Evidence of novel type of ribosome in eukaryotic intermediate flatworm

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In all organisms, messenger-directed protein synthesis is catalyzed by ribonucleoprotein particles called ribosomes. A ribosome is typically composed of one small and one large subunit which contain one short (18S) and one long (28S) rRNAs, respectively. Surprisingly, in this study, three similar size rRNAs (18-21S) were revealed in the electrophoresis profile of the total RNAs of tapeworm *Spirometra erinaceiuropaei*. Northern blot analysis shows that one of the three bands belongs to 18S rRNA, and the other two bands are of 28S rRNAs, implying structurally distinct ribosomes in this intermediate animal. Furthermore, similar, but not identical profiles were observed in two other tapeworms *Diphyllobothrium hottai* and *Diphyllobothrium Nipponkaiizeme*. Relevant to this finding, in flatworm *Paragonimus westermani*, 18S rRNAs were found much more numerous than 28S rRNAs. Moreover, consistent with this biochemical finding, transmission electron microscopy examinations show that the ribosomes isolated from *Spirometra erinaceiuropaei* are composed of either one ball or two similar size subunits (balls), while the structure of ribosomes isolated from control liver tissue exactly match the conventional large and small subunit ribosome model. Our study provides direct biochemical and biophysical evidence of structurally distinct novel type of ribosomes in intermediate eukaryotic flatworms. These finding may be important for re-recognition of biological protein synthesis and evolutionary process of living things.

Key words: Ribosome; rRNA; intermediate animal; evolution

The different subunits of ribosomes contain rRNAs of different lengths as well as a number of different proteins [1-3]. It is generally believed that all ribosomes contain one shorter (18S) and one longer (28S) major rRNA molecules which are equally distributed into small and large subunits of ribosomes, respectively [1-3]. This is essentially important for ribosome structure and biological protein synthesis. To our knowledge, no exception has been reported to date. Nevertheless, recent studies showed that there are structurally distinct, stage-specific ribosomes in *Plasmodium* [4, 5], and that, in insect vectors, heterogeneous ribosome populations of *Plasmodium* are present [6]. In spite of extensive studies in prokaryotic and eukaryotic organisms, the biochemical and biophysical studies on parasites are still not enough [7-10]. In particular, ribosome structure of flatworm has been rarely reported. Here, we report evidence of structure difference of ribosomes between intermediate flatworm and mammal.

In this study, to our surprise, under usual electrophoresis and by ethidium bromide staining, only one (approximately 21S) single band of ribosomal RNA was detected in the profiles of total RNAs of tapeworm *Spirometra erinaceiuropaei*, whereas, in the control tissues e.g. mouse liver, spleen and nematode *Anisakis simplex*, there were indeed two major rRNA bands (Fig. 1A). When increasing the RNA amount of SEP total RNAs for electrophoresis, the pattern of the bands remained essentially unchanged (Fig. 1B). It is worth pointing out that these results did not vary with RNA isolation methods, and that the rRNAs isolated directly from ribosomes of these animals displayed similar electrophoresis profile (data not shown). To further investigate this phenomenon, we examined the RNA electrophoresis profiles of the related intermediated animals. As a result, similar rRNA electrophoresis patterns were obtained in tapeworm *Diphyllobothrium hottai (D. hottai)*, *Diphyllobothrium Nipponkaiizeme* (*D. Nipponkaiizeme)* and flatworm *Paragonimus westermani (P. westermani)* (Fig. 1C). This, at least, suggests the relative generality of the characteristic rRNA components in tapeworms and flukes. To characterize the components of the single rRNA band, the cDNA probes for both 18S and 28S rRNAs were cloned from the plerocercoids of *S. erinaceiuropaei*. Northern blot analysis shows that the single band hybridized with both *Spirometra erinaceiuropaei* plerocercoid (SEP, larva of the worm) 18S and 28S cDNA probes, and also cross-hybridized with rat 18S rRNA probe (Fig. 1A, lower panels). A very faint longer band at the usual 28S rRNA position was found in *Paragonimus westermani*, and the rRNAs in the shorter band are much more numerous than those in the faint longer one (Fig. 1C). To further clarify the components of the single band in the SEP RNA, the running distance of electrophoresis was extremely (3-4 times of the usual) extended. Consequently, the single band in *S. erinaceiuropaei* was shown to be composed of three sub-bands with very similar sizes (Fig. 2A). And the bands in fluke *Paragonimus westermani* and liver tissue remained unchanged (Fig. 2A). In addition, Northern blotting shows that the upper and lower bands hybridized with 28S rRNA, and the middle band specifically hybridized only with 18S rRNA (Fig. 2B). These results show that the tested flatworms have basic rRNA components to the results reported to date in prokaryotic and eukaryotic cells [1-3]. Here, for the application of various controls, it is impossible that these results reflect differential stabilities of 28S and 18S rRNA gene transcripts during the processes of RNA isolation, electrophoresis, and/or Northern blotting. Therefore, the current concept of the 1 to 1 ratio (18S to 28S) of small to large subunit cannot explain the phenomenon existing in the flatworms tested. These data suggest that, other than mammals and bacteria, there likely still be unknown type of ribosomes in flatworms. To test this hypothesis, the

ribosomes isolated from SEP and control mouse liver tissues by ultracentrifugation were subjected to high resolution electron microscopy. As a result, the ribosome from liver consist of one small subunit and one large subunit (Fig. 3A), whereas the ribosomes from SEP are either composed of one large ball or two similar size balls (Fig. 3B).

In the present study, we present several lines of direct evidence for the structurally distinct ribosomes in flatworm. First, in the examined SEP, three similar size rRNAs (18-21S) were observed—one 18S and two 28S bands; and in *Paragonimus westermani*, only two bands were observed—but the rRNAs in 18S band is much more numerous than that in the 28S band (Fig. 1B and Fig. 2A). These results suggest that the components in these worms are markedly different from the usually known rRNA components in prokaryotic bacteria and eukaryotic mammalian cells [1-3]. Second, more importantly the structurally distinct ribosomes have been directly observed and verified by electron microscopy. It was demonstrated that the ribosomes of SEP take the shape of one single ball or two fused similar size balls (subunits). Correspondingly, the ribosomes from mouse liver control are indeed composed of one small subunit and one large subunit (Fig. 3). Third, considering the varied components of rRNA in different species of worms as shown above, the differences in ribosome structure may likely not be restricted to the *Spirometra* and *Paragonimus* lines of descent. Therefore, in this study, we have provided direct biochemical and biophysical evidence supporting the notion that there exist structurally distinct ribosomes, at least, in some of the flatworms. The ribosome difference between these intermediate parasitized animals and bacteria and mammals might provoke us to reconsider the fundamental mechanism difference of biological protein synthesis between bacteria, intermediate animals and mammals. These newly identified ribosomes are designated collectively as **In**termediate **A**nimal (**INA-Wang**) ribosome: INA ribosome. Further identification of **INA-Wang** ribosome using more precise biochemical and biophysical methods, for instance x-ray crystallography, may provide more solid evidence.

METHOD SUMMARY: Plerocercoids of *Sperometra erinaceieuropaei* (SEP) were collected from two species of snakes, *Elaphe quadrivirgata* and *Rhabdophis tigrinus*, captured in Shimane Prefecture, Japan and identified by microscopy [11]. The adult worms were obtained by orally infecting the dogs with SEP. A 689-bp fragment of 28S SEP rRNA probe (Accession number: AB027761) was obtained from SEP cDNA library constructured with pSPORT1 plasmid according to the introduced protocol, and

sequenced on Pharmacia auto-sequencer. A 210-bp cDNA probe of SEP 18S rRNA was amplified from total RNA of SEP by PCR (Accession number: D64072) and identified by sequence analysis. Rat 18S rRNA probe has been described elsewhere [12, 13]. For RNA isolation and Northern blot analysis [14, 15], ICR mice were obtained from Shimizu Experimental Animal Inc. (Shizuoka Prefecture, Japan). *Anisakis simplex* and *Paragonimus westermani* were collected from infected fish and dog lungs, respectively. Prokaryotic free organism bacterium *E. coli* (strain XL-1 blue), were bought from Promega Inc. (USA), and grown in LB medium. Total RNAs were isolated from the organisms or ribosomes by Isogen kit (Nippon Gene, Kyoto, Japan) by standard method as described [11-13, 16], unless specifically indicated. Electrophoresis was carried out under both denatured and non-denatured conditions. Standard methods were used for Northern blotting [12, 13]. For transmission electron microscopy observation, ribosomes were isolated as described with little modifications[17]. SEP from snakes were homogenized for 10 times in a homogenizing medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 7.6) on ice and then centrifuged at 750 g at 4ºC for 10 min. The supernatant was collected and subjected to a further centrifugation at 20,000g at 4ºC for 20 min, resulting in two portions: the supernatant and the pellet. For isolation of ribosomes, the supernatant was layered on top of 2 ml of 0.5 M sucrose cushion over 2.0 M sucrose cushions for ultracentrifugation as described above. After centrifugation in the Bechman ultracentrifuge for 10 hr at 45,000 rpm (120,000 g) at 4°C, with the brake on for maximum deceleration rate, the supernatant on top of the translucent pellet of ribosomes was removed. The pellet was quickly rinsed twice with distilled water. The ribosome pellet was resuspended in a proper amount of TBS containing 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6 (KMT) buffer. As control, fresh liver tissues taken from the 16-hour-fastened mice underwent the same procedure as SEP. For transmission electron microscopy examination, ribosomes were treated and observed essentially as described[7]. Ultracentrifugation-purified ribosomes (1mg/ml) were polymerized on electron microscope grids attached to glass slides. The grids (200 mesh, Pelco, CA) were coated sequentially with: 0.25% Formvar; 1% polylysine (Mr 20,000.00, Sigma); and finally 1% bovine serum albumin at 1 mg/ml (Sigma). The grids were washed thoroughly with deionized water after being coated with the albumin solution, and allowed to dry until use. A drop of SEP ribosome solution containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6 were polymerized on the treated mesh. After 30 min, the slides and adherent grids were gently washed in KMT buffer. The grids were fixed with 1% glutaradehyde diluted from a 25% stock solution in KMT buffer for 10 min. After fixation the grids were

dipped in KMT buffer, and then placed in 0.5% OsO_4 in KMT for 5 min. They were then: dehydrated through increasing concentrations of ethanol; critical-point-dried from ethanol with liquid CO_2, shadowed with gold; and finally viewed in a Hitachi 750 transmission electron microscopy at an accelerating voltage of 75 keV. As mammal control, ribosomes from mouse liver tissues underwent exactly the same process.

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Legend to the figures

Figure 1. Different ribosomal RNA component between flatworm, mammalian tissues and nematode. **A**. Ethidium bromide (EB) staining of total RNA electrophoresis gel (top panel) and Northern blotting analysis of rRNA transcripts in different tissues (lower panels). Single band (arrows) of ribosomal RNA of SEP was detected by EB staining (0.01%, 20 min) after usual electrophoresis of 5 μg/lane total RNAs. Lane 1-5 represent murine liver tissue, spleen tissue, SEP (isolated by acid guanidinium thiocyanate-phenol-chloroform (GTC) method [16, 18], SEP (isolated by Isogen kit [11], NipponGene Co., Kyoto, Japan), nematode *Anisakis simplex* (isolated by Isogen kit), respectively. For Northern blotting, total RNAs were denatured in 50% formamide and 2.2 M formaldehyde at 57°C for 14 min. Aliquots containing 5 μg of total RNA were loaded onto a 1% agarose gel containing 2.2 M formaldehyde and electrophoresed using a running buffer containing 50 mM MOPS-acetate (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. The cDNA probes for SEP 18S or 28S rRNA or rat 18S rRNA were labeled with [α-^{32}P]dCTP (ICN, Biomedical Inc, CA, USA) by random priming as described in the TaKaRa BcaBEST Labeling Kit (TakaRa Biomedicals, Tokyo, Japan). Northern hybridization was performed according to the standard method as described [11, 13]. **B**. Observation of the EB staining patterns upon increasing the SEP RNA amount from 1 to 6 μg per lane. **C**. EB staining analysis of flatworms and roundworms. Eight μg of total RNAs from the indicated tapeworm *Diphyllobothrium hottai (D. hottai)*, *Diphyllobothrium nipponkaienzeme (D. nipponkaienzeme)*, fluke *Paragonimus westermani (P. westermani)*, and roundworm *Anisakis simplex (A. simplex)*, were denatured, respectively, then subjected to electrophoresis, and finally stained as shown in Panel A.

Figure 2. Further analysis and identification of the single ribosomal RNA band. **A**. Extremely long distance electrophoresis of total RNAs to determine if the single rRNA band is composed of a single component. Eight μg of total RNA were denatured, and underwent a long electrophoresis (15 cm) and then stained with EB as described in Fig.1. A similar rRNA electrophoresis pattern is observed in liver tissue, *E. coli*, and nematode *Anisakis simplex (A. simplex)*. The original single band of SEP resolved into three sub-bands. The number of rRNA bands in *D. hotti* and fluke *Paragonimus westermani* (P. *westermani*) remain unchanged. **B**. Northern blot analysis of the sub-bands. The rRNAs were stained with EB, transferred onto member and then hybridized with SEP 18S rRNA and 28S rRNA probes,

respectively. L and S represent RNAs from liver tissue and SEP, respectively. Both upper (10.3 cm) and lower bands (10.7 cm) hybridized only with SEP 28S rRNA; and the middle band (10.5 cm) hybridized only with SEP 18S rRNA probe.

Figure 3. Electron microscopy examination of ribosomes. Plerocercoid ribosomes were isolated as described [17], treated by shadowing, and observed under transmission electron microscopy essentially as described [7]. Briefly, ultracentrifugation-purified ribosomes (1 mg/ml) were polymerized on electron microscope grids attached to glass slides. The grids were coated, washed, and allowed to dry until use. A drop of SEP ribosome solution was polymerized on the treated mesh. The slides and adherent grids were gently washed in TBS. The grids were fixed with 1% glutaradehyde, dipped in KMT buffer, and then placed in 0.5% OsO_4 for 5 min. They were dehydrated through increasing concentrations of ethanol, critical-point-dried from ethanol with liquid CO_2, shadowed with gold, and then viewed in a Hitachi 750 TEM. Liver ribosomes, as controls, underwent the same protocol. **A**. Liver ribosomes with one small and one large subunits (indicated by arrows); **B**. The ribosomes from SEP take the shape of either a single ball (subunit, up) or two similar size balls (subunits, down).

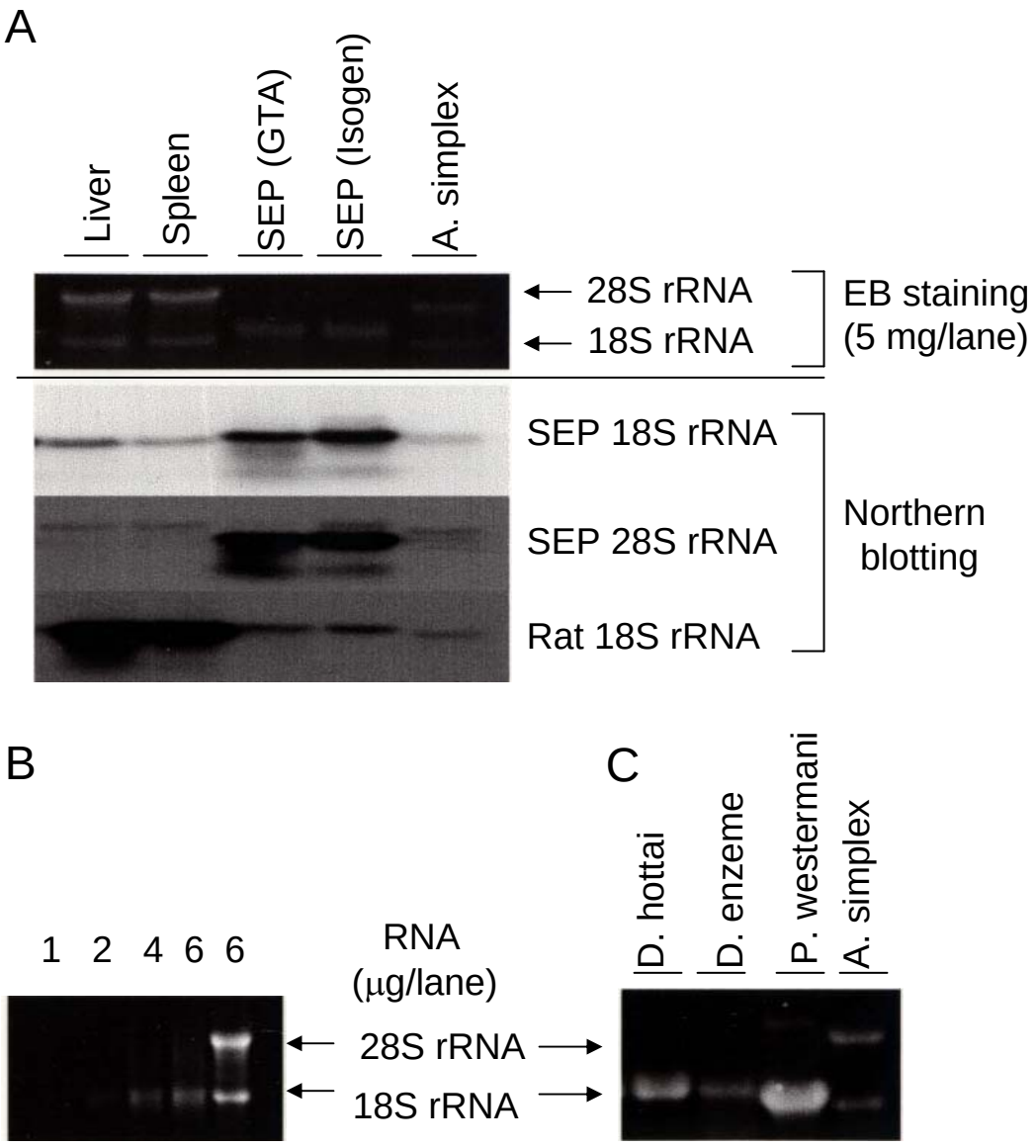

Figure 1

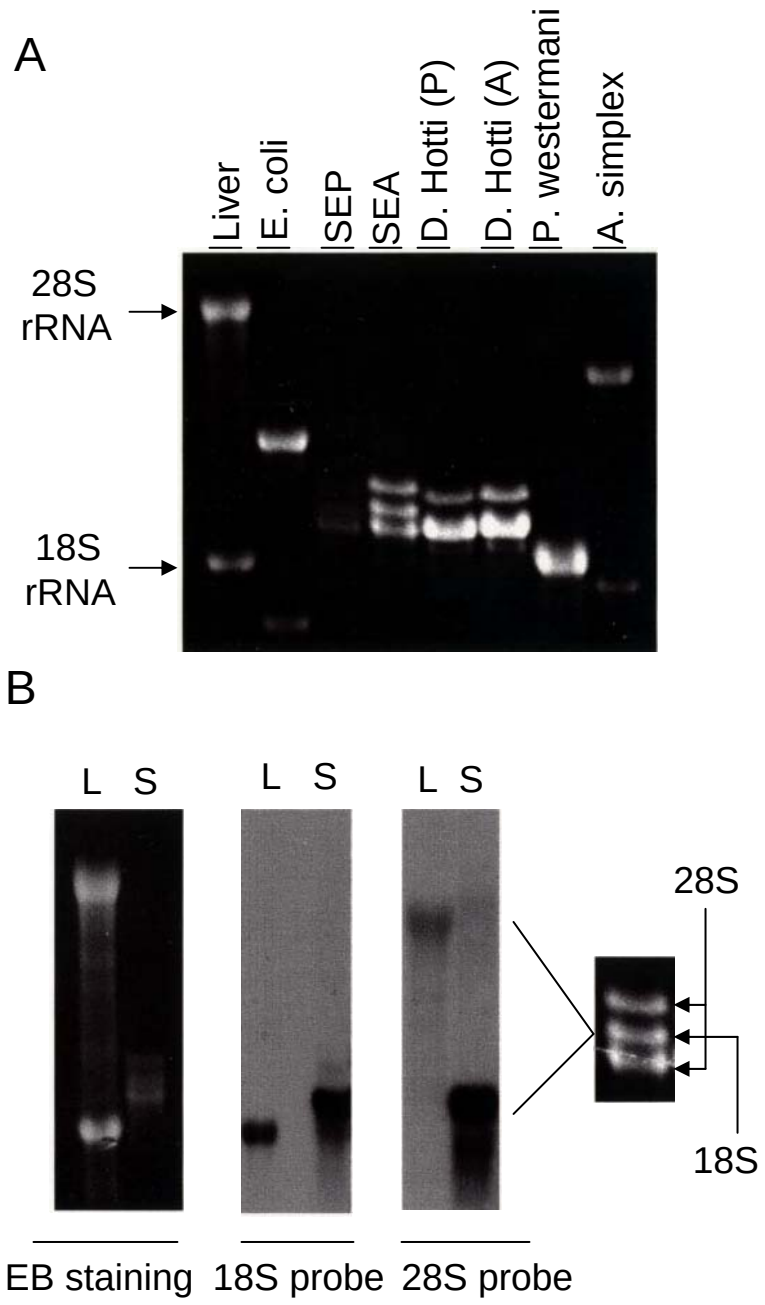

Figure 2

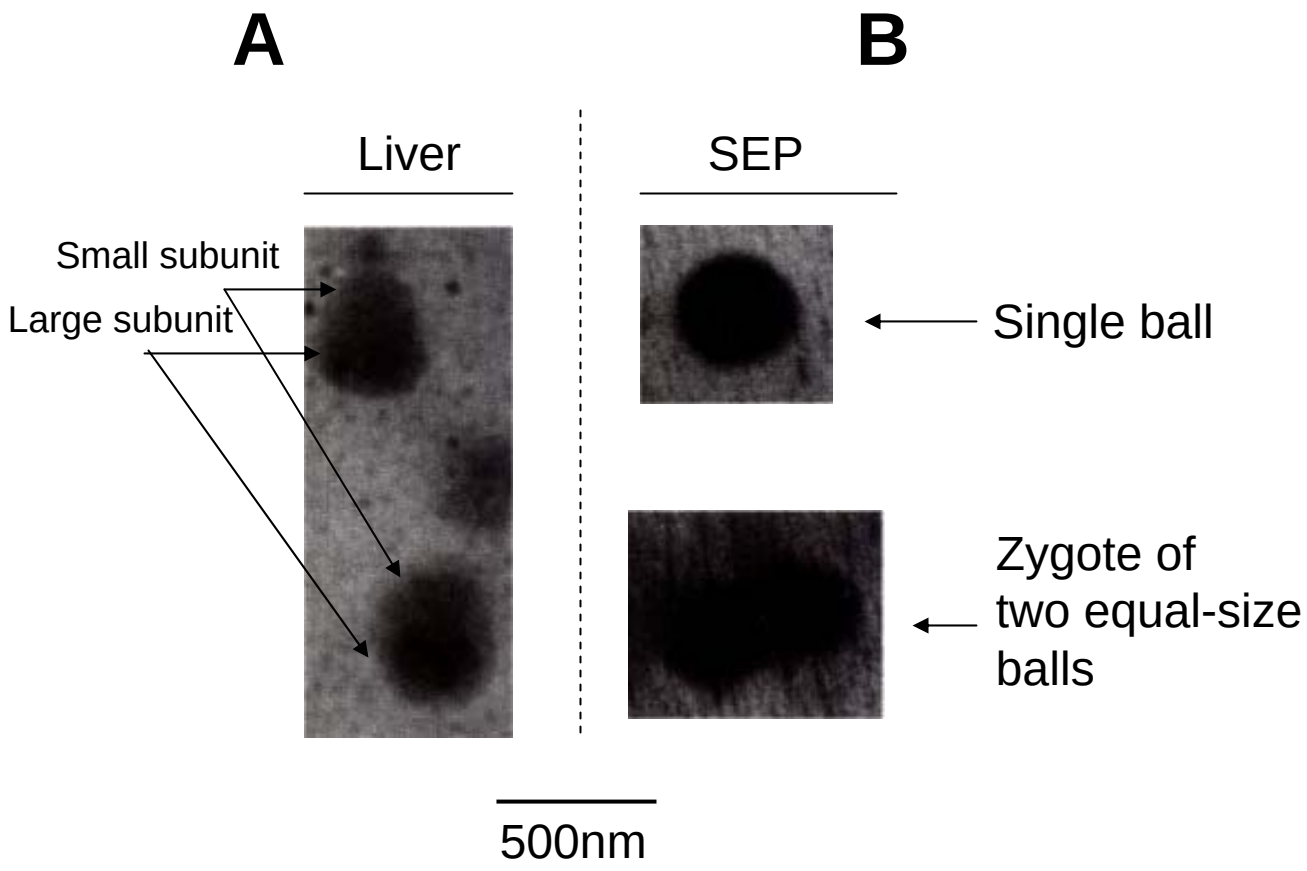

Figure 3