DIFFERENTIAL EFFECTS OF ETHIDIUM BROMIDE ON CYTOPLASMIC AND MITOCHONDRIAL PROTEIN SYNTHESIS

Narayan G. AVADHANI and Robert J. RUTMAN
Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania Philadelphia, Pennsylvania, 19174, USA

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1. Introduction

The phenanthridine dye ethidium bromide (EB) is well-known for its ability to bind to DNA molecules. In eukaryotic cells, even at very low concentrations, EB is known to affect mitochondrial specific events [1]. In mouse BHK cells 0.1 to 5 μg/ml EB specifically inhibits mitochondrial DNA synthesis [2]. This drug is also known to inhibit mitochondrial specific RNA synthesis [1,3], probably by binding to the DNA template thus interfering with chain initiation [4,5]. Consequently EB has been widely used as a tool to study mitochondrial specific transcription [6,7,8].

Isolated mitochondria from various cell types are known to incorporate both synthetic and natural polynucleotides which participate in intramitochondrial translation [9–12]. Using an in vitro mitochondrial system from Xenopus, Grivell and Metz [12] recently demonstrated that EB directly interferes with the intramitochondrial phenylalanine incorporation directed by imported poly (U). This observation coincides with another indirect observation that EB may have an inhibitory effect on mitochondrial protein synthesis [13]. We have verified these indirect results using a highly active in vitro protein synthesizing system from Ehrlich ascites mitochondria [14]. Our results show that EB specifically inhibits mitochondrial but not cytoplasmic in vitro protein synthesis by preventing polysome formation.

2. Materials and methods

Ehrlich ascites hypotetraploid cells were grown for seven days in the peritoneal cavity of Swiss colony mice and used for preparing cytoplasmic and mitochondrial in vitro systems. Mitochondria were prepared by a two step digitonin washing technique and lysed with nonidet NP40 (Shell Oil Co.) under controlled conditions [14]. 25 000 *g* supernatant fraction (S-25) from lysed mitochondria contains organelle specific factors and ribosomes highly active in in vitro protein synthesis. Details of preparation of S-25 system from Ehrlich ascites mitochondria have been described elsewhere [14]. The cytoplasmic protein synthesizing system was prepared according to Aviv et al. [15]. Both cytoplasmic and mitochondrial protein synthesis assays were run in 0.05 ml final volumes at 35°C for 45 min. The cytoplasmic protein synthesizing system contained 30 mM Tris–HCl (pH 7.8), 120 mM KCl, 8mM Mg $(CH_3COO)_2$, 7 mM 2-mercapto ethanol, 1 mM ATP, 0.25 mM GTP, 0.3 mM CTP, 4,5 mM phosphoenol pyruvate, 1.6 μg pyruvate kinase and cytoplasmic extract containing 230–250 μg protein. The assay mixture for mitochondrial protein synthesis contained 30 mM Tris–HCl (pH 7.5), 120 mM KCl, 15 mM Mg $(CH_3COO)_2$, 7 mM 2-mercaptoethanol, 0.5 mM ATP, 0.1 mM GTP, 0.3 mM CTP, 3 mM phosphoenol pyruvate, 1.6 μg pyruvate kinase and 20 μl of mitochondrial S-25 containing 250 μg protein. Both the systems also contained 0.25 OD 260 nm poly (U), 0.5 OD 260 nm tRNA (yeast) and 0.5 μCi [^{3}H] phenylalanine (25 Ci/m mole, New England Nuclear). For both mitochondrial and cytoplasmic systems the above described conditions were found to be optimal for poly (U) directed phenylalanine incorporation. Other details were as described before [14,15].

3. Results and discussion

The direct effects of EB on poly (U) directed phenylalanine incorporation in mitochondrial and cytoplasmic in vitro systems are shown in fig. 1. At the highest amount of EB used, i.e., 10 μg, the cytoplasmic system is inhibited by only about 10%. In contrast, EB has a pronounced inhibitory effect on the mitochondrial system and even at the 1 μg level, protein synthesis is inhibited by about 60%. Considering the very low concentrations of EB needed to inhibit mitochondrial DNA and RNA synthesis, the concentrations of EB needed to affect the protein synthesis in the present experiments are high. However, similar effects have been noted by others [4,5].

In order to further examine the inhibitory effects of EB on the mitochondrial and cytoplasmic systems experiments were carried out using ^{3}H-labeled poly (U) and the reaction mixture was analyzed. As seen in fig. 2A, within 7–8 min of the reaction in both mitochondrial and cytoplasmic systems counts are seen in polysomal region. The cytoplasmic system containing 0.5 μg EB also shows a typical polysomal pattern (fig. 2B) comparable to that seen in the control experiment without EB (see fig. 2A). In contrast, the mitochondrial system with 0.5 μg EB does not show a polysome-like pattern on a sucrose gradient (fig. 2B). These results conclusively show that EB specifically inhibits mitochondrial protein synthesis without any detectable effect on the cytoplasmic system in vitro.

Although these experiments show that the inhibitory effect of EB on the mitochondrial system is probably due to interference with some stage of the initiation process thereby preventing polysome formation, the precise effect is not yet known. Since it is known that EB has an affinity fo double-stranded RNA, it is possible that it binds to mRNA and prevents the initiation of protein synthesis. However, the fact that there is no EB inhibition of poly (U) directed cytoplasmic protein synthesis suggests that inhibition of the mitochondrial system is not due to the binding of the drug to mRNA but may involve binding of the drug to mitochondrial ribosomes. The exact mechanism of action will have to be tested using radio-labeled EB and natural mRNA's

Several studies in the past showing either the inhibitory effects of EB on amino acid incorporation by isolated mitochondria [16,17] or reduced amounts of mitochondrial polysomes or polysomal RNA contents

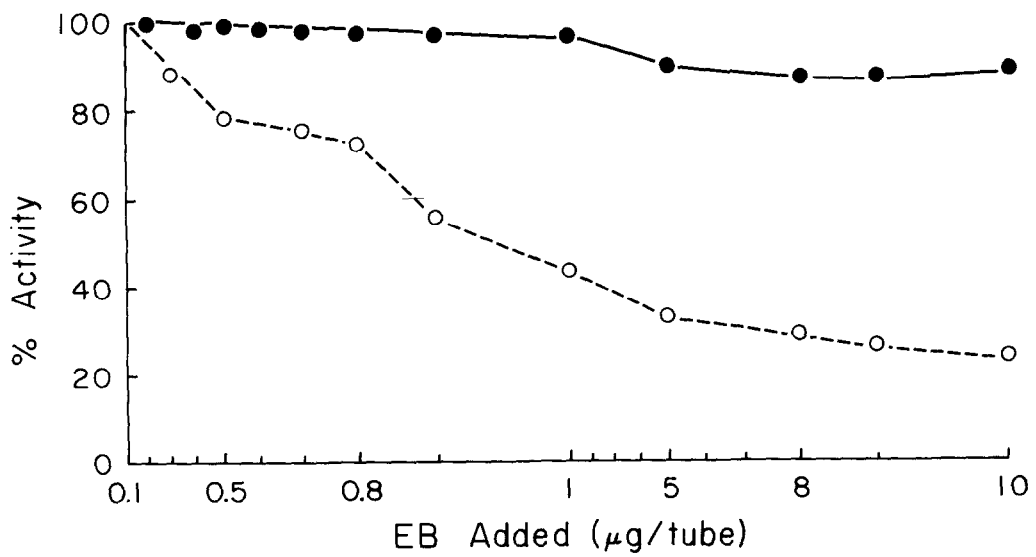

Fig. 1. Effect of EB on mitochondrial (o--o--o), and cytoplasmic (•–•–•) protein synthesis. Details of protein synthesis assays were as in Materials and methods. Amino acid incorporation activity in tubes with 0.1 μg EB were considered to be 100%. Actual CPM at this concentration were 225 000 CPM for cytoplasmic and 189 000 for mitochondrial systems. Tubes without EB gave 226 000 CPM for cytoplasmic and 205 000 CPM for mitochondrial systems.

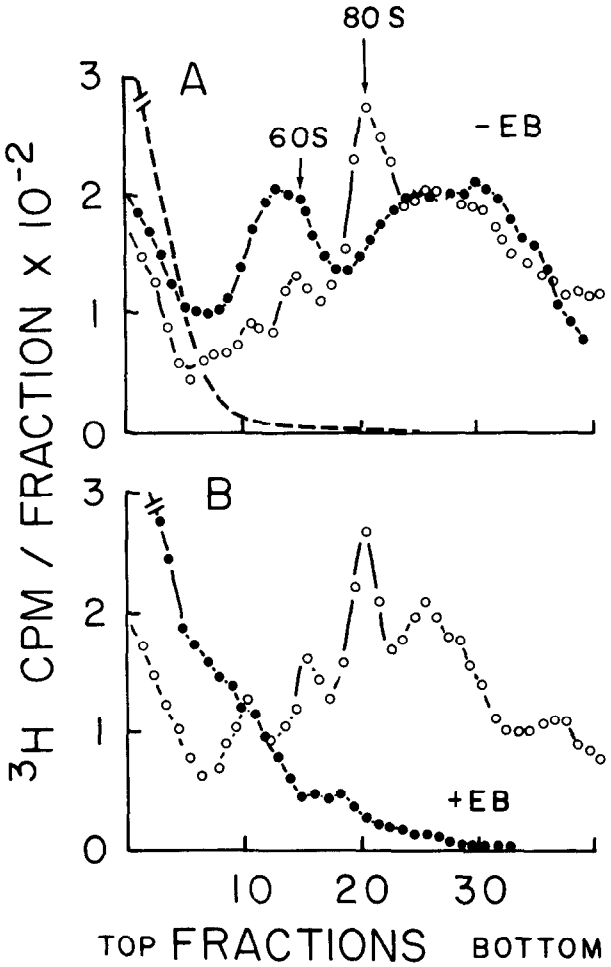

Fig. 2. Effect of EB on polysome formation in mitochondrial (●—●—●) and cytoplasmic (○—○—○) systems. The reaction was run as described in Materials and methods, except that 2 μg (7.0 × 10 CPM) ^{3}H-labeled poly (U) (Miles Laboratories) were added. After 7.5 min reaction, aliquots containing 5–6000 CPM were layered on 4.5 ml of 10–36% linear sucrose gradients and centrifuged at 145 000 *g* for 2 hr. Gradients were fractionated and 4 drop fractions were counted with 10 ml cab-o-sil mixture. Fig. A represent control mitochondrial and cytoplasmic systems without EB. The dashed line shows the sedimentation profile of poly (U) alone. Fig. B shows the sedimentation profiles of mitochondrial and cytoplasmic systems with 0.5 μg EB. Cytoplasmic ribosomes were used as markers.

[6,7] have been interpreted in favor of the hypothesis that mitochondria are autonomous with respect to their mRNA synthesis. However, because of the direct effect of the drug on mitochondrial specific protein synthesis, these arguments will have to be re-evaluated.

In conclusion, our data confirm and extend the observations of Grivell and Metz [12] and Lederman and Attardi [13] that EB inhibits mitochondrial specific protein synthesis.

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