Changes in polysome content during development after diapause of *Bombyx mori* embryos

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Undegraded polysomes could be extracted from eggs of *Bombyx mori* by cutting egg shells with a blade in a buffer containing high salt. The polysome content as measured by this method increased steeply during post-diapause development, which was commenced by long term chilling followed by hot HCl treatment of diapausing eggs. At the 24th hr of the post-diapause development the polysome content became 2.6-times the level at 0 h. The alteration of the polysome content paralleled that of the incorporation of [^{14}C]leucine into acid-insoluble fractions investigated by modified Takami's embryonic culture system.

Polysome content *Polysomal ribosomes* *Post-diapause development*
Bombyx *embryos* *Protein synthesis* *Embryonic fragment culture*

1. INTRODUCTION

Eggs of *Bombyx mori* enter into diapause at an early embryonic stage if they are destined to hibernate. Morphogenesis is suppressed during diapause and wintering, but it starts soon after diapause is terminated. It was shown that wintering *Bombyx* eggs contain polysome-like aggregates [1] which were reported to be inactive in protein synthesis [2]. Histologically, ribosomal particles are observed between whirling lamellae of rough endoplasmic reticulum (ER) in diapausing *Bombyx* eggs but the whirling structures disperse after the resumption of development [3]. Ribosome preparations isolated from diapause eggs of *Bombyx* exhibit marked dissociability in high salt and after the termination of diapause the proportion of non-dissociable ribosomes rises [4]. These findings appear to imply that activated ribosomes increase as diapause breaks.

At present, little information exists about the net changes of polysomes and about the proportion of ribosomes forming polysomes, in relation to the termination of diapause in *Bombyx* eggs. Here, we measure the amounts of polysomes and total ribosomes at, and after, the onset of post-diapause development. This was made possible by applying a technique devised for obtaining undegraded polysomes from eggs with rigid shells [5]. The polysome content was found to increase parallel to the incorporation of a labeled amino acid into acid-insoluble fractions investigated with fragmented embryos cultured in vitro.

2. MATERIALS AND METHODS

Freshly deposited eggs of a strain Kinshu × Showa were kept at 25°C until day 3, by which time the dumbell-shaped embryos (at the early gastrula stage) had entered diapause. Then the eggs were kept at 5°C for 3 m in order to terminate diapause. These will be called chilled eggs. The chilled eggs were treated with hot HCl (spec. grav. 1.10, 48°C, 6 min) to allow synchronous post-diapause development (98% of the eggs hatched on day 10 of incubation, at 25°C). The period shortly after the hot-HCl treatment is termed 0 h of age. Embryos were staged according to Takami and Kitazawa [6].

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Polysomes were extracted and analyzed as reported elsewhere [5]. In brief, 1000 eggs were cut with a blade at 4°C in 2 ml of 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ and 500 mM KCl + polyvinylsulfate and diethyl pyrocarbonate as RNase inhibitors. The cut eggs were disturbed by passage through a Pasteur pipette and centrifuged at 900 × *g* for 10 min. The supernatant was made 1% with respect to sodium deoxycholate and centrifuged at 18 000 × *g* for 15 min. An aliquot of the supernatant (lysate) was fractionated in an 11 ml 15–50% (w/w) linear sucrose density gradient, made up with the extraction buffer minus RNase inhibitors at 49 000 × *g* for 3 h with a Hitachi 40T rotor. Absorption of the gradient was traced with an ISCO UV monitor at 254 nm. Polysomes and ribosomal subunits were quantified by determining planimetrically the areas of relevant regions above base lines, which were established by blank gradients run with the post-ribosomal supernatant. The areas of polysomal regions relative to total ribosomal regions (polysomes + subunits) were expressed in terms of percent polysomal ribosomes. Polysomes were validated by their sensitivity to EDTA (10 mM for 1 h in the cold) and RNase (Worthington, × 1 crystallized, 5 μg/ml for 10 min in the cold) added to the lysates. By either of the treatments polysomes were dissociated quantitatively to subunits or monosomes.

RNA was extracted from eggs by the phenol method and electrophoresed in gels as in [7]. The areas of peaks were measured planimetrically to calculate the ratios of rRNA (28 S + 18 S) and 'sRNA' (4 S + 5 S) to total RNA. The bulk RNA and DNA contents in the total eggs or in the lysates were determined as in [8] and [9], respectively, after the fractionation was made as in [10]. The rRNA content was estimated to be the product of the bulk RNA content and the ratio of rRNA to total RNA.

For incorporation of radioactive precursors, 5 eggs were immersed in 1 ml of the physiological saline prescribed for embryonic culture of *Bombyx* [11], and cut by using fine scissors so that each embryo was divided in two, through the antero–posterior axis. The cut eggs were gently passed through a Pasteur pipette to detach cut tissues from shells, and the whole suspension was mixed with [^{14}C]leucine (NEN, 0.25 μCi/mmol), penicillin (100 units/ml) and streptomycin (50 μg/ml). After incubation at 25°C the tissues were washed with ice-cold saline and homogenized in 2 ml of 0.5% sodium dodecyl sulfate. The homogenate was mixed with 1 vol. of 10% trichloroacetic acid and boiled for 10 min. The precipitate was washed with 5% trichloroacetic acid, dried and counted for radioactivity in 10 ml of 35% Triton X-100, 65% toluene, and 6 mg 2,5-diphenyloxazole/ml.

3. RESULTS

As seen in table 1, the DNA content increased slightly until 24 h and then rapidly until 72 h after the onset of post-diapause development. The bulk RNA content rose gradually. The relative amount of rRNA varied slowly showing a broad peak at 72 h. The large proportion of 'sRNA' for the chilled and 0-h eggs as shown in table 1 may not be due to artefacts produced during extraction of RNA, since the extraction seemed to be valid on the basis that the ratios of 28 S RNA to 18 S RNA in the electropherograms were 2:1 for all samples.

We have found that intact polysomes could be extracted from *Bombyx* eggs only by a mild method without using homogenizers [5]. Under these conditions (see section 2) the lysates contained 65–75% of total egg RNA for all the stages of eggs tested, suggesting a constant recovery of polysomes in the lysates. The high salt added to the extraction buffer may be effective for cell lysis, since 500 mM KCl gave a higher recovery of RNA in the lysates than lower [KCl]. KCl at >500 mM broke polysomes. During extraction, 500 mM KCl may be diluted to 380 mM with the internal water of eggs [12]. Deoxycholate added to the 900 × *g* supernatant increased the recovery of RNA in the lysates. Nonidet P-40 and Triton X-100 were less effective. Ribosome monomers (80 S) free from mRNA and tRNA would be dissociable into subunits in 300–500 mM KCl [13,14]. Here, 500 mM, but not 300 mM, KCl in the sucrose gradient could diminish 80 S peaks. Usually degradation of polysomes would give undissociable 80 S bound with mRNA fragments, and the minimum occurrence of such 80 S at early stages of development (fig. 1a,b) suggests that degradation of polysomes into monosomes was negligible. At later stages the 80 S peak was consistently observed (fig. 1c), implying degradation of polysomes, or the occurrence of functional monomers. We assume these 80 S frac-

Table 1

Changes in content of nucleic acids and in ratio of rRNA, sRNA and polysomes during post-diapause development

Eggs		Content/egg		Relative content		Polysomal ribosomes: total ribosomes (%)
Age (h)	Stage	DNA (ng)	RNA (μg)	rRNA (%)	'sRNA' (%)	
Chilled		25	0.93	66.9	33.1	34
Post-diapause						
0	14	25	0.93	66.9	33.1	35
10		28	0.96	76.0	24.0	66
20		32	1.00	85.0	15.0	75
24	16	34	1.02	88.5	11.5	75
48	17	65	1.18	85.9	14.1	74
72	18	94	1.42	91.3	8.7	73
96	20	103	1.68	87.0	13.0	67
120	21	–	2.20	83.1	16.9	60
144	24	–	2.25	89.9	10.1	45
168	25	–	3.15	85.7	14.3	35

Analyses of nucleic acids were as in section 2. The ratios of polysomal ribosomes were derived from the planimetrical measurements of profiles as in fig. 1

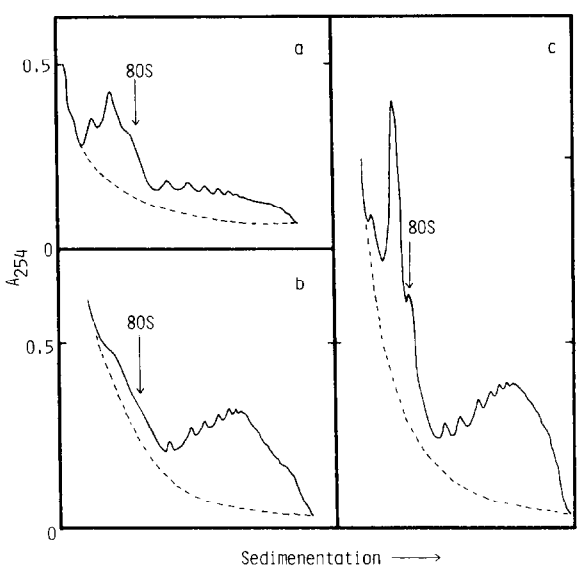

Fig. 1. Polysomal profiles of eggs at 0 h (a), 19 h (b) and 120 h (c) of post-diapause development. Lysates of eggs were sedimented in 15–50% sucrose gradient in the presence of 500 mM KCl (see section 2).

tions are polysomal ribosomes.

The gradient sedimentation pattern of polysomes for chilled eggs (not shown) was very similar to that of polysomes for 0-h eggs (fig. 1a). Eggs of later stages exhibited increased polysomal peaks (fig. 1b,c). The polysomes with 8 or more ribosomes could be clearly distinguished (fig. 1a–c). The ratio of polysomal ribosomes increased 2.2-fold up to 20 h, remained at about 75% up to 48 h and decreased to 35% after 168 h (table 1). The polysome content was calculated by the ratio of polysomal ribosomes, multiplied by the total ribosome content, which was assumed to be twice the rRNA content. Fig. 2 shows that the amount of polysomes was 2.6 × the 0-h amount during the first 24 h and gradually increased thereafter. The content of ribosomal subunits changed inversely to that of polysomes (fig. 2). These overall findings were reproducible in several series of experiments.

The eggs were cut at intervals during post-diapause development and the fragmented embryos, together with yolky substances, serous cells and broken shells, were incubated with [^{14}C]leucine. This label was incorporated into acid-insoluble fractions linearly for the first 2 h of incubation. Thereafter, the incorporation leveled off until 6 h. When [^{3}H]thymidine or [^{3}H]uridine was mixed with [^{14}C]leucine during incubation, ^{3}H was incorporated into acid-insoluble fractions (not boiled)

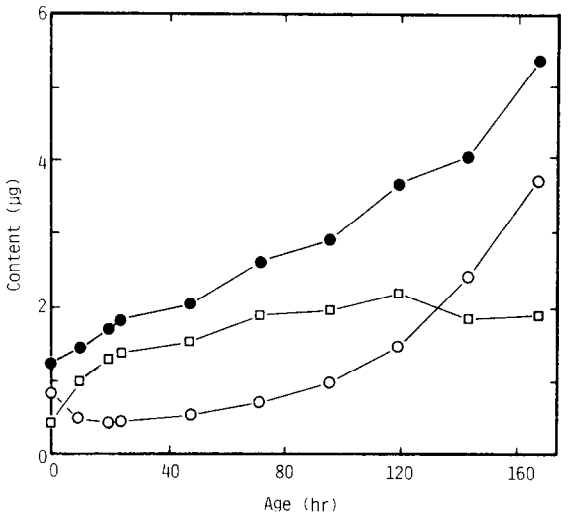

Fig. 2. Changes in amount of total ribosomes (—●—), polysomes (—□—) and ribosomal subunits (—○—)/egg during post-diapause development. Data shown in fig. 1 and table 1 were recalculated (see text). Each plot was the average of 3 analyses.

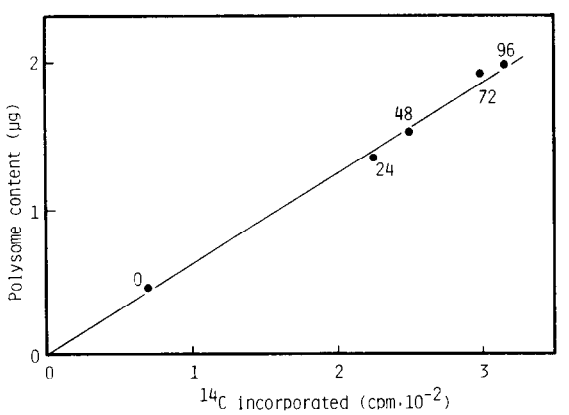

Fig. 3. Apparent correlation between polysome content and [^{14}C]leucine incorporation into acid-insoluble fractions of whole eggs from 0–96 h of post-diapause development. The polysome content shown in fig. 2 was replotted against the amount of radioactivity incorporated for 2 h. Incorporation data were the averages of 3 expt.

in a similar manner to ^{14}C, implying that synthetic activities were reserved in the cultured fragments. The value of the 2-h incorporation of ^{14}C roughly correlated with that in the polysome content after 96 h of post-diapause development (fig. 3).

4. DISCUSSION

The net content of polysomes increased markedly during the early period of development after the embryonic diapause. This rapid increase of polysomes, in addition to the dispersion of whirling lamellae of ER as in [3], may be characteristic of a post-diapause process of *Bombyx*. This increase was mainly ascribed to the mobilization of ribosomal subunits into polysomes, and to a lesser extent to a net increase of total ribosomes.

The polysome profile was almost the same between eggs after long-term chilling and those at the beginning of development. The size of polysomes remained almost unchanged. These facts may imply that the overall degradation of polysomes does not take place with the dispersion of ER materials occurring after the termination of diapause. Whether the polysomes change in translational activities at the break of diapause is uncertain.

For the labeling of developing eggs we applied a culture system instead of the microinjection method [4,7,10], considering an egg is composed of a complex population of cells that are compartmented. Moreover, the post-diapause embryos cultured in a medium under the presence of yolky substances are known to grow at a rate similar to that in vivo at least until stage 20, the period before katatrepsis [11,15]. Here, we fragmented eggs at each stage indicated, and labeled the acid-insoluble fractions for short periods. The incorporation in this system might not be a healing effect of injured embryos, since fragmented embryos grow without regeneration [15]. Although not corrected for possible changes in pool size of leucine, the rate of incorporation of [^{14}C]leucine thus investigated changed parallel to the increase of the polysome content (fig. 3). It is thus very likely that the polysome content mirrors the changes in the rate of protein synthesis at the early stages of post-diapause development in *Bombyx mori*, as was the case for the developing embryos of *Xenopus laevis* [16] and the sea urchin [17].

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