour period following virus infection metabolism becomes progressively abnormal.

The time course of inhibition of mitochondrial protein synthesis by some inhibitors of mitochondrial RNA synthesis is shown in Figure 3. Actinomycin inhibits mitochondrial RNA synthesis along with all other cellular RNA synthesis. Cordycepin (3'deoxyadenosine), also a potent inhibitor of mitochondrial RNA synthesis (8), has a mode of action very different from actinomycin (9). Both agents cause relatively slow decay with a half-life of at least 3 hours. In contrast, ethidium bromide, which appears to be a specific inhibitor of mitochondrial synthesis (10), causes a rapid decay of protein synthesis with a half-life of less than 30 minutes.

The inhibition of protein synthesis by ethidium bromide is probably not simply due to suppression of mitochondrial RNA synthesis by the drug since the other RNA inhibitors result in a slower decay. Rather,

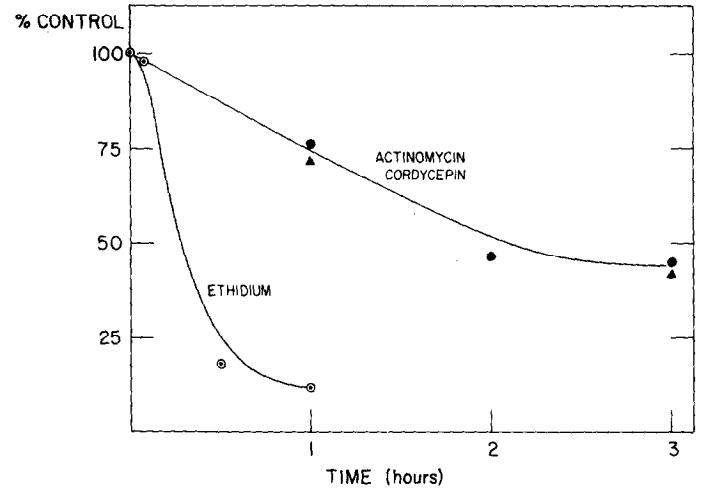

Figure 3: Effect of actinomycin, cordycepin and ethidium bromide on mitochondrial protein synthesis.
4×10^7 cells were incubated for the times indicated in either 1µg/ml EBr., 20µg/ml cordycepin or 5µg/ml actinomycin D. They were then concentrated 20-fold in leucine-free medium. Emetine was added to 80µg/ml and 5 minutes later ^{3}H-leucine was added for 10 minutes as in Fig. 2b. Mitochondria were isolated, treated with detergent and analyzed as described in Materials and Methods. Each point in the figure represents the fraction of activity remaining in the form of nascent radioactivity on 95S structures after the above treatments.

a more general effect, possibly a disruption of mitochondrial structure, is suggested. Ethidium also inhibits mitochondrial DNA synthesis, with the dose response similar to inhibition of mitochondrial RNA (11).

Mitochondrial protein synthesis has been studied in single cell eukaryotes (12). An autonomous protein synthesis system in the mitochondria of mammalian cells is now well established. While the mitochondrial protein synthesizing structures resemble cytoplasmic ribosomes in some respects, they also have several unique properties. They appear to be composed of dissociable subunits, each with its distinctive RNA species (2). However, no 5S RNA has been detected (13). The buoyant density of the particles in CsCl is very light (1.40gm/cc) implying a high protein to RNA ratio (3). Finally, exposure to EDTA

does not appear to release the nascent polypeptide chain (3). It is very likely that the mitochondrial structure differs from mammalian and bacterial ribosomes in several important aspects.

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