Post-transcriptional regulation of endoribonuclease VI expression during *Artemia* development

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Expression of endoribonuclease VI activity during early larval development of the crustacean *Artemia* has been studied in vivo in the presence of different inhibitors of transcription and translation. All the transcription inhibitors tested (α-amanitin, cordycepin and actinomycin D) did not affect the expression of the activity. However, protein synthesis inhibitors (cycloheximide and anisomycin) prevented the appearance of the activity. These data strongly indicate that the control of the expression of endoribonuclease VI is carried out post-transcriptionally.

Artemia *Endoribonuclease VI* *Development* *Transcription inhibitor* *Translation inhibitor* *Post-transcriptional regulation*

1. INTRODUCTION

Artemia (class crustacea, order anostraca) can undergo a developmental program in which a cryptobiotic state is reached under some environmental situations. When optimal conditions of hydration, temperature and oxygenation are met, cryptobiotic gastrulae resume development giving rise to free swimming larvae (nauplii) (see [1] for reviews and specific information on *Artemia* development). Several enzyme activities are expressed de novo during early larval development, including several proteases [2] and a ribonuclease [3], which has been characterized as endoribonuclease VI [4]. It is not clear which are the mechanisms that regulate the expression of these enzymatic activities. In a preliminary study [5] it was suggested that both types of enzymes were regulated post-transcriptionally. This communication presents our studies on the in vivo induction of endoribonuclease VI in the presence of different inhibitors of transcription and translation.

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid

2. MATERIALS AND METHODS

Artemia cryptobiotic gastrulae (cysts) were from San Francisco Bay Brand, Division of Metaframe. Poly(U), soybean trypsin inhibitor, actinomycin D, anisomycin and cordycepin were from Sigma. Cycloheximide was from Calbiochem. α-Amanitin was from Boehringer and proteinase K from Merck.

To obtain nauplii, cysts were treated as in [6] and incubated at 30°C in sterilized 0.25 M NaCl containing 20 mg/l penicillin and 100 mg/l streptomycin. Hatched nauplii were harvested in a separation funnel. If longer incubations were needed, sterilized sea water plus antibiotics was used. Between 5000 and 10 000 larvae were incubated in 25 ml of medium at 30°C in petri dishes or tissue-culture plastic bottles (Corning) in the induction experiments. Nauplii were allowed to acclimatize for 1 h before addition of inhibitors. At the end of

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the incubation time larvae were collected by filtration, washed with water (or 10 mM sodium phosphate buffer, pH 7.0, if $^{32}P_i$ was incorporated) and immediately frozen in dry ice. Nauplii were stored at −20°C until use (normally overnight).

To measure endoribonuclease VI activity, larvae were homogenized in 1 ml of 50 mM Tris-HCl (pH 7.5) 10 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 20% glycerol and 100 μg/ml soybean trypsin inhibitor. Homogenates were centrifuged at 27 000 × *g* for 20 min and endoribonuclease VI activity assayed as described elsewhere [4]. 1 unit of activity is defined as the amount of enzyme that renders acid-soluble 1 μmol of UMP/min under the assay conditions with poly(U) as substrate.

To study $^{32}P_i$ incorporation into RNA, total nucleic acids were extracted as follows; larvae were homogenized in 1 ml of 20 mM HEPES (pH 7.6) 70 mM KCl, 9 mM $Mg(CH_3COO)_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol plus 250 μg heparin/ml, treated with 1% diethylpyrocarbonate and sterilized [7]. Homogenates were made 0.5% sodium dodecylsulfate and incubated with 50 μg/ml proteinase K for 1 h at 37°C. The homogenates were extracted at least 3 times with phenol-chloroform-isoamyl alcohol (25:24:1) and the aqueous phase precipitated with 2.5 volumes of ethanol at −20°C overnight. Nucleic acids were resuspended in sterile water. After measuring the absorbance at 260 nm, radioactivity incorporated into RNA was estimated by pancreatic ribonuclease digestion. Protein was determined by the method in [8].

3. RESULTS AND DISCUSSION

Authors in [3] showed that endoribonuclease VI is first expressed after hatching during *Artemia* development. We have shown that this activity continues to increase at least until late larval stages (14 days after hatching) [4].

Table 1 shows the effect of the incubation of *Artemia* nauplii with different inhibitors on the increase of the levels of endoribonuclease VI activity after hatching. Experiment 1 shows that α-amanitin, cordycepin and actinomycin D have no effect on the levels of the enzyme, whereas cycloheximide completely prevents the increase of the activity. The concentrations used of the different inhibitors were those that give 50% lethality in 24 h of nauplii hatched at 18 h (J. Sebastián, unpublished results). Experiment 2 in table 1 shows that increasing the concentration of actinomycin D in the incubation medium does not

Table 1

Effect of different inhibitors on endoribonuclease VI expression

Experiment	Inhibitor	Activity ($U/10^5$ nauplii)		
		$t = 0$	$t = 6$	Δ
1	Control	5.42	8.22	+2.80
	α-Amanitin (5 μg/ml)		8.17	+2.75
	Cordycepin (100 μg/ml)		8.16	+2.74
	Actinomycin D (100 μg/ml)		7.49	+2.07
	Cycloheximide (50 μg/ml)		4.68	−0.74
2	Control	0.88	4.67	+3.79
	Actinomycin D (100 μg/ml)		3.57	+2.69
	Actinomycin D (200 μg/ml)		3.81	+2.93
	Actinomycin D (400 μg/ml)		3.83	+2.95
3	Control	1.17	7.93	+6.76
	Cycloheximide (50 μg/ml)		1.37	+0.20
	Anisomycin (50 μg/ml)		0.56	−0.61

In experiment 1 nauplii were harvested at 21 h after resumption of development. In experiments 2 and 3 larvae were harvested at 13 h. In all cases, inhibitors were added 1 h later (zero time) and incubation was followed for 6 h at 30°C. Δ refers to the difference of activity between $t = 6$ and $t = 0$

produce any effect on the extent of endoribonuclease VI expression. Experiment 3 shows that anisomycin, another translation inhibitor, has the same effect as cycloheximide. In experiments 2 and 3 endoribonuclease VI activity at zero time is lower than in experiment 1 because nauplii were harvested at 13 h instead of 21 h, to get a more synchronous population and therefore a more clear effect of the inhibitors (the increase in activity in experiment 1 is 1.5-fold whereas in experiments 2 and 3 it is of 5- and 7-fold, respectively).

Figure 1 shows the time course of the expression of endoribonuclease VI. Cycloheximide inhibits the increase of the levels of the enzyme from the first hour of incubation. Nauplii incubated with actinomycin D show a parallel induction curve with that of the control larvae.

Of the 3 transcription inhibitors tested, we chose actinomycin D to make positive control experiments to exclude the possibility that the inhibitor is not affecting endoribonuclease VI expression because of a failure in the transport of the inhibitor inside the cells (although the lethality results mentioned above argue against this possibility). Table 2 shows that actinomycin D suppressed RNA synthesis in conditions in which endoribonuclease VI expression is not affected. Experiment 1 was carried out in parallel with that shown in fig. 1, with nauplii harvested at 13 h. As authors in [9] had shown that $^{32}P_i$ incorporation into RNA is increased at later time in development, in experiment 2 (table 2) larvae were harvested at 21 h and the labeling period was increased to 4 h. Although there is more incorporation, both experiments clearly show that actinomycin D effectively inhibits $^{32}P_i$ incorporation into RNA.

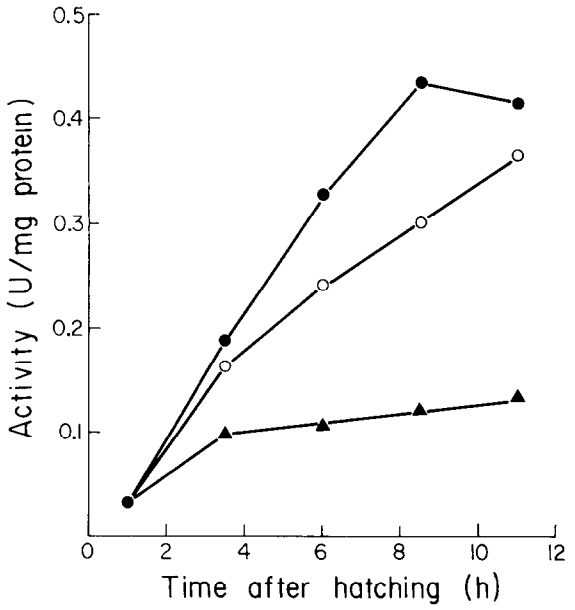

Fig.1. Induction of endoribonuclease VI activity. Nauplii were harvested at 13 h as described in section 2. Inhibitors were added 1 h later (zero time): (●) control without inhibitors; (○) incubation in the presence of 100 μg/ml actinomycin D; (▲) incubation in the presence of 50 μg/ml cycloheximide.

We have demonstrated that the expression of endoribonuclease VI is dependent on protein synthesis. There are however, several alternative explanations to our data: de novo protein synthesis could be needed to make a factor(s) that could unmask the enzymatic activity already present in encysted embryos; this factor could also activate a 'pro-enzyme' or could inactivate an inhibitor complexed with the enzyme in encysted embryos; alternatively, translation of endoribonuclease VI mRNA takes place at the time of the expression of the activity.

We have looked for the presence of masked activity in encysted embryos by making homogenates

Table 2

Incorporation of $^{32}P_i$ into RNA during *Artemia* development (total cpm)

Experiment	Pulse time (h)	Actinomycin D	
		−	+
1	19–21	95	75
	21–23	1785	70
2	25–29	27 865	90
	29–33	7641	95

In experiment 1 nauplii were harvested at 13 h (same experiment shown in fig.1); in experiment 2 larvae were harvested at 21 h after resumption of development. 1 h later actinomycin D was added at 100 μg/ml in 25 ml of medium. Cultures were labeled with 10 μCi/ml of carrier-free $^{32}P_i$ (Radiochemical Center, Amersham). Radioactivity incorporated into RNA was estimated as described in section 2. The total amount of RNA extracted in the different cultures varied between 120 and 200 μg.

in the presence of the chaotropic agents potassium thiocyanate and potassium iodide [10]. No activity was found in the encysted embryos under such conditions (not shown).

The results presented here strongly support the idea that the expression of endoribonuclease VI is controlled post-transcriptionally, although we cannot rule out the pro-enzyme hypothesis. We believe however, that the fact that the expression of other enzymes, such as neutral proteases [5] and an aminoacyl-tRNA hydrolase found in *Artemia* larvae [5,11], have the same sensitivity to translation inhibitors, makes this hypothesis unlikely. Further support to post-transcriptional regulation of endoribonuclease VI comes from the fact that *Artemia* encysted embryos contain stored mRNAs, as messenger ribonucleoprotein particles, both cytoplasmic and membrane-bound [12,13]. These mRNAs could code for the different enzymes and proteins that are expressed after hatching.

The final evidence about the post-transcriptional regulation of the expression of endoribonuclease VI will come with the use of antibodies against the enzyme, which will allow to detect endoribonuclease VI protein at different times of development. We are now in the process of making such antibodies.

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