EXTRACELLULAR RIBOSOMES DURING METAMORPHOSIS OF THE BLOWFLY CALLIPHORA VICINA R. D. -- A REAPPRAISAL OF THEIR AUTHENTICITY

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SUMMARY. Extracellular ribosomes during adult development of the blowfly Calliphora vicina were previously considered to occur naturally in vivo. A variety of radioisotopic experiments performed at different stages of metamorphosis now demonstrates that the specific activities of extra- and intracellular ribosomes are consistently very similar. Further, in addition to previously described physico-chemical similarities, extra- and intracellular ribosomal protein profiles are now shown to be essentially identical. These results vitiate the notion of a natural pool of extracellular ribosomes, the occurrence of which is now ascribed to an experimental artifact, resulting from unusual cell fragility.

In an earlier paper (1) we presented what appeared to be convincing evidence for the occurrence of Erib[2] during metamorphosis of the blowfly Calliphora vicina R.D. (= C. erythrocephala (Meig.)). These earlier observations, which we have repeatedly confirmed, left little doubt that under very mild isolation procedures presumably ensuring minimal cell damage, free ribosomes were present in the 20,000 g supernatant derived from the internal contents of developing adults. The possibility that such Erib were the product of cell damage (lysis) was nevertheless not excluded. Isotopic experiments described below now point to artificial cell rupture as the probable explanation for the occurrence of Erib. Apparently these do not occur naturally in vivo during pharate adult development, but are artificially released into the isolation medium during handling.

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[2] Abbreviations: Erib, extracellular ribosomes; Irib, intracellular ribosomes.

MATERIALS AND METHODS

Labeled ribosomes at various stages of the life cycle were prepared from aseptic larvae grown on a sterile, semi-defined synthetic diet (2,3) to which both labeled uridine and lysine (New England Nuclear) had been added. 15-20 aseptic eggs were added to 12-13 ml medium in 50 ml Erlenmeyer flasks, or about 100 eggs to 75 ml medium in 250 ml flasks. Ribosomes were also labeled by injection of isotope. The tracer was concentrated to about 1.0 μCi/μl and neutralized. Larvae were chilled in ice, anaesthetized with solid CO_2 vapor, and individually injected with 1.0 μl of isotope dorso-laterally in the 3d posterior segment using a fine glass needle (diameter of tip 35-40 μ) attached by polyethylene tubing to an "Agla" micrometer actuated syringe (Burroughs Wellcome Co.). Unanesthetized developing adults ("pupae") were injected similarly, through a small hole made with a fine steel needle in the rigid, puparial cuticle. Erib and Irib were prepared and quantitated as previously described (1,2). Ribosomal proteins were isolated and subjected to two-dimensional polyacrylamide gel electrophoresis (4). For radioactivity measurements, aliquots of CCl_3 COOH-precipitated ribosomes were collected on glass fiber discs, treated with NCS (New England Nuclear) solubilizer, and counted in 10 ml of scintillation fluid consisting of xylene-Triton X-100 (2:1 v/v) containing 7 g/l Omnifluor (NEN). All samples were corrected for quenching.

RESULTS

Labeled diet experiments. Blowflies were raised on semi-defined diets containing varying amounts of [G-^{3}H]lysine and [U-^{14}C]uridine. The specific activities (dpm/mg) of isolated ribosomes prepared from three developmental stages showed a progressive decrease during the transformation of the larva through the developing (pharate) adult to the adult fly. Of particular relevance, however, is that the ratios of specific activities of RNA in Erib particular relevance, however, is that the ratios of specific activities of RNA in Erib to RNA in Irib, and of the corresponding ribosomal proteins,

Table 1

Radioactivity of ribosomes isolated from developmental stages of the blowfly.

Stage	$\frac{\text{s.a.}^{*}\ \text{Irib}}{\text{s.a. Erib}}$			
	Precursor fed		Precursor injected	
	[^{3}H]Lys	[^{14}C]Urd	[^{3}H]Lys	[^{14}C]Uri
Larva	(96)#	(60)	(54)#	(109)
2-day pharate adult	1.05	1.04	1.11	1.08
6-day pharate adult	1.02	1.00	1.03	1.02
Adult fly, 1-day post emergence	(52)#	(47)	(38)#	(76)

For precursor fed larvae were grown on synthetic diet containing 100 μCi [^{3}H]lysine and 7.5 μCi [^{14}C]uridine in 80 ml medium. For precursor injected, early 3rd instar larvae were individually injected with 1.4 x 10^{6} dpm of [^{3}H]lysine and 0.5 x 10^{6} dpm [^{14}C]uridine.

*s.a. (specific activity) = dpm/mg ribosomes based on 10 $A_{260\ nm}$ = 1 mg ribosomes.

#At these stages of development little or no Erib exist, and hence no ratio can be calculated. The values in parentheses are the actual specific activities of the purified ribosomes.

were essentially unchanged during pharate adult development. The results of one such experiment, typical of several, are shown in Table I.

Isotope injection experiments. Young (feeding) and mature (post-feeding) 3rd instar larvae were injected with a mixture of [G-^{3}H]lysine and [U-^{14}C]uridine. Labeled Erib and Irib were subsequently isolated from developing adult and adult flies, and their specific activities determined. Five such experiments gave reproducible results independent of larval age at the time of injection. A typical experiment shown in Table 1 demonstrates that again there were no differences in specific activity ratios

between pharate adult Erib and Irib-RNA's or the respective ribosomal proteins. These results are therefore similar to those of the feeding experiments described above.

In other experiments doubly labeled ribosomes prepared from larvae grown on radioactive diets containing [G-^{3}H]lysine and [U-^{14}C]uridine were injected into fully grown "cold" larvae at the post-feeding (wandering) stage. Ribosomes were then isolated during adult development and their radioactivity determined. The percentage of injected radioactivity recovered in ribosomes progressively declined during metamorphosis, more rapidly at first, and subsequently more slowly. The course of the decline followed neither first nor second order kinetics. A very slow, steady decrease in the percentage of recoverable radioactive ribosomes commenced on the 2nd day after pupariation and continued through adult emergence. Adults contained approximately 10% of the initially injected radioactivity in ribosomes, of which about half could be obtained only after homogenization in the presence of 1% deoxycholate indicating that they were membrane-bound. There was no change in the initial ribosomal $^{3}H/^{14}C$ ratio of 1.9 - 2.0 at any time, but the reduced ribosomal radioactivity was accompanied by appearance of tritiated CCl_3COOH-precipitable material (presumably protein), and of ^{14}C counts in CCl_3COOH-soluble form in ribosome-free supernatants. These findings are indicative of ribosomal degradation.

The crucial experiments, however, were those involving injection of tritiated uridine into pharate adults[1)] at a stage when Erib were most abundant. Since rRNA is normally synthesized by the nucleolus within intact cells (5), it would be predicted that the RNA of pre-existing Erib would either be unlabeled, or would certainly have a lower specific activity than Irib-RNA. Accordingly, insects were injected either on the 3rd or 5th day after pupariation, and 22 hr later Erib and Irib were isolated.

1)Earlier experiments involving injection of pharate adults were vitiated due to unacceptably high mortality. By using the finer needles described, the mortality was reduced to about 10%.

Table 2

Radioactivity of ribosomes following injection of [^{14}C] uridine into pharate adult blowflies.

Pharate adult age (days after pupariation)	Specific Activity (dpm/$A_{260\ nm}$)		s.a. Irib / s.a. Erib
	Irib	Erib	
3	13,919	14,230	0.98
5	16,240	16,823	0.97

Pharate adults were injected with 0.5 - 0.6 μCi [^{14}C]uridine (specific activity: 470 μCi/μMole). Ribosomes were isolated 22 hr after injection.

The results (Table 2) show that again the specific activities of Erib and Irib were virtually identical, a finding contrary to prediction had Erib existed.

Ribosomal proteins. The number of pharate adult ribosomal protein "spots" on two dimensional gel electrophoresis was 51 for Irib and 54 for Erib, the protein-profiles being virtually identical except for three weak Erib "spots," (Fig. 1). These three Erib proteins may be due to soluble proteins which can bind strongly to ribosomes. This possibility is strengthened by our unpublished finding that two of the three Erib proteins were absent from adult fly ribosomes, and were replaced by two new protein "spots" not observed in either Erib or Irib of the developing pharate adult.

DISCUSSION

Our isotope experiments demonstrate that Erib and Irib do not exist as two biologically distinct pools within the developing insect. Aside from the unlikely possibility of extracellular de novo biosynthesis of Erib, there are two possible explanations for Erib and Irib having the

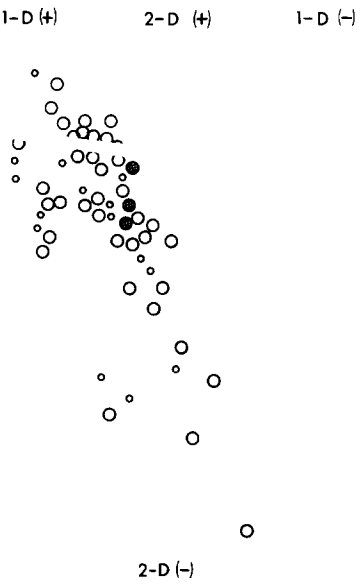

Fig. I. Two-dimensional electropherogram of C. vicina intracellular and extracellular ribosomal proteins. Electrophoresis in the 1st dimension: 8% acrylamide, pH 8.6, running time 24 hours; 2nd dimension: 18% acrylamide, pH 4.6, running time 22 hours. Large circles, major protein "spots;" small circles, weak protein "spots;" cross-hatched circles, three Erib proteins not present in Irib.

same specific activities following injection of labeled precursor at a stage when the Erib titer is maximal: (1) If Erib and Irib were in extraordinarily rapid equilibrium, such that within 22 hr the approx. 40% of the entire ribosomal pool which is extracellular at the time when precursor was injected had equilibrated with all the labeled ribosomes of the Irib pool. Injection of pre-labeled ribosomes into mature larvae does indicate that Erib may in fact be converted to membrane bound Irib, but to a much smaller extent and at too slow a rate to account for the assumed equilibrium. This possibility must therefore be discounted. (2) The 2nd, and more plausible possibility, which explains all the findings, is that Erib are an experimental artifact due to accidental cell breakage at the time of extraction *in vitro*.

Evidence for unusual cell fragility during metamorphosis is provided

by the following preliminary experiment. The contents of a group of 4-day pharate adult "pupae" were gently squeezed out through the amputated anterior end of the puparium into cold embryonic Drosophila cell tissue culture medium (6). After 10 min the suspension was centrifuged at 70 g; the cell-free supernatant was re-centrifuged at 20,000 g, decanted, and centrifuged at 105,000 g. The absorbance of the resulting ribosomal pellet was about half of that obtained from a comparable group of insects treated in the conventional manner. Whether the difference was due to the nature of the extraction medium, or to the much lower initial g force -- fragile cells would likely be broken at 20,000 g -- was not determined. The role of an ill-defined "membranolytic factor" with maximal activity at about the 5th day of development (7) may partially explain the cell fragility.

None of our results negates the possibility of some naturally occurring Erib, which indeed might be anticipated from the cellular events during metamorphosis to be released in small numbers.

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