PROTEIN SYNTHESIS IN RELATION TO EMBRYONIC DIAPAUSE OF THE SILKWORM, BOMBYX MORI

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CONTENTS

GENERAL INTRODUCTION

Diapause is a developmental arrest involving many physiological and metabolic changes. In insect, diapause may take place at the early embryonic, late embryonic, larval, pupal or adult stage. The stage at which diapause occurs is intrinsically determined for each species and its beginning and termination are developmentally programmed in the life cycle. Diapause is thus distinguished from the simple quiescence or developmental stoppage forced to occur by extreme environmental conditions such as low and high temperatures (Lees, 1955). Diapause in this strict sense has also been found to occur in the embryos of mammalians and marsupials (Berger, 1966) and study on diapause may be of value for the progress of general developmental biology.

Insect diapause is categorized into the two types, obligate and facultative. In the former type, diapause occurs inevitably. In the latter type, whether diapause takes place or not is dependent upon the environment. Both types of diapause are under the control of the environmental or seasonal factors, light, temperature, nutrition and moisture. The organism receives the seasonal information at the induction phase, which more or less precedes the actual onset of the diapause phase. During the interval from the induction phase to the diapause phase some memory of the seasonal information must be kept

in the body and modulate the endocrine systems including the brain, corpus cardiacum, corpora allatum, prothoracic gland and suboesophageal ganglion; these in turn control the start of diapause (Harvey, 1962).

The type of diapause is directly related to the insect voltinism. The obligate diapause is found in the univoltine insects, whereas the facultative diapause is of the bivoltine, trivoltine insects and so on (insects of the polyvoltine type, which are adapted to high-temperature regions, do not enter diapause)(Beck, 1980).

The silkworm, *Bombyx mori*, has many variants in nature of diapause. For the bivoltine silkworms, the mode of response to the environmental stimuli, which bring about the modulation whether diapause occurs or not, have been investigated in detail (e.g. Kogure, 1933; Kobayashi *et al.*, 1986). In contrast to many species of insects in which the induction phase and the diapause phase are very close (often in the same developmental stage), these phases are far separated in *B. mori*; the seasonal stimuli received at the late embryonic stage determine, and those received during the post-embryonic stages modulate, the diapausability of the next generation. During oogenesis in the maternal body, oocytes are influenced by the diapause hormone secreted from the suboesophageal ganglion (Fukuda, 1951; Hasegawa, 1951). The release of this hormone from the ganglion is dependent upon the central nervous system, which memorizes the seasonal information (Matsutani and Sonobe, 1987). If the

release occurs, the deposited eggs enter diapause at the early gastrula stage, the period preceding the onset of organogenesis. Diapause continues when the eggs are kept at 25° C, the temperature adequate for development of non-diapause organisms. On the other hand, the eggs are awakened from diapause by overwintering or by the artificial chilling at 5° C for a prolonged period, sometimes followed by the hot-HCl treatment. Eggs thus activated begin the post-diapause development if they were transferred to 25° C and in time undergo the visual organogenetic processes.

The eggs of *B. mori* have been investigated for the diapause-specific metabolism (Chino, 1957, 1958; Harvey, 1962; Yamashita and Hasegawa, 1966; Ichimasa and Hasegawa, 1973; Kurata *et al.*, 1979; Yamashita and Hasegawa, 1983; Suzuki and Miya, 1983; Suzuki *et al.*, 1984; Sonobe and Okada, 1984; Osanai and Yonezawa, 1986; Kai *et al*, 1987). Also from the view-point that protein synthesis is a major controlling step for gene expression, studies like those cited below have been conducted with diapausing *Bombyx* eggs: For the early stages including the period of the onset of diapause, the incorporation of radioactive amino acids into acid insoluble fraction of the whole eggs were measured (Kawaguchi and Fujii, 1984; Sonobe and Odake, 1986), indicating that the rate became lower as the diapause begins. The patterns of two-dimensional (2D) gel electrophoresis of *in vivo* protein synthesis at the comparable stages were available in a report in which the product of $+^{pnd}$ (the

pigmented and non-diapausing egg) gene was analyzed (Sonobe and Odake, 1986). By using univoltine European races, *in vitro* translational products of RNAs and the proteins synthesized *in vivo* at the onset of diapause were resolved by 2D gel electrophoresis (Dorel and Coulon, 1988). These studies revealed the production of a certain stage-specific proteins. The eggs during diapause were found to have RNAs which are active in *in vitro* translation systems derived from wheat germ and rabbit reticulocyte lysate (Saito *et al.*, 1985a; cf. also Grzelak *et al.*, 1979) but contain a small amount of polysomes (Szyszko and Lassota, 1977; Saito *et al.*, 1982); these situations last until the termination of diapause after the long-term chilling (Saito *et al.*, 1982, 1985a). Thus the eggs at the dormant phases of diapause and wintering are believed to be low in rate of protein synthesis, while containing a store of template active messages. During the post-diapause development, the rate of amino acid incorporation and the amounts of polysomes were increased about 3 to 4-fold, suggesting that the rate of protein synthesis is augmented; this augmentation seemed to be dependent, at least in part, upon the increased translatability of mRNAs (Saito *et al.*, 1982, 1985a). In spite of these fluctuations, the overall patterns of RNA translation products *in vitro*, as analyzed by 1D gel electrophoresis, remained unchanged during the first 20 hr of the post-diapause development, the time during which the augmentation in rate of protein synthesis was rapid (Saito *et al.*,

1985a).

The purpose of the present study is to analyze more fully the protein synthesis in relation to diapause of *B. mori*. As materials, the eggs during the early period after oviposition, at the time near the onset of diapause, and during the first stages of the post-diapause development were principally used. This is because previous studies cited above have shown that the activity of protein synthesis is markedly fluctuated at these periods, and in expectation that these materials would provide much information relevant to diapause switches. Some of the eggs were treated with hot-HCl at 24 hr after oviposition to arrest the entrance of diapause. As to the post-diapause eggs, the following sorts of treatments were applied: (1) the eggs chilled for a prolonged time of 180 days to terminate diapause and then directly transferred to 25° C; (2) the eggs similarly handled but treated with hot-HCl before the transfer to 25° C. Eggs at other phases, e.g. during early periods of chilling, will be occasionally used as comparisons.

First, 2D gel electrophoretic patterns of egg proteins were observed after staining of the gels (Chapter I). Fluorography of *in vitro* translation products of RNAs were then analyzed after 2D gel electrophoresis (Chapter II). The overall stained and fluorographic patterns exhibited very small changes through the stages and phases tested, indicating that the bulk populations of proteins and mRNAs

are rather stable even during the period at which marked modulations in protein synthetic activity, and in physiology, are taking place. On the other hand, some spots of translation products of RNAs appearing in the post-diapause eggs were specific to the hot-HCl treatment. These are likely to be the so-called heat- or stress-shock proteins. Thus, gene expression following this treatment was investigated by the Northern blotting technique (Chapter III). This was followed by the fluorography of *in vivo* synthesized proteins to see the mode of utilization of mRNAs in the cells (Chapter IV), indicating again that the patterns of fluorographic spots did not undergo extensive fluctuation among the eggs tested. On the other hand, the rate of amino acid incorporation into the whole proteins of the eggs exhibited changes depending on the stage (Chapter V). The significance of results of these observations are debated in General Discussion.

Chapter I

Analysis of egg proteins by two-dimensional gel electrophoresis

INTRODUCTION

Proteins contained in the eggs of *B. mori* have previously been analyzed in the study of yolk protein metabolism (Izumi *et al.*, 1980; Irie and Yamashita, 1980; Zhu *et al.*, 1986). These analyses, however, were mostly conducted by 1D gel electrophoresis, and well-resolved 2D patterns for egg proteins of *B. mori* were scarcely reported. The first chapter of the present study was devoted to the establishment of basal patterns of egg proteins of this species as analyzed by 2D gel electrophoresis followed by staining. This revealed a display of the previously known major yolk proteins (Izumi *et al.*, 1980; Ono *et al.*, 1975; Irie and Yamashita, 1980; Zhu *et al.*, 1986). Also a number of minor components were seen. The results of the present work provided landmarks for the survey of fluorographic patterns in the subsequent chapters. As to the whole pattern of the spots no large developmental fluctuation was exhibited except for a certain numbers of minor spots, which appeared in the post-diapause development. The presence of diapause

specific proteins has not been found to date.

MATERIALS AND METHODS

Preparation of diapause eggs and artificial non-diapause eggs

The silkworm eggs used were those given by the moths of a hybrid between a Japanese race (N137) and a Chinese race (C137). It should be noted here that the presently used eggs were thus of the second filial generation; this may offer the problem that the individual egg would be heterologous in genetic background, possibly lowering the reproducibility of analysis if respective eggs were compared. However, the author dared to utilize the samples in the present study because of their availability. Care was taken to combine the eggs given by several moths and to use 30 (randomly chosen) eggs for extraction at a time.

To obtain eggs the parent pupae were kept at 25° C, and after emergence the moths were randomly mated for 4 hr. The mother moths inseminated were chilled at 5° C overnight to synchronize the start of oviposition (this treatment did not affected the initiation of diapause and the hatchability of the eggs). The moths were allowed to oviposit at 25° C under dark conditions. A batch of eggs deposited for 30 min were used.

The eggs deposited by the hybrid used entered diapause. In previous studies on hibernating *B. mori* eggs, both the

activity of thymidine incorporation into DNA and the rate of respiration begin to be lower than those of non-diapause egg on day 2 after oviposition (Sonobe and Odake, 1986) and it can be inferred that diapause (at least in terms of these biochemical indices) commences at about 48 hr after oviposition. Thus eggs at 20 hr after oviposition used in the present experiment will be termed pre-diapause eggs. Other eggs were begun to be chilled at 5° C on day 2 after oviposition (shortly after the onset of diapause), and kept at this temperature for a prolonged period. Under the present conditions, 180 days were enough to terminate diapause. These eggs will be termed the cold-treated eggs. They began the post-diapause development when kept at 25° C. Some of the cold-treated eggs were soaked in hot-HCl (sp. gr. 1.10) at 46° C for 5 min. In general such treatment is utilized to activate insufficiently chilled eggs, or to make solely the post-diapause development more synchronous. The latter was the case in the present experiment. Eggs at each stage were kept at -80° C until used for extraction.

Extraction and electrophoretic analysis of egg proteins

Eggs (the number for one analysis was set to 30) were homogenized in 300 ul of the lysis solution containing 9.5 M urea (Wako), 2% (W/V) Nonidet P-40 (Sigma), 2% (V/V) Ampholine (pH 3.5 - 10, Pharmacia) and 5% (V/V) 2-mercaptoethanol (Wako) and centrifuged for 5 min at 5,000 x g. An aliquot of the supernatant was subjected to 2D gel electro-

phoresis under the conditions essentially as described previously (O'Farrell, 1975). The first dimensional run was done with isoelectric focusing gels made in glass tubings (130 x 4 mm inside diameter), sealed at the bottom with Parafilm. The gel mixture contained 3.95% acrylamide, 0.05% *N,N'*-methylenebisacrylamide, 2% (W/V) Nonidet P-40, 2% (V/V) Ampholine (pH 3.5 - 10), 0.01% (W/V) ammonium persulfate and 0.07% (V/V) *N,N,N',N'*-tetramethylethylenediamine. For the running buffer 0.02 M NaOH and 0.01 M H_3PO_4 were used in the anode and cathode, respectively. The gels were preliminarily run at 200 volts for 30 min then at 400 volts for 30 min, and thereafter samples were loaded onto the gels and electrophoresed at 800 volts for 12 hr followed by 1,600 volts for 15 min. The gels were soaked in the solution containing 10% (W/V) glycerol, 5% (V/V) 2-mercaptoethanol, 2.3% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl buffer, pH 6.8, and 1 mM phenylmethylsulfonyl fluoride (Sigma) for 1 hr at room temperature and then stored at -70° C. The gels were each thawed, fixed with 1% agarose containing the running buffer (25 mM Tris-HCl buffer, pH 8.3, 192 mM glycine and 0.1% SDS) and loaded on top of a slab gel (15 x 12 cm) for the second dimensional run (Laemmli, 1970). The latter slab was composed of a separation gel with 9.73% (W/V) acrylamide and 0.27% (W/V) *N,N'*-methylenebisacrylamide in 0.375 M Tris-HCl buffer, pH 8.8, with 0.1% SDS, connected with a stacking gel with 2.48% polyacrylamide and 0.62% *N,N'*-methylenebisacrylamide in 0.125 M Tris-HCl buffer, pH

6.8, plus 0.1% SDS. Electrophoresis was conducted at room temperature at a constant voltage of 75 volts in the running buffer described above. Then the gels were stained by a silver staining kit (Wako) and dried.

RESULTS

Protein patterns in eggs before, during and after diapause

Eggs were extracted for proteins with a buffer containing urea. The supernatants were analyzed by O'Farrell type 2D gel electrophoresis. Fig. 1 shows the silver-stained protein patterns for different stages. The all electropherograms obtained gave high resolution at the region of 5 to 7 in pI and 20 to 100 in kDa. At the alkaline region many spots became smear along the direction of pI and only a part of spots were steadily distinguished. No spots were found at the region below pI 4. The major yolk proteins were detected as large spots. As drawn in the composite chart (Fig. 2), which will be discussed below, these were identified to be vitellin heavy chain (Vt.H), vitellin light chain (Vt.L), egg specific proteins (ESPs) and 30 kDa proteins (30K) based on their molecular weights and pIs that have been reported previously (Izumi *et al.*, 1980; Ono *et al.*, 1975; Irie and Yamashita, 1980; Zhu *et al.*, 1986).

As shown in Fig. 1a, the eggs at 20 hr after oviposition (a pre-diapause stage) exhibited up to 143 countable spots in addition to the major yolk proteins. The eggs on

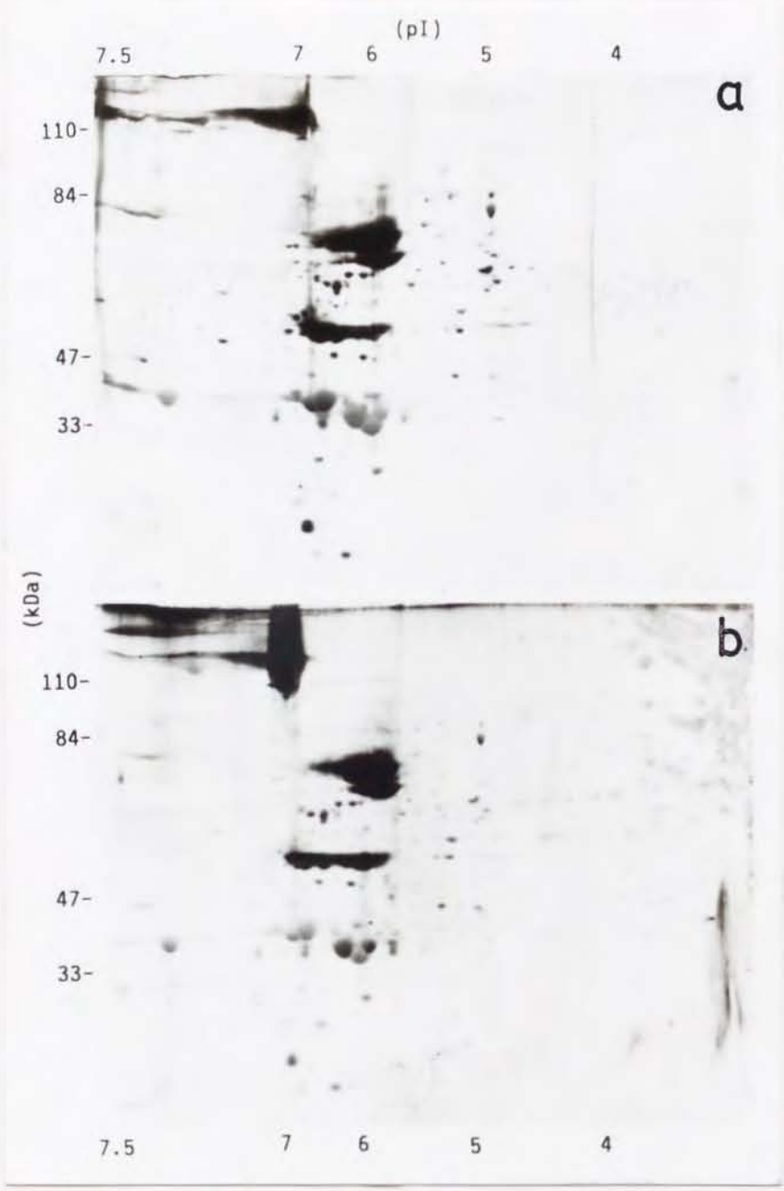


Fig. 1. Silver stained patterns of egg proteins. Eggs at each of different stages were extracted by the solution containing urea and non-ionic detergent. The extract was separated by 2D gel electrophoresis (O'Farrell, 1975) followed by silver staining as described in MATERIALS AND METHODS. The 1st dimension was isoelectric focusing on a pH gradient from 4 to 7. The 2nd dimension was done in the presence of SDS. Each gel had the sample equivalent to one egg. a, Pre-diapause eggs at 20 hr after oviposition; b, eggs chilled at 5°C for 35 days (chilling started on day 2); c, post-diapause eggs at 6 hr after hot-HCl treatment (preceded by chilling at 5°C for 180 days); d, post-diapause eggs at 72 hr (do.). The spots indicated by arrow heads changed in intensity with stages.

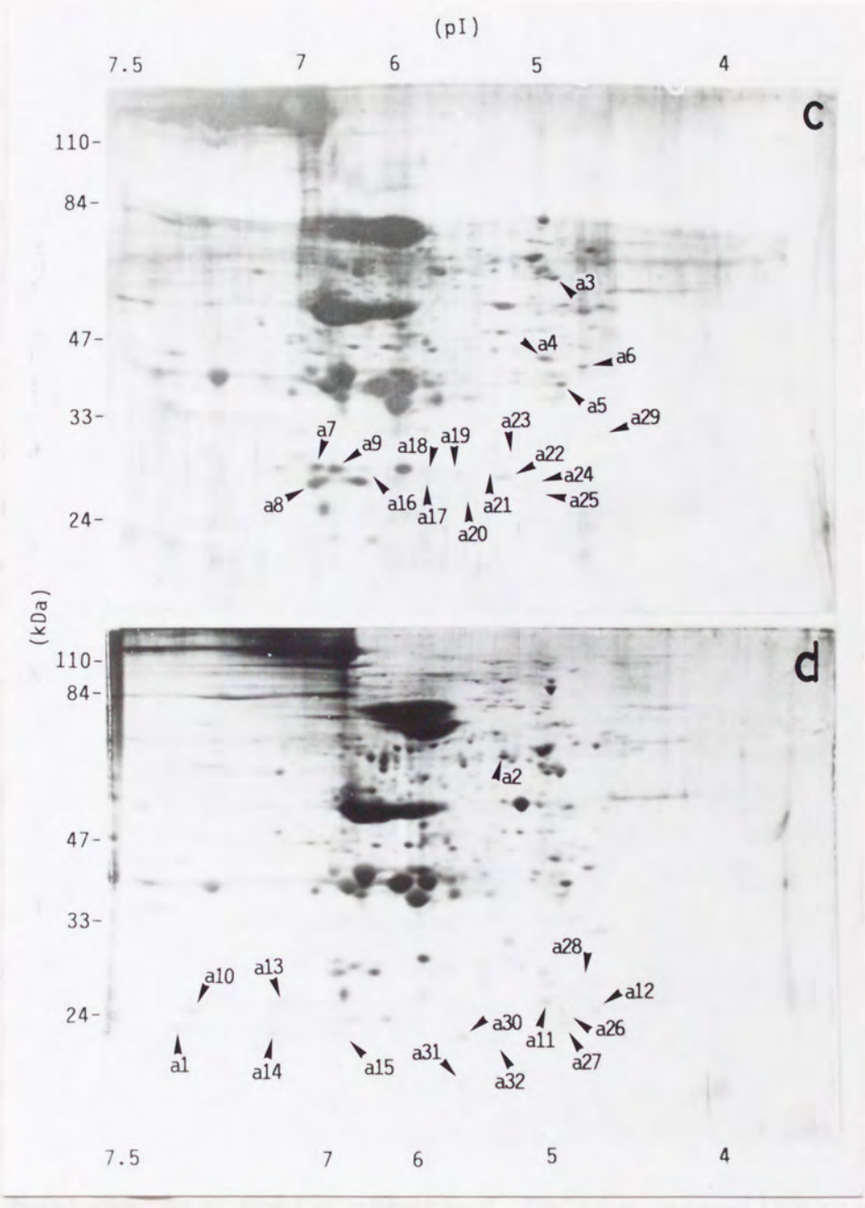


Fig. 1 (continuation).

day 35 after the onset of chilling at 5° C (these eggs could not recover the normal development after the transfer to 25° C so that these were regarded to be still in a diapause state), the above mentioned 143 spots were again detected (Fig. 1b), giving an almost identical pattern to that of the pre-diapause eggs.

The eggs at the post-diapause development (after the hot-HCl treatment preceded by cold activation) again displayed the patterns basically similar to those shown above, although new spots appeared, changing the number to 159 at 6 hr of post-diapause development (Fig. 1c) and to 173 at 72 hr (Fig. 1d). The spots accompanying arrow heads and numbers a1 to a32 were those occurred in the post-diapause stages.

Summary of the results

The basic display of the stainable spots is illustrated in Fig. 2. The major yolk proteins did not change in distribution throughout the present experiments. The numbers of the countable spots are summarized in Fig 3. Little difference in number were observed between the pre-diapause eggs and those on day 35 of chilling. The number increased in the post-diapause eggs, whereas approximately 80% of the total spots were steadily detected in all the stages tested.

Most of the spots observed in the pre-diapause and chilling stages were more than 30 kDa in size. On the other hand, the spots newly appeared in the post-diapause stages

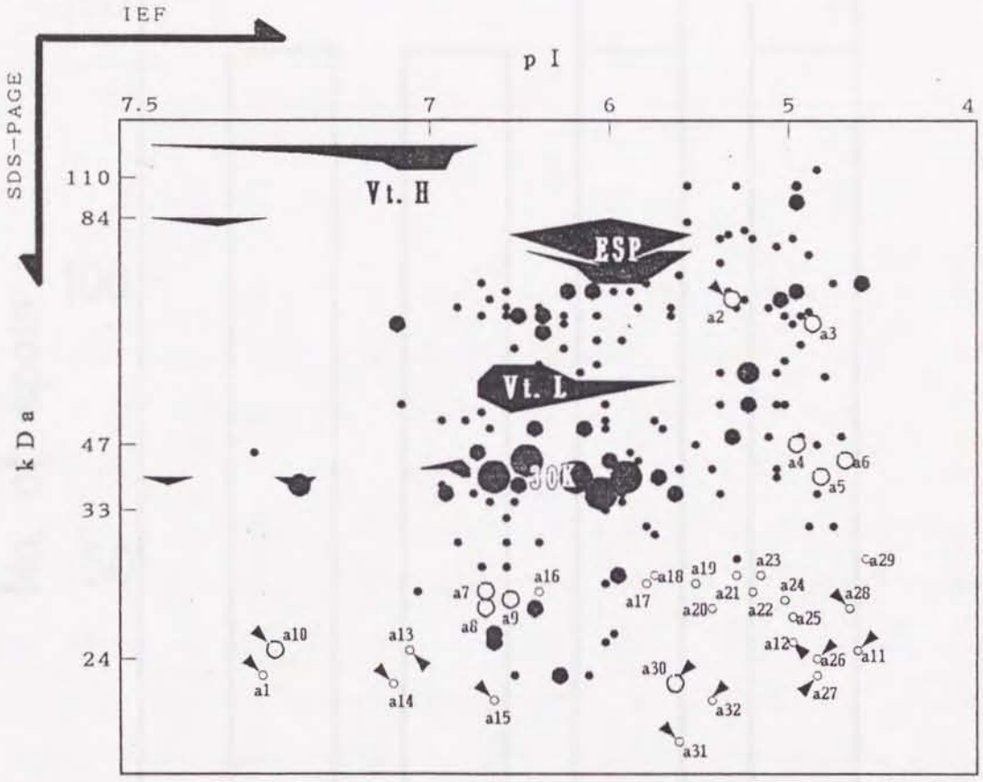


Fig. 2. Schematic representation of stained patterns. The results shown in Fig. 1a to d are composed. Silver stained spots are represented by circles. Solid circles represent generally occurring spots. Open circles indicate the spots appeared first in Fig. 1c (6 hr of post-diapause development), and those with solid arrow heads were seen only in Fig. 1d (72 hr of post-diapause development). Vt.H, vitellin heavy chain; Vt.L, vitellin light chain; ESP, egg specific protein; 30K, the 30 kDa proteins. Horizontal and vertical lines represent isoelectric point and molecular weight, respectively.

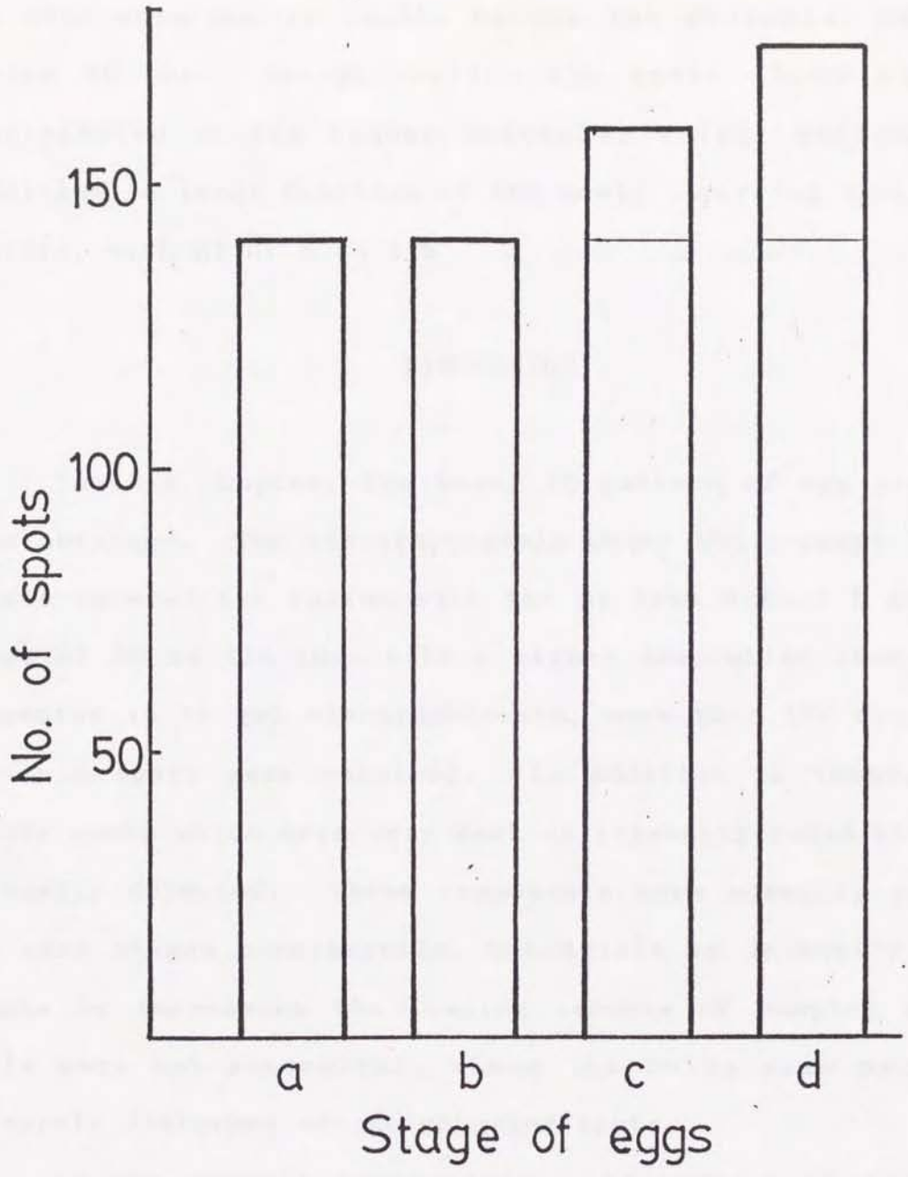


Fig. 3. Changes in number of stained spots on 2D gel electrophoresis. The numbers of spots counted on Figs. 1 and 2 are summarized. a, Pre-diapause eggs at 20 hr after oviposition; b, eggs chilled at 5°C for 35 days (chilling started on day 2); c, at 6 hr after hot-HCl treatment (preceded by chilling at 5°C for 180 days); d, at 72 hr (do.).

(drawn by open circles in Fig. 2, with the numbers from a1 to a32) were mostly small, having the molecular weights below 30 kDa. Exceptionally, the spots a2 to a6 were distributed at the higher molecular weight region. In addition, a large fraction of the newly occurring spots were acidic, with pI of 6 to 4.5

DISCUSSION

In this chapter, the basal 2D pattern of egg proteins was obtained. The electrophoresis under the present conditions covered the region with the pI from 4 to 7.5 and the size of 20 to 110 kDa, with a higher resolution than which expected in 1D gel electrophoresis; more than 170 countable protein spots were resolved. In addition to these, many other spots which were very week in intensity could be occasionally detected. These components were possibly present in some stages consistently, but trials to intensify these spots by increasing the loading amounts of samples to the gels were not successful, since the bulky yolk proteins severely disturbed the neighboring spots.

In the present experiments, the number of proteins could be counted in each stage and 80% of these were steadily seen. It can be concluded that the overall patterns were almost similar between the pre-diapause and post-diapause eggs, although some new spots appeared in the latter eggs.

At the pre-diapause stage, some changes in protein

synthesis must occur as seen in the previous experiment done to detect the gene expression in relation to the *pnd* mutant (Sonobe and Okada, 1984), but even highly sensitive silver staining applied in the present study did not detect any fluctuation in protein quality.

A number of spots, although they were minor ones, were newly detected during the post-diapause development after the hot-HCl treatment. These results are not surprising since this is the period when the protein synthesizing activity rapidly recovers (Saito, 1982, 1985a). Also the occurrence of these spots could be interpreted by the phosphorylation of some pre-existing proteins (Takahashi, 1987) or by the limited degradation of yolk proteins (Indrasith *et al*, 1987; 1988). This is reminiscent of the electrophoretic analysis of proteins in *Antheraea yamamai*, where specific proteins were found after diapause, which takes place in the fully grown embryo (Furusawa *et al.*, 1989), and in this case the fluctuation could be explained by the degradation of yolk proteins.

There were few reports about protein patterns directly referring to the diapause phase in *B. mori*. The present analysis of the eggs on day 35 of chilling (these may be still in a diapause state without any signal of the normal developmental ability as discussed above) again did not reveal the presence of specific proteins. On the other hand, electrophoretic investigation of haemolymph proteins have indicated proteins peculiar to the adult diapause in

Rhagoletis cerasi (Turchetto *et al.*, 1987), *Leptinotarsa decemlineata* (de Loof and de Wilde, 1970) and *Gastrophysa atrocyanea* (Ichimori *et al.*, 1990), although the precise function and mode of appearance of these proteins remain to be elucidated.

At any rate, the present investigation established the basic 2D pattern of egg proteins for the first time in *B. mori*. Many of the spots, including those of the major yolk proteins, may serve as indices in the comparisons and identifications of spots in the fluorographic analyses that would be conducted in the subsequent chapters.

Chapter II

Patterns of *in vitro* translational products of RNAs

INTRODUCTION

It is generally accepted that diapause accompanies a decrease in rate of gross protein synthesis, and this decrease would contribute to the conservation of energy during the dormant phases (Harvey, 1962). However, whether a consensus model about the regulation mechanism of protein synthesis in diapause, e.g. alteration in the ribosomal complex, the amino acid pool, the energy supplying system like ATP or the availability of mRNAs, can exist or not is still an open question.

In *B. mori* the protein synthesis in relation to diapause has been studied. The eggs of this species enter diapause at the early gastrulation. During the cryptobiotic processes including diapause and wintering, ribosomes were mostly dissociated into subunits remaining a part of them as polysomes (Saito *et al.*, 1982). RNAs did not lose translatability (Saito *et al.*, 1984) and little loss in amount of respective amino acids was observed during diapause (Suzuki *et al.*, 1984; Sonobe and Odake, 1984; Osanai and Yonezawa, 1986). The rate of protein synthe-

sis seems to increase about 3-fold during the first 20 hr of post-diapause development on the basis of the observation that the polysome content and the rate of incorporation of a radioactive amino acid into total proteins increased in parallel (Saito *et al.*, 1982). These increases were not accompanied by the comparable augmentation in amount of translatable mRNAs and ribosomes, which gradually reach maximum by the end of embryogenesis (Saito *et al.*, 1982, 1985a). From these results, it is suggested that the availability of mRNAs (and of ribosomes) is limited during diapause, and that some translational control mechanism is involved in the regulation of protein synthesis at the onset of post-diapause development.

The quantity and quality of proteins synthesized in cells are dependent upon those of translatable mRNAs present and it is highly possible that the mRNAs change in terms of species, as well as physicochemical modification at each mRNA molecule, affecting their translational activity, during some critical periods of development.

The author is therefore interested in the problem as to whether the species of mRNAs fluctuate or not in relation to diapause. Previously a study at this point of view has already been conducted (Saito *et al.*, 1985a, b), but the results were with using 1D gel electrophoresis, and overall revision by the 2D gel analysis is needed; this will be done in the present chapter. Again an *in vitro*

translation system was used for this purpose, because the system is able to give knowledge about the sorts of active mRNAs that are present in the cells.

The results of the present chapter show that the 2D patterns of the main translation products of RNAs mostly do not differ at the stages before and after the onset of post-diapause development. Even the patterns for the pre-diapause eggs were highly similar to those for the post-diapause eggs. On the other hand, the eggs in which the post-diapause development was evoked by the hot-HCl treatment (followed by the chilling for 180 days) exhibited several specific spots, which were not found in the eggs activated solely by the long-term chilling. These products may belong to the so-called heat- or stress-shock proteins.

MATERIALS AND METHODS

Preparation of eggs

Silkworm eggs were prepared as described in MATERIALS AND METHODS of Chapter I. Newly deposited eggs were used at 20 hr after oviposition (termed the pre-diapause eggs, see Chapter I). A part of the deposited eggs were treated with hot-HCl (sp. gr. 1.075; 46° C, 5 min) at 20 hr; by this treatment *B. mori* eggs can be prevented from entering diapause; developmental arrest were not observed in such eggs when kept at 25° C for development. These eggs will thus be

termed artificial non-diapause eggs. The eggs kept at 5° C from day 2 after oviposition were used on day 180 (the chilled eggs). A part of the chilled eggs were transferred to 25° C directly or after hot-HCl treatment to allow the post-diapause development as described in Chapter I. The eggs at different stages were stocked at -80° C until extraction of nucleic acids.

Extraction of egg RNAs

Eggs (1 g) were frozen in liquid nitrogen and brayed with a pestle. The homogenate was mixed with 10 ml of the lysis buffer containing 0.1 M Tris-HCl buffer, pH 9.0, 0.1 M NaCl, 0.01 M ethylendiamine tetraacetic acid (EDTA), 1% SDS, 0.01 mg/ml polyvinylsulfate and 10 units/ml heparin and stirred for 15 min at room temperature. One volume of phenol, saturated with 0.1 M Tris-HCl buffer, pH 9.0, 0.1 M NaCl, 0.01 M EDTA and 0.1% (W/V) 8-hydroxyquinoline, was added to each sample, which was shaken for 1 min and centrifuged at 20,000 x g for 30 min; the aqueous phase was transferred to a new tube. This phenol treatment was repeated three times and then each sample was extracted three times with phenol-chloroform (1:1) followed by centrifugation at 20,000 x g for 30 min. The resultant aqueous supernatants were added with 2.5 volumes of ethanol and stocked at -70° C. Before *in vitro* translation experiments the pellets were washed by 70% ethanol, air dried and dissolved in double distilled water

(DDW). The concentration of total nucleic acids in DDW were estimated by the absorption at 260 nm.

In vitro *translation of egg RNAs*

A rabbit reticulocyte lysate system, N-90Y (Amersham), was used for translation under the conditions as described by Pelham and Jackson (1976). The reaction mixture (45 ul) contained 2 uCi L-[^{35}S]methionine (1,201 Ci/mmole, ICN), 3 ug total nuclear acids dissolved in DDW and 40 ul N-90Y. The mixture was incubated at 30° C for 45 min and a 10 ul aliquot was subjected to 2D gel electrophoresis. The procedure of the latter was as described in MATERIALS AND METHODS of Chapter I. In the present Chapter, the gels after electrophoresis were fixed in a mixture of ethanol, acetic acid and water (4 : 1 : 5), and then treated with Enhance (New England Nuclear, NEN) followed by washing with water for 30 min. After drying the gels, fluorography was conducted as described previously (Bonner and Laskey, 1974) with X-Omat AR films (Eastman Kodak) in the presence of an intensifying screen (Eastman Kodak). Exposure lasted for 2 weeks at -70° C.

RESULTS

RNAs were extracted from *B. mori* eggs and translated in an *in vitro* system derived from the rabbit reticulocyte lysate. Although the RNA preparations may contain a large

amount of rRNAs and be contaminated with DNA, these stimulated the translation system efficiently. In general, RNA translation experiments have been conducted after isolating poly(A)-plus RNAs by using oligo(dT)cellulose or poly(U)-Sepharose 4B. However, high-molecular-weight RNA fractions obtained from the silkworm eggs under the present conditions were unstable and the use of the total nucleic acid fractions without further purification steps was favored. Moreover, this has another advantage of avoiding the loss of messages without poly(A)-tails or with short poly(A)-tails. The translation products were separated by 2D gel electrophoresis and visualized by fluorography. Up to 134 spots were countable. Out of these, the 30 spots that exhibited more or less changes at different stages as shown below were numbered (from b1 to b30) on the fluorograms for the sake of comparison. The remaining 104 spots without numbers were steadily observable in all the fluorograms shown below.

The patterns of translation products of RNAs from pre-diapause and artificial non-diapause eggs

From the pre-diapause eggs at 20 hr after oviposition and the artificial non-diapause eggs at 5 and 12 hr after the hot-HCl treatment, RNAs were extracted and their *in vitro* translation products were compared. Fig. 4a shows the pattern for the pre-diapause eggs; this contained 133 countable spots. At 5 hr of development (Fig. 4b), the 3

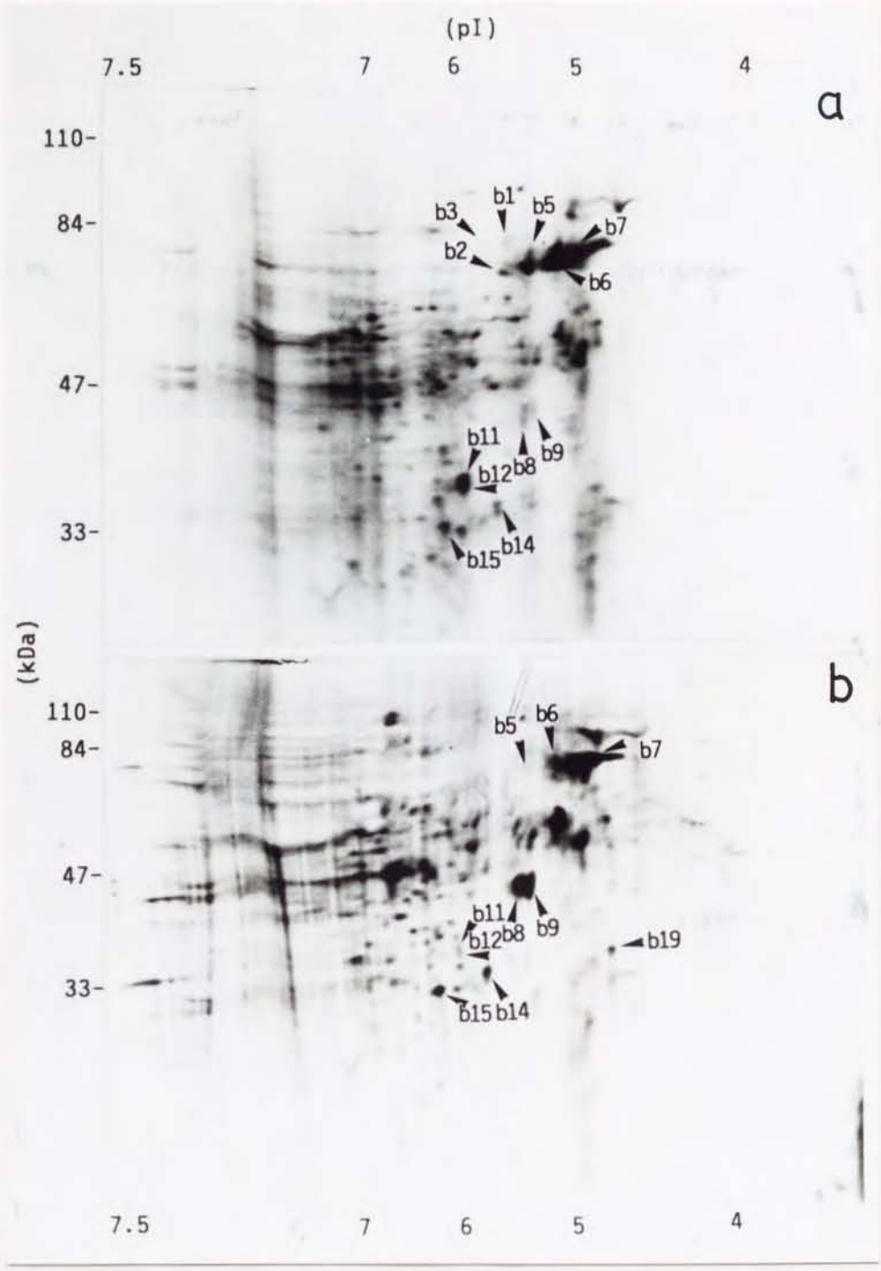


Fig. 4. Patterns of *in vitro* translation products of RNAs extracted from pre-diapause eggs and artificial non-diapause eggs. Total nucleic acids of eggs were extracted by SDS-phenol method and translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The products were separated by 2D gel electrophoresis followed by fluorography. a, Eggs at 20 hr after oviposition; b, artificial non-diapause eggs at 5 hr after hot-HCl treatment; c, artificial non-diapause eggs at 12 hr after hot-HCl treatment.

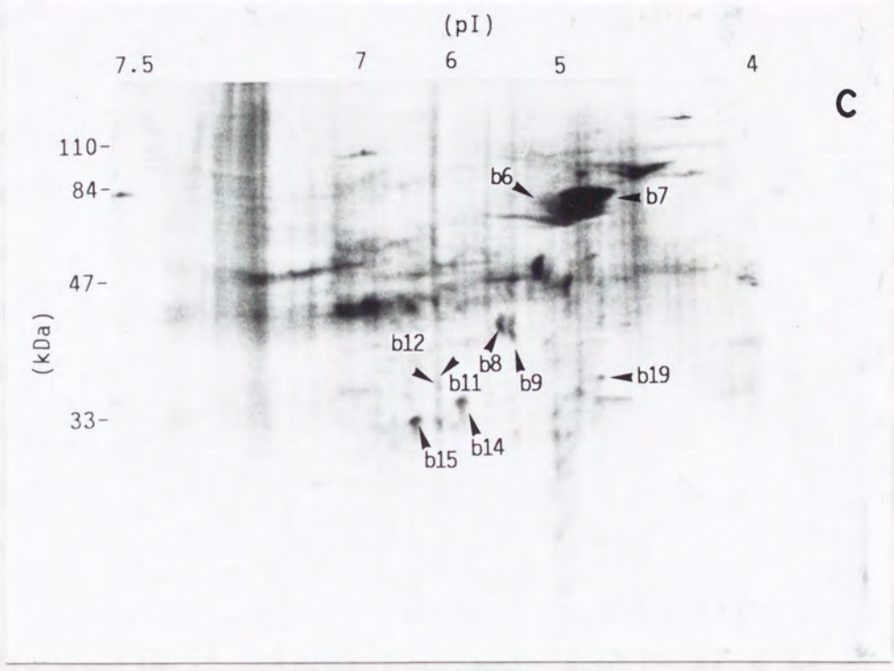


Fig. 4 (continuation).

spots b1, b2 and b3 disappeared, while a spot numbered b19 occurred. The intensities of the spots b5, b11 and b12 decreased, whereas those of b8, b9 and b14 increased. At 12 hr after the hot-HCl treatment (Fig. 4c), 3 spots (b8, b9 and b19) became weak.

Patterns of translational products of RNAs from post-diapause eggs

The *in vitro* translational products of RNAs were analyzed for the eggs after the cold treatment, i.e. before the onset of, and during, the post-diapause development. The patterns are illustrated in Fig. 5. Most spots were common to those from the pre-diapause and artificial non-diapause stages (cf. Fig. 4). However, precise comparison revealed some changes. The chilled eggs displayed a rather weak pattern but it still contained 129 spots, being smaller only by 4 than that of the pre-diapause eggs. The spots unseen in the present fluorogram include b1, b2, b3 and b5. The spots b6, b11, b12, b15 and so on were still detected but their intensities were low. At 6 hr of the post-diapause development, which commenced at 25° C without hot-HCl treatment, the basic distribution pattern (Fig. 5b) remained unchanged compared to the chilled eggs, but some spots including b9 increased in intensity. Little changes were observed at 24 hr of development (Fig. 5c), although b29 was slightly intensified. At 72 hr of the post-diapause development (Fig. 5d), when the eggs were at

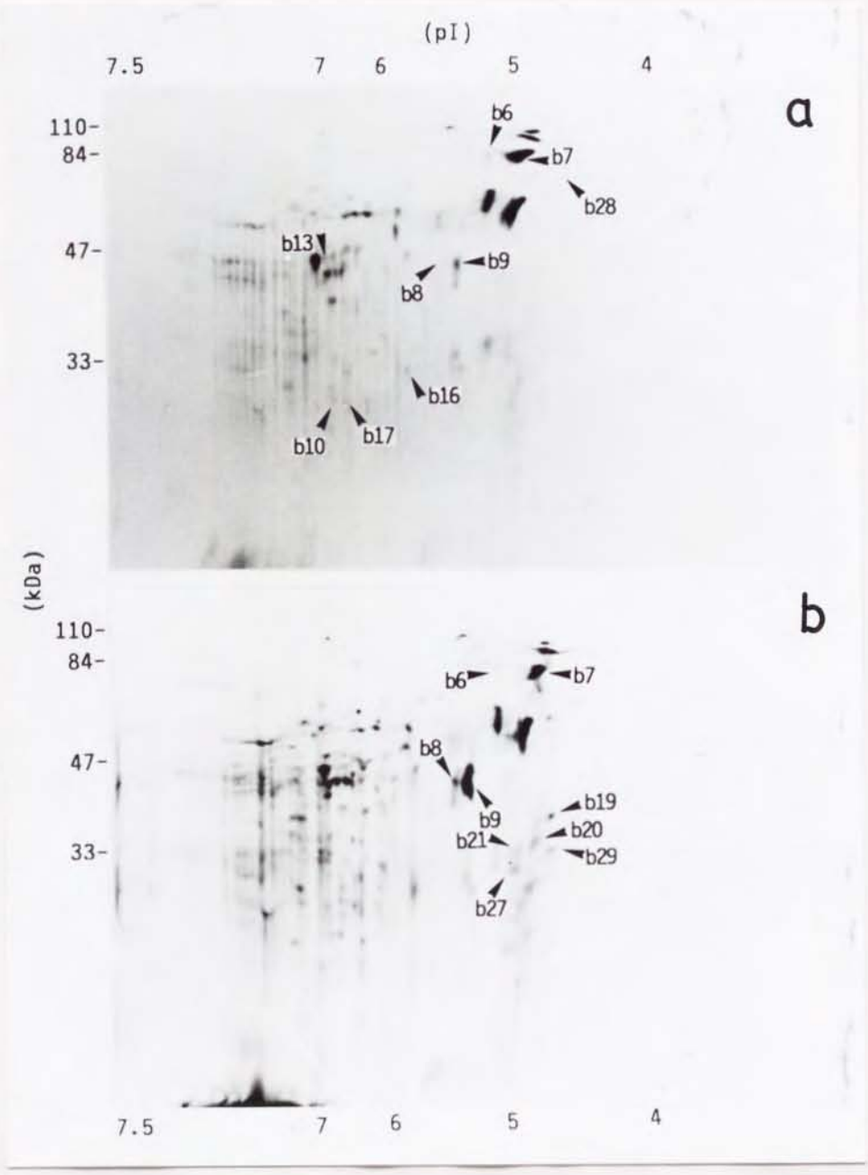


Fig. 5. Patterns of *in vitro* translation products of RNAs extracted from chilled eggs and post-diapause eggs received no hot-HCl treatment. a, Eggs chilled for 180 days (chilling started on day 2); b, eggs at 6 hr of post-diapause development evoked by transfer to 25°C without hot-acid treatment; c, do. at 24 hr; d, do at 72 hr. Other procedures were as described in Fig. 4.

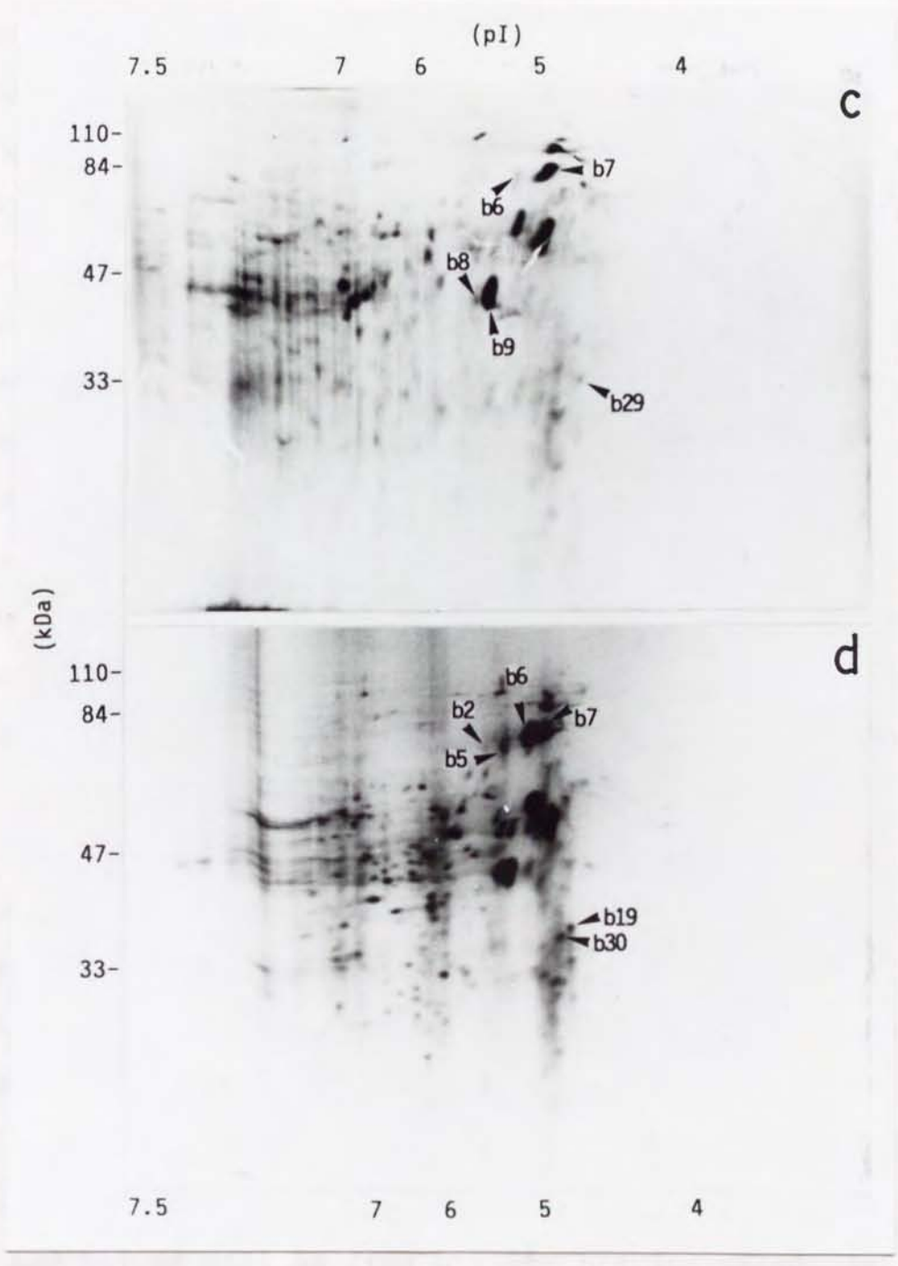


Fig. 5 (continuation).

the early stage of visual organogenesis (neural groove invagination), many spots including b5, b6, b8, b9, b15, b19, b20, b21, b22, b27 and b30 became marked.

Patterns of translational products of RNAs from hot-HCl treated post-diapause eggs

The eggs chilled for 180 days and then treated with hot-HCl. These were kept at 25° C to start the post-diapause development and analyzed for the *in vitro* translation products of RNAs (Fig. 6). Again this resulted in a distribution patterns basically similar to those shown above. However, at 6 hr (Fig. 6a) a remarkable difference was found in comparison to the pattern from the eggs of the comparable stage (6 hr) of post-diapause development but without the hot-acid treatment (cf. Fig. 5b). The hot-HCl sample gave the strong spots of b1, b2 and b3 as well as b5 and faint b4; these were absent without the hot-HCl treatment. The total spots became 134. In addition, the spots b6, b10, b13, b17 and so on were intense in the HCl treated sample. On the other hand, the spot b7 became weaker.

It is worth noting that b1, b2, b3 and b4 again disappeared at 24 hr of development (thus the total number was again 130), although b6 remained to be marked (Fig. 6b). In the 24 hr sample, 15 more spots these including b8, b9, b16 and b17 as well as from b18 to b28 increased in intensity. This made a contrast to the result at the com-

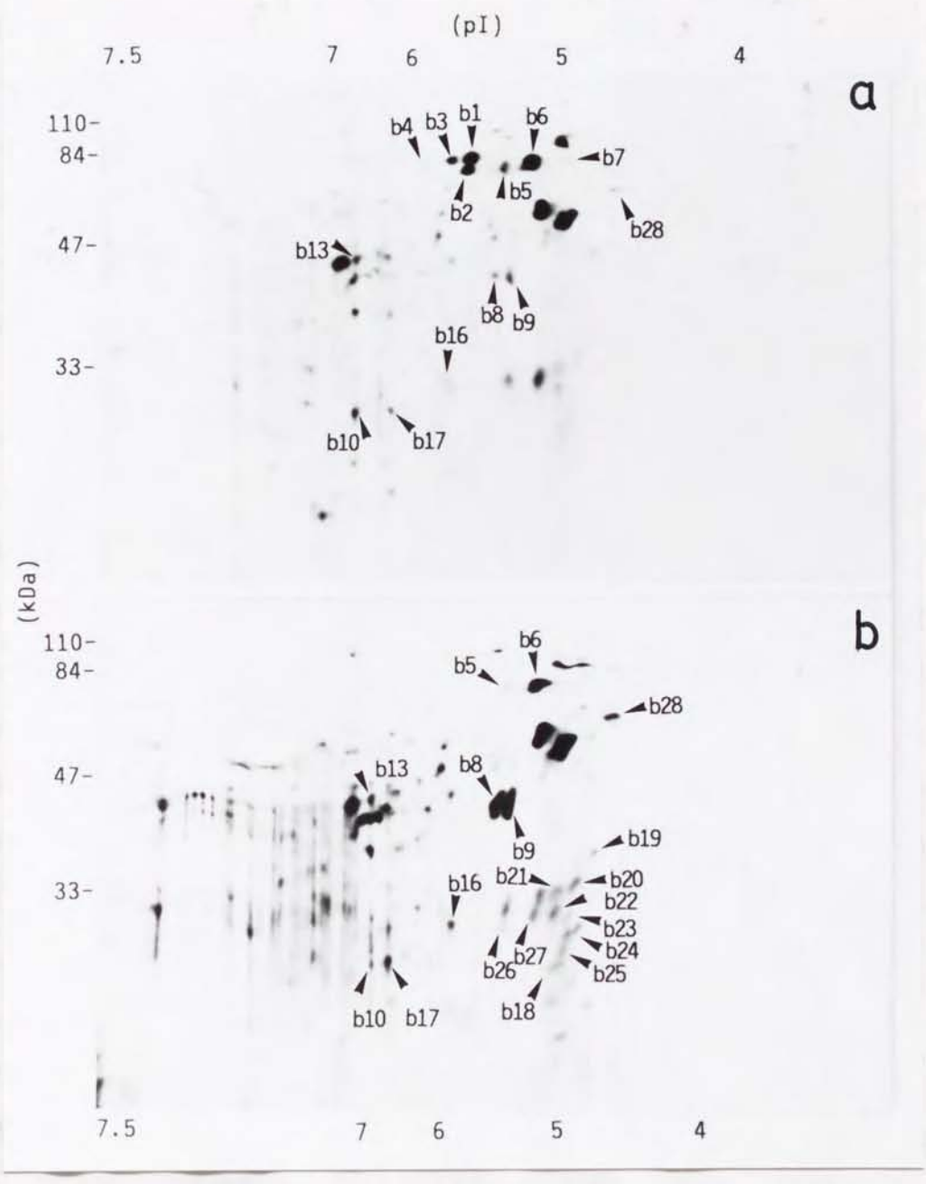


Fig. 6. Patterns of *in vitro* translation products of RNAs extracted from post-diapause eggs received hot-HCl treatment. a, eggs at 6 hr of post-diapause development evoked by transfer to 25°C followed by hot-acid treatment; b, do. at 24 hr. Other procedures were as described in Fig. 4.

parable stage (24 hr) without the hot-acid treatment (cf. Fig. 5c), in which the numbered spots were rather weak except for b7 and b9. One might argue that this contrast between Figs. 5c and 6b was spontaneous, simply due to the experimental divergence. This may not be the case, since all procedures including the cell-free synthesis, electrophoresis and fluorographic exposure were done under comparable conditions. Moreover, the 105 spots other than those marked by the numbering were similar in intensity between Figs. 5c and 6b indicating the above difference to be valid.

Summaries of the changes of in vitro *translational products*

The present results of the distribution patterns of RNA translation products are summarized in Fig. 7. The 30 spots that are indicated by open circles (and accompanied by the numbers from b1 to b30) are those which show variation at some stages. The changes of these numbered spots were summarized in Table 1. As already described, the remaining 105 spots were constant in intensity in all the samples tested. Thus, in total 135 different spots were observable. Table 1 includes also the total numbers of the countable spots at each stage. In the pre-diapause eggs 133 spots were distinguishable (135 minus 2 due to the lack of b4 and b19); this gave the second largest number. The number differed only slightly among the different samples, becoming 129 in minimum in the chilled eggs whereas

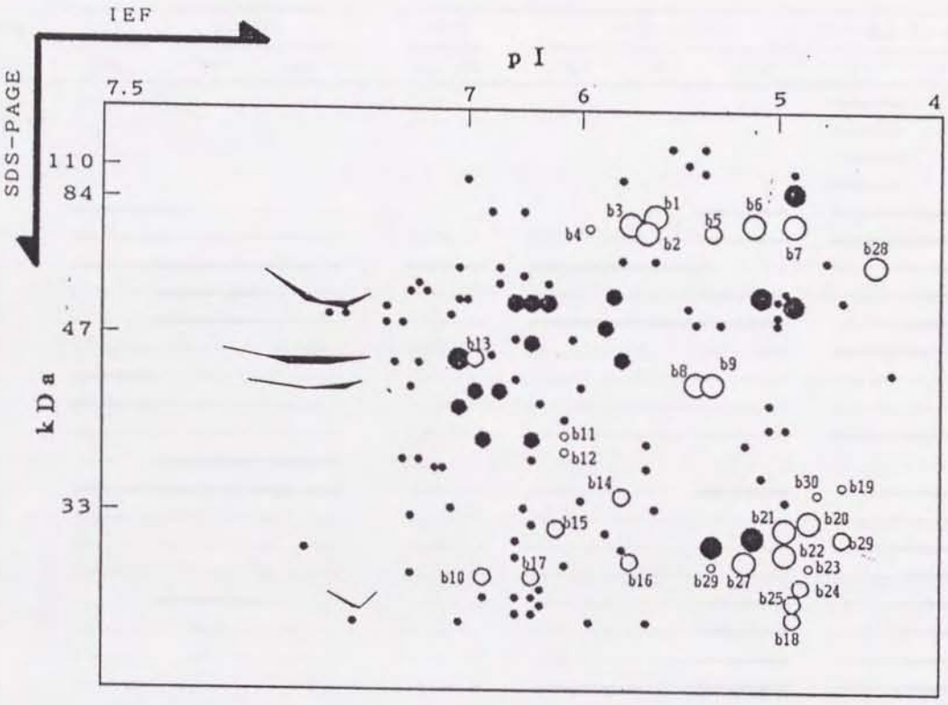


Fig. 7. Schematic representation of fluorographic patterns of *in vitro* translation products. The results shown in Figs. 4, 5 and 6 are composed. Solid circles represent generally occurring spots with constant intensities. The spots drawn with open circles changed in intensity at different stages, or occurred in a stage-specific manner. Horizontal and vertical lines show isoelectric point and molecular weight, respectively.

Table 1. Changes of fluorographic spots of *in vitro* translation products

Products	Pre-diapause eggs[a]	Artificial non-diapause eggs[b]		Post-diapause eggs					
	25℃	Hot-HCl		5℃[c]	5℃→25℃[d]			5℃→Hot-HCl→25℃[e]	
	20hr	5hr	12hr	day 180	0hr	24hr	72hr	6hr	24hr
b1	—							—	
b2	—							—	
b3	—							—	
b4								—	
b5	—						—	—	—
b6	—						—	—	—
b7	—	—	—	—	—	—			
b8		—					—		—
b9		—			—	—	—		—
b10								—	—
b11	—								
b12	—								
b13								—	—
b14		—	—						
b15	—	—	—				—		—
b16									—
b17								—	—
b18									—
b19		—					—		—
b20							—		—
b21							—		—
b22							—		—
b23							—		—
b24									—
b25									—
b26									—
b27							—		—
b28									—
b29					—	—	—		—
b30							—		—
Total No.	133	131	131	129	129	129	130	134	130

The fates of spots that showed fluctuation (b1 to b30) on Figs. 4, 5 and 6 are summarized. The total numbers of spots including b1 to b30 were also shown. Fine line indicates that each spot was observable. Bold line means that the spot was stronger at the concerning stage than at other stages examined (note that the boldness does not stand for the intensities relative to other spots on the map). a, Eggs at 25℃ after oviposition; b, eggs treated with hot HCl at 20 hr after oviposition and then incubated at 25℃ for different periods; c, eggs chilled at 5℃ for 180 days (chilling started on day 2 after oviposition); d, eggs transferred to 25℃ after chilling and incubated at 25℃. e, eggs treated with hot HCl after chilling and incubated at 25℃ for different periods.

130 in the post-diapause eggs without hot-acid treatment.

Many of the numbered spots became strong at the stages 6 hr and/or 24 hr of the post-diapause development preceded by the hot-HCl treatment. It is remarkable that the spots which occurred temporally and in time disappeared completely were only up to 6 in number (b1, b2, b3 b4 and b5), occupying below 3% of all of the countable spots. The spots b1, b2, b3 and b4 were seen markedly at 6 hr of development after the hot-HCl treatment and 3 of them (b1, b2 and b3) were also seen, although faintly, at the pre-diapause stage (it is uncertain whether b4 exist in pre-diapause eggs or not). In contrast, b5 was observed in most of the stages but not in the eggs during chilling. On the 2D gel, the spots that showed a stage specificity locate within the range between pIs 5 and 6 and at about 70 kDa, making a cluster.

The components b11 and b12 were intensified preferentially at the pre-diapause stage and b14 was specifically intensified to the artificial non-diapause eggs. The spot b7 was observed as a major component at the stages except for the later post-diapause period, when it exhibited a complex mode of changes; decreasing in intensity at 24 hr in the eggs not treated with hot acid, while being constantly weak in the hot-acid treated eggs. The spots b5, b6, b8, b9, b15, b19 to b22, and b26 were intensified at the organogenetic stages in the post-diapause development, but many components including these became marked already at 24

hr in the eggs treated with the hot-HCl treatment. The spots intensified at the later post-diapause periods were clustered at the acidic and low-molecular-weight region. Some of them may be identical to the stained spots reported in Chapter I, in particular the components Nos. a17 to a32, which have the low molecular weights below 30 kDa, and with pI of 6 to 4.5.

In comparison to the pre-diapause eggs at 20 hr after oviposition, those during prolonged chilling (180 days), in which diapause was terminating but development was still arrested by low temperature, had only 8 (6% of all) spots decreased in their intensity. These changes would occur at early periods of chilling. Only one spot, b7, was maintained as a major spot. This is the component that decreased after the initiation of post-diapause development and this may be abundant mainly in the eggs at an inactivated state.

DISCUSSION

The patterns of *in vitro* translation of egg RNAs were analyzed by 2D gel electrophoresis, and 129 to 134 chief spots (up to 135 different kinds of spots) were detected. Very small changes in the overall distribution patterns of the spots were observed upon the comparisons between the pre-diapause eggs and the artificial non-diapause eggs, and between the two kinds of post-diapause eggs, one evoked

solely by chilling and another by chilling followed by hot-acid treatment. The 30 spots numbered from b1 to b30 were elicited changes, but most of these components were consistently detected in all the fluorograms tested, fluctuating only in intensity. Also, as will be discussed below, temporally occurring and disappearing components were observed (b1, b2, b3, b4, b5 and b14); but they occupy only less than 3% of the total population of countable spots. Consequently, it is concluded that the most of main species of mRNAs, which could be detected in the present technique, do not change throughout the stages investigated. That is, modulation in complexity of mRNA pool would be small if any.

This is rather surprising in view of the fact that the eggs analyzed were at the periods when many lines of physiological fluctuation would be taking place; these may include the start of artificial non-diapause development, the break of diapause by chilling, the onset of post-diapause development and the start of visual organogenesis. Thus the suppression and stimulation of protein synthesis in relation to diapause and development is controlled mainly by changing the utilization of pre-existing set of mRNA molecules, not by the extensive interchanges in terms of mRNA species. In this connection *in vivo* patterns of protein synthesis are to be analyzed. This problem will be investigated later in the present article (Chapter IV).

The spots b1, b2, b3 and b4 were detected markedly at 6

hr in the post-diapause eggs after hot-HCl treatment but not when the latter treatment was omitted. These products were considered to be so-called heat-shock proteins on the basis of their mode of appearance, depending upon the hot-acid treatment. Their size (about 70 kDa) and pI (5 to 6) also resemble the heat-shock proteins of other species (Ashburner and Bonner, 1979; Lindquist, 1986; Giudice, 1989). In general, heat-shock proteins are inferred to be of stress-response molecules (Ashburner and Bonner, 1979; Lindquist, 1986; Giudice, 1989) and subsequently the term "stress-shock" proteins will be used. The b5 spot, although its size and pI are close to those of b1 etc., would not be stress-shock protein, since it appeared even in the eggs without hot-HCl treatment. If the b1, b2, b3 and b4 were in fact stress-shock proteins, these may have a role in modulating physiological conditions altered by the hot-HCl treatment. This would explain the reason why the eggs are fully normal even after encountering with the extreme circumstance of low pH and high temperature. The validity of the hot-HCl-dependent difference of egg RNAs will also be investigated by the Northern blot hybridization technique in the following chapter.

A product similar to the presently observed, tentative stress-shock proteins has already been reported for eggs during the post-diapause development that was evoked by chilling followed by hot-HCl treatment (Saito *et al.*, 1986). In this case the product was found as a single band

in 1D gel electrophoresis at the size of also about 70 kDa (pI not reported). However, whether it occurs or not in eggs without hot-acid treatment has been unknown. The result described in this chapter is the first indication of the hot-acid dependency for the post-diapause eggs. Moreover, the present 2D analysis permitted to show the presence of such substances in a cluster of a few different components.

It is worth noting that these "stress-shock" proteins were not detected in the artificial non-diapause eggs; even these were after the hot-HCl treatment. This is in contrast to the situation of some European races of *B. mori*, in which a temporal spot with a size of 70 kDa has been markedly detected upon 2D gel analysis of *in vitro* translation products of RNAs from the eggs that had received the hot-HCl treatment shortly after oviposition (Dorel and Coulon, 1988; they confirmed the appearance of a similar spot also in *in vivo* incorporation experiments). This was reported only for the eggs shortly after oviposition and no information is available for the post-diapause eggs of the European races. In the experiments described in this chapter, the 4 products with lower molecular weights (b8, b9, b14 and b19) increased its intensity in artificial non-diapause stage. These spots were without response to the hot-acid treatment and, in this sense, did not resemble the b1, b2, b3 and b4 proteins. It is therefore concluded that, in the presently analyzed race of the temperate

zone, stress-shock proteins are scarcely produced shortly after oviposition. The distinctness between the present and the above-cited races would be due to some intrinsic nature of the stress response, which may possibly be related to the voltinism of the races.

On the whole, the results of the present chapter indicate that the different stages of eggs displayed almost similar patterns of RNA translation products as analyzed by 2D gel electrophoresis. However, in the post-diapause eggs that had been soaked in HCl at a high temperature gave a cluster of temporally occurring products. These may have a role in the physiological recovery from the stress-shock.

Chapter III

Detection of mRNA for stress-shock proteins by Northern blot hybridization

INTRODUCTION

Chapter II reported the temporal occurrence of specific translatable RNA species at a period of the post-diapause development. This was found to be dependent upon the hot-HCl treatment, which must be a severe stress to the eggs. In fact, HCl was reported to be permeable into the eggs via the chorion (Yoshimi *et al.*, 1990), probably making the internal pH lower. Moreover, a high temperature is needed as an effective stimulation to the eggs. Nevertheless, thus-treated eggs were completely normal, undergoing the highly synchronized development and hatch in time. It is known that a families of proteins are synthesized transiently in many organisms after various kinds of stress, e.g. an elevated temperature. These proteins were first called heat-shock proteins and recently stress-shock proteins as a generic term (Ashburner and Bonner, 1979; Lindquist, 1986; Giudice, 1989). The latter name is based on the idea that the occurrence of such proteins is related to the recovery from distortion due to stress. It is therefore reasonable

to suppose that the hot-HCl treatment to activate diapause eggs are forced to produce a certain stress-shock proteins.

A group of proteins named *hsp*70 (the heat-shock protein with the size of 70 kDa), isolated from various sources including *Drosophila melanogaster*, have the molecular weight and pI at nearly 70 kDa and 6, respectively, upon 2D gel electrophoresis (e.g. Buzin and Petersen, 1982). The presently found hot-HCl-dependent *in vitro* translational products, named b1, b2, b3 and b4 in Chapter II, were reminiscent of the *hsp*70 of various organisms in terms of size and pI. This prompted the author to detect, by using an authentic *hsp*70 probe, the presence of specific messages produced in response to the stress. In this chapter, RNA preparations from the eggs which have been received the treatment with hot acid are subjected to the Northern transfer followed by the hybridization with a genomic clone for *hsp*70 from *D. melanogaster*. Consequently, RNAs from the eggs after the hot-HCl treatment alone exhibited a signal for this probe, providing evidence supporting, although indirectly, the notion that the above mentioned translational products are the stress-shock proteins.

MATERIALS AND METHODS

Isolation of egg RNAs for hybridization

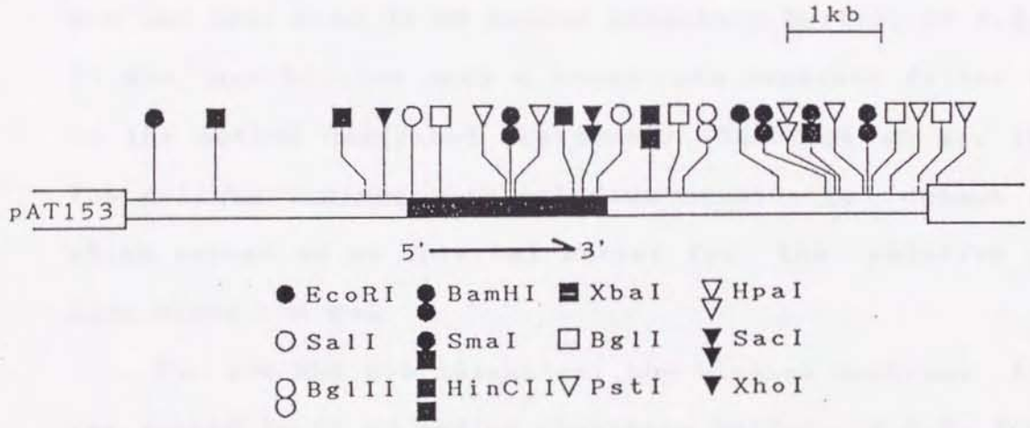
Total RNAs for the Northern blot hybridization were extracted from the eggs according to the method with cesium

trifluoroacetate (CsTFA, Wako) as essentially described previously (Perbal, 1988). For each preparation, 1 g of eggs were frozen in liquid nitrogen, powdered by braying with a pestle and stirred for 15 min at room temperature in 10 volumes of a solution containing 5.5 M guanidium isothiocyanate (Wako), 25 mM sodium citrate, 1% 2-mercaptoethanol and 0.5% sodium lauryl sarcosine (Wako). The mixture was adjusted to pH 7.0. The extract was centrifuged at a low speed and the supernatant was transferred onto a cushion of 1.51 g/ml CsTFA and 0.1 M EDTA, pH 7.5, in ultracentrifuge tubes, which were spun at 23,000 rpm for 20 hr at 15°C in a Beckman rotor SW28. The total RNA fractions were recovered as pellets; these were taken up and washed with 70% ethanol plus 0.3 M sodium acetate. The suspension was allowed to stand for chilling at -20°C for 24 hr and then washed again with the same solution. Finally, RNAs were precipitated with ethanol, air dried, dissolved in DDW and stocked at -70°C until use.

Probe for Northern hybridization analysis

A genomic clone named 56H8RA for the *D. melanogaster hsp*70 gene, which locates at the chromosomal site 87A7 and is characterized by having no intron, was supplied by the courtesy of Dr. A. Kuroiwa of the Tokyo Metropolitan Institute for Neurosciences. The clone had been inserted into the *Eco*RI site of the plasmid pAT153 (see the scheme illustrated on the next page). In this experiment, the plasmid

was digested with *Sal*I and the resulting fragments were separated by agarose gel electrophoresis. This gave 3 fragments, of which the middle sized fragment contained the most part of the coding region of the *hsp*70 sequence (indicated by an arrow in the scheme), and was used for the present blotting analysis as a probe. About 2 ug of the *hsp*70 probe was radioactive by the primer extension method



Restriction map of pAT153 plasmid containing the heat-shock locus 87A7. Filled sections represents the cording region. The polarity of transcription was indicated by an arrow. The symbols indicate the restriction sites. Cutting with *Sal*I forms 3 restriction fragments, of which the middle sized one contains most of the coding region of *hsp70* DNA; this was used as a probe.

with using [alpha-^{32}P]dCTP (Amersham) and a random primer DNA labeling kit (Takara).

Northern blotting analysis of egg RNA

The RNA solution was heated on a boiling water bath for 5 min, rapidly cooled on ice and subjected to electrophoresis at 15 to 100 mA for 12 hr on 1% agarose gel made up in 40 mM sodium phosphate buffer, pH 7.5, plus 12% formaldehyde. As a running buffer, 40 mM sodium phosphate buffer, pH 7.5, containing 12% formaldehyde was used. After electrophoresis, the end region of the gel facing the cathode was discarded and the remainder was washed with DDW for 5 min and then with 25 mM sodium phosphate buffer, pH 6.5, for 20 min, and blotted onto a Genescreen membrane filter (NEN) by the method described previously (Sambrook *et al*, 1989). The gel was stained with ethidium bromide to detect rRNA, which served as an internal marker for the relative position along the gel.

For the RNA hybridization, the blotted membrane filter was soaked in 25 mM sodium phosphate buffer, pH 6.5, for 15 min and incubated at 42° C for 3 hr in 15 ml of the prehybridizing solution containing 50% formamide (Nakaraiteque), 5 x SSC (5 times concentrated standard saline citrate: 0.15 M NaCl and 0.015 M sodium citrate), 50 mM sodium phosphate buffer, pH 6.5, 250 ug/ml heat-denatured salmon sperm DNA (ssDNA), 1 x Denhardt's solution [0.02% bovine serum albumin fraction V (Sigma), 0.02% polyvinylpyrrolidone and 0.02%

Ficoll (Pharmacia)], 0.1% SDS and 0.1% sodium pyrophosphate (Nakaraiteque). The filter was incubated at 65° C for 20 hr by soaking in 15 ml of the hybridization mixture. The latter consisted of 50% formamide, 5 x SSC, 1 x Denhardt's solution, 20 mM sodium phosphate buffer, pH 6.5, heat-denatured ssDNA, 0.1% SDS, 0.1% sodium pyrophosphate, 50 mM sodium phosphate buffer, pH 6.5, and an aliquot of labeled probe DNA. After incubation the filter was washed 2 times with 2 x SSC, 0.1% SDS and 0.05% sodium pyrophosphate for 20 min at room temperature, and then once with 2 x SSC, 0.1% SDS and 0.05% sodium pyrophosphate for 3 min at 55° C. The filter was dried in air and exposed for 3 days at -70° C to an X-Omat AR film (Eastman Kodak) with an intensifying screen (Eastman Kodak).

RESULTS

Detection of hybridizable RNA with D. melanogaster hsp*70 probe*

Total RNA fractions were isolated by the CsTFA method from *B. mori* eggs. This method was applied in order to reduce the contamination of DNA, which would interfere the hybridization of RNA. The RNAs were subjected to electrophoresis and the gel was analyzed for the Northern blot hybridization with a genomic clone of *hsp*70 from *D. melanogaster* as a probe. The results are illustrated in Fig. 8. A best signal of hybridization was seen on the membrane when

the washing step following hybridization was conducted with a high salt buffer at a low temperature, i.e. under mild stringency conditions, as described in MATERIALS AND METHODS. These facts indicate the presence of homology between the sequences of *B. mori* RNA(s) and the *D. melanogaster* probe, but the degree of similarity is low. The RNAs for this analysis were isolated from the eggs at 4 different stages: at day 180 of chilling (Lane a), at 6 hr of the post-diapause development at 25° C after chilling (Lane b), at 6 hr and at 24 hr of the post-diapause development at 25° C after chilling followed by hot-HCl treatment (Lanes c and d, respectively). The 6 hr-eggs that have accepted the stress by hot HCl exhibited the strongest band of hybridization as seen on Lane c. Other RNAs gave only a trace band (Lanes a, b and d).

The hybridization band, strong or trace, obtained for all RNAs had a size slightly smaller than that of 18S rRNA (arrow on Fig. 8). A high temperature applied to the RNA preparation before electrophoresis must have dissociated 28S rRNA (to give fragments with a size of roughly 18S), since the silkworm 28S rRNA, like those of other insects, intrinsically has a hidden intramolecular break (Kurata and Sakaguchi, 1978); this explains why 28S rRNA was not detected in this electrophoretic gel.

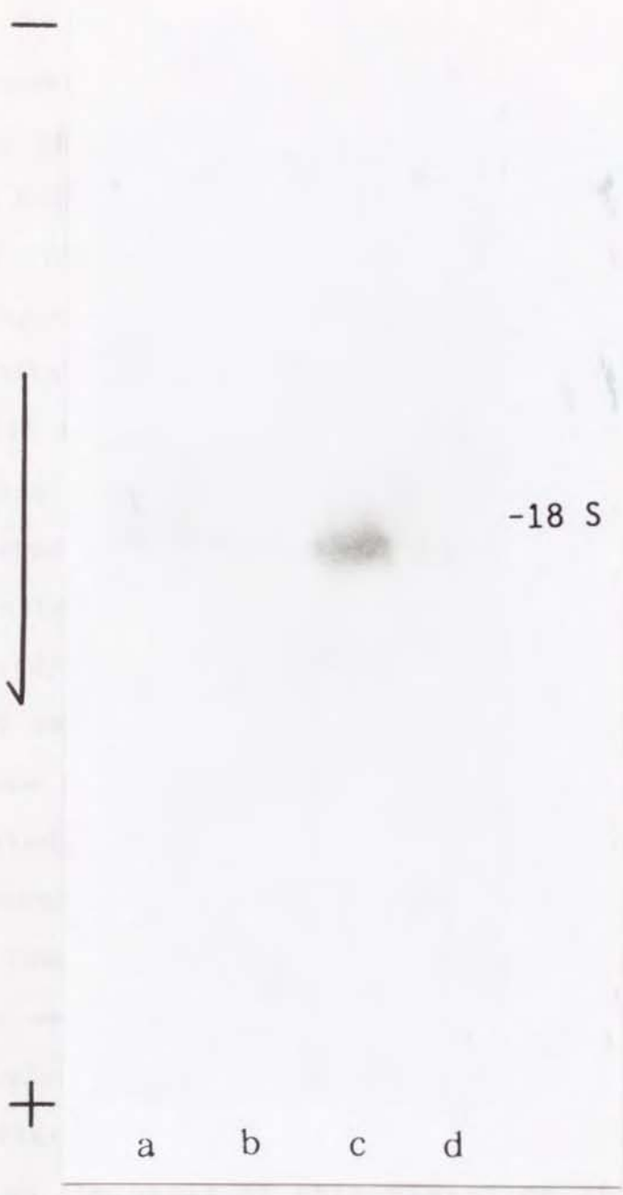


Fig. 8. Northern blotting analysis of total RNAs extracted from post-diapause eggs. RNAs were subjected to agarose gel electrophoresis in the presence of formaldehyde and then the gel was blotted to a Genescreen membrane filter. The RNAs on the membrane were hybridized with *D. melanogaster hsp*70 DNA at a mild stringency as detailed in MATERIALS AND METHODS. Lane a, RNAs from eggs on day 180 of chilling; Lane b, at 6 hr of post-diapause development at 25°C after chilling, Lane c, at 6 hr of the post-diapause development at 25°C after chilling followed by hot-HCl treatment; Lane d, do. at 24 hr.

DISCUSSION

A radioactive band, i.e. a signal of hybridizability to a probe for the *hsp*70 gene, was markedly seen in the RNA preparation from the *B. mori* eggs at 6 hr after the hot-HCl treatment. It is uncertain whether the band consisted of either a single component or more. The *in vitro* translational products b1, b2, b3 and b4, found in Chapter II, were also specific to the hot-HCl treated post-diapause eggs, both occurring 6 h after the hot-acid stress. Therefore, it is suggested that the strong band detected by the present Northern blotting analysis contains mRNA(s) coding for the b1, b2, b3 and/or b4 proteins.

A trace signal was also found in both chilled eggs and post-diapause eggs without the hot-acid treatment. This fact suggested the presence of a small amount of a message(s), probably due to the basal expression. This may code for a kind of heat-shock "cognate" protein ("*hsc*70") known for *D. melanogaster* (Ingolia and Craig, 1982).

In the electrophoresis illustrated above, the hybridizable band migrated slightly faster than 18S rRNA. A rough estimation on the basis of this migration indicated that size of the RNA(s) to be about 2 kb or less. If the silkworm stress-shock protein(s) has a molecular weight of 70 kDa, the relevant mRNA(s) should possess a size at about 2 kb or more depending upon the length of the 5'-non-coding region and poly(A)-tail (e.g. 2.55 kb in case of *D. melano-*

*gaster hsp*70 (Buzin and Petersen, 1982)). Accordingly, the size of RNA detected by hybridization in *B. mori* RNA seemed to be slightly smaller than expected. This may be explained if limited degradation had occurred in the present RNA preparation. Provided that this is the case, the results are taken to support the afore-mentioned idea that the RNA(s) detected by hybridization contains the mRNA(s) coding for any of the products named b1 to b4. Definite conclusion should be drawn after additional investigation including the direct hybridizability test between the probe and the b1 to b4 mRNAs.

It is supposed that the temporal production of hot-HCl dependent molecules in *B. mori* is needed to the normal progress of the post-diapause development, which may be attained after recovery from the shock. Because, the function of the stress-shock proteins has been discussed previously in reference to a protection from thermal killing in *D. melanogaster* (Mitchell *et al.*, 1979).

Chapter IV

Patterns of proteins newly synthesized *in vivo*

INTRODUCTION

The results described in Chapter II indicated that the major population of translatable mRNAs present in the eggs did not change largely at the periods including the pre-diapause stages, the onset of post-diapause development and chilling. Most of the translation products were consistently detected in all the samples tested, although a part of them fluctuated only in intensity. Consequently the major members of mRNAs remain the same under the circumstance where extensive developmental switchover is taking place. These results, in addition to those previously reported (Saito *et al.*, 1982, 1985a, b), seemed to imply that translational level control of protein synthesis is involved in the cells.

Therefore, the patterns of de novo synthesized proteins *in vivo* is to be analyzed together with the *in vitro* RNA translation experiments. Here, a radioactive amino acid was microinjected into the eggs, and proteins were extracted and resolved by 2D gel electrophoresis followed by fluorography to analyze the patterns of labeled proteins. As materials

developing artificial non-diapause eggs at the stages until the onset of visual organogenesis were used, since these permit a direct comparison to the acid non-treated, dormant eggs, which were also investigated in parallel.

MATERIALS AND METHODS

Preparation of eggs

The materials were prepared as described in Chapter I. Eggs deposited were treated with hot HCl at the age of 20 hr and allowed to develop at 25° C without entrance to diapause. Also eggs chilled from day 2 after oviposition without hot-acid treatment were used on day 11 of chilling. Like the eggs on day 35 of chilling as described in Chapter I, the day-11 eggs did not undergo the normal development when transferred to 25° C and could be referred to as a kind of diapause eggs.

Microinjection of radioactive precursor into eggs and analysis of labeled proteins by 2D gel electrophoresis

Eggs were each adhered to a glass substrate of a petri dish by using a glue applied to one of the flat surfaces of the eggs. Then at the center of another flat surface was stabbed by a sharp steel needle. Through the resulting hole 50 nl of [^{35}S]methionine (1,150 Ci/mmole, 11.3 mCi/ml, ARC) was injected per egg by using a thin glass capillary. The eggs were then incubated at 25° C for 60 min. For one analy-

sis 30 labeled eggs were collected and homogenized in the buffer containing 2.0% (V/V) Ampholine (pH 3.5-10), 2.0% (W/V) Nonidet P-40, 9.5 M urea and 5% (V/V) 2-mercaptoethanol. The mixture was centrifuged at 15,000 x g for 5 min at room temperature, and aliquots of the supernatant were subjected to 2D gel electrophoresis under the same conditions as those in Chapters I and II. The radioactive spots of newly synthesized proteins were visualized by fluorography as described in Chapter II. Coomassie brilliant blue staining was conducted to see the major egg proteins.

RESULTS

Patterns of newly labeled proteins in vivo

Eggs were microinjected with [^{35}S]methionine and the patterns of proteins labeled for 60 min were analyzed 2D gel electrophoresis followed by fluorography for the purpose of analyzing the de novo synthesized proteins *in vivo*. In the present study, the eggs during continuous development (artificial non-diapause eggs; at 6, 12, 24, 36, 48 and 72 hr after hot-HCl treatment) and the eggs on day 11 of chilling were compared (Figs. 9 and 10). The relative positions of the fluorographic spots were highly distinguishable by referring to the strong spots of major yolk proteins (Chapter I), which served as convenient landmarks (the stained patterns are omitted, but the positions will

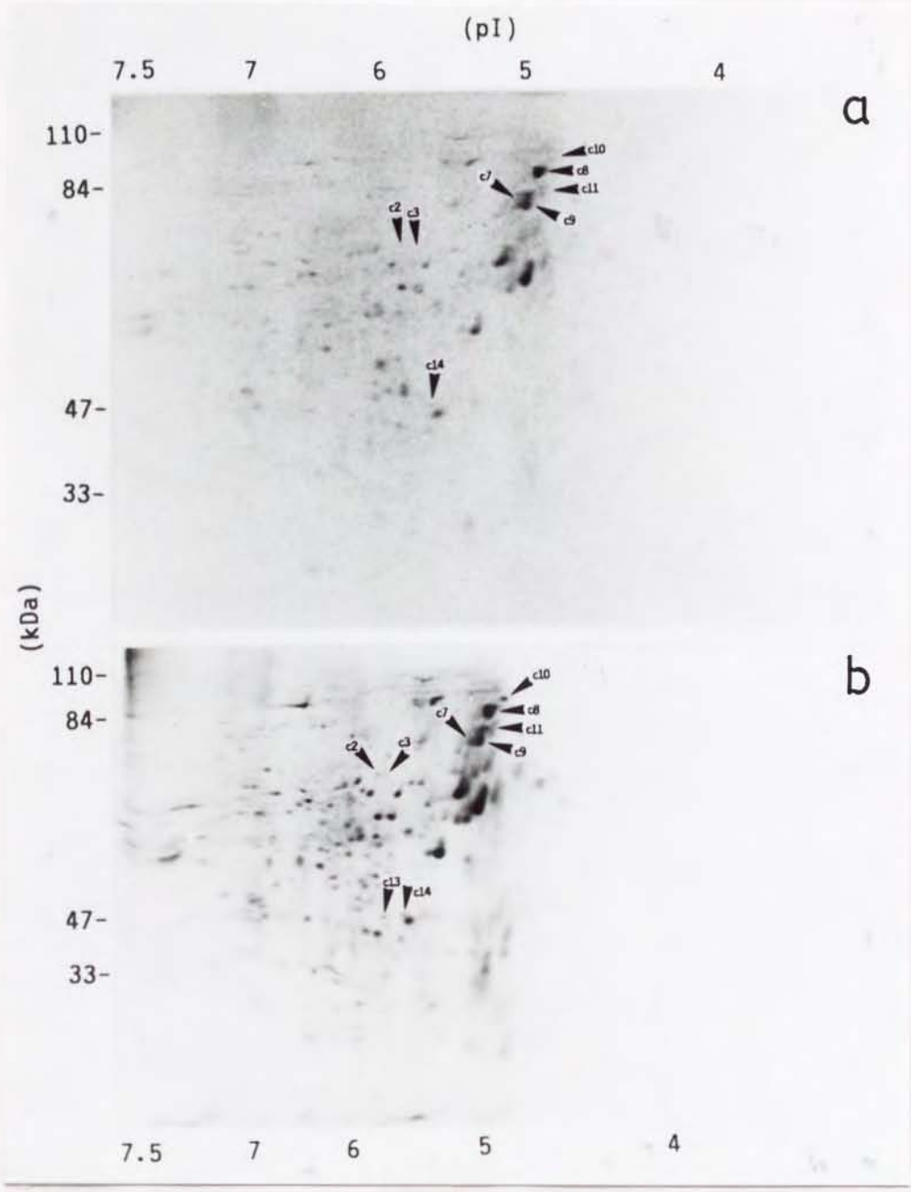


Fig. 9. Patterns of *in vivo* synthesized proteins in artificial non-diapause eggs. Each egg was microinjected with [^{35}S]methionine and incubated for 60 min at 25°C. After labeling, the eggs were extracted for proteins, which were resolved by 2D gel electrophoresis and the spots were visualized by fluorography. a, Eggs at 6 hr after hot-HCl treatment (made at 20 hr after oviposition); b, do. at 12 hr; c, do. at 24 hr; d, do. at 36 hr; e, do. at 48 hr; f, do. at 72 hr.

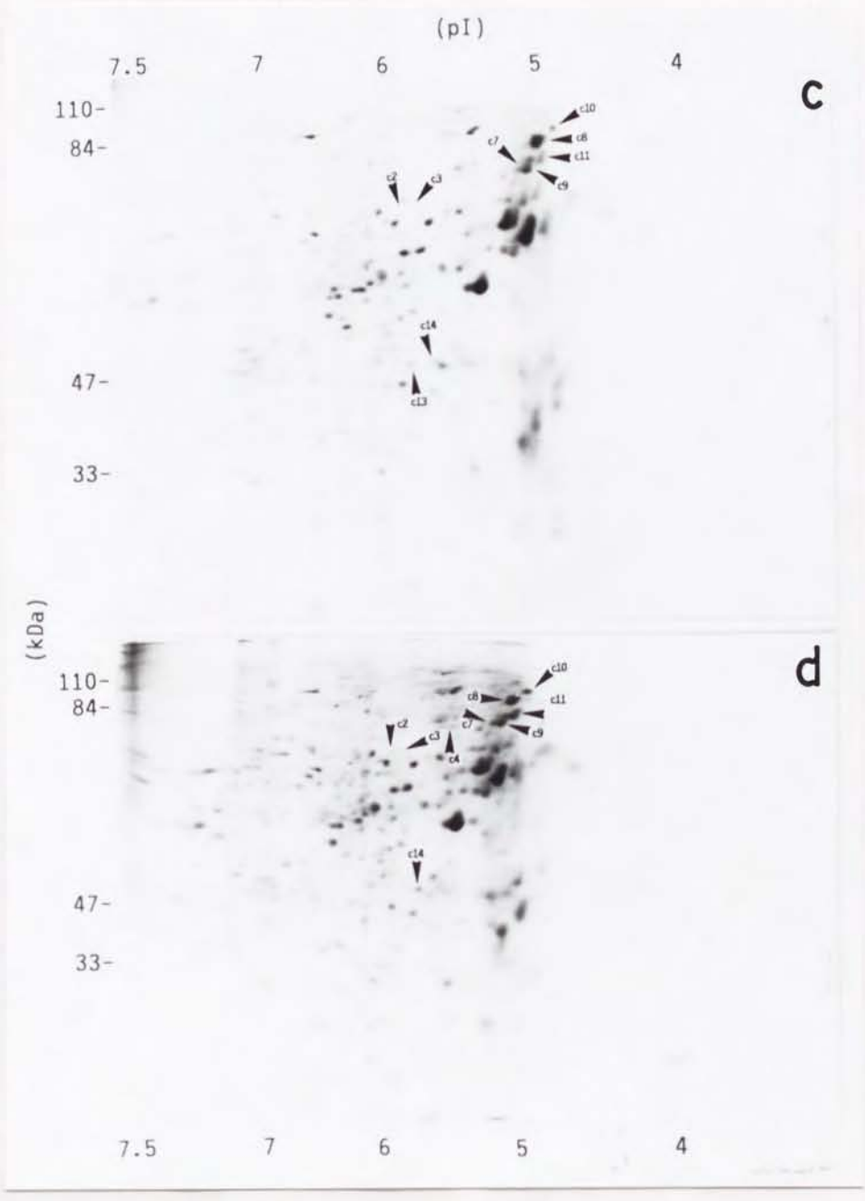


Fig. 9 (continuation).

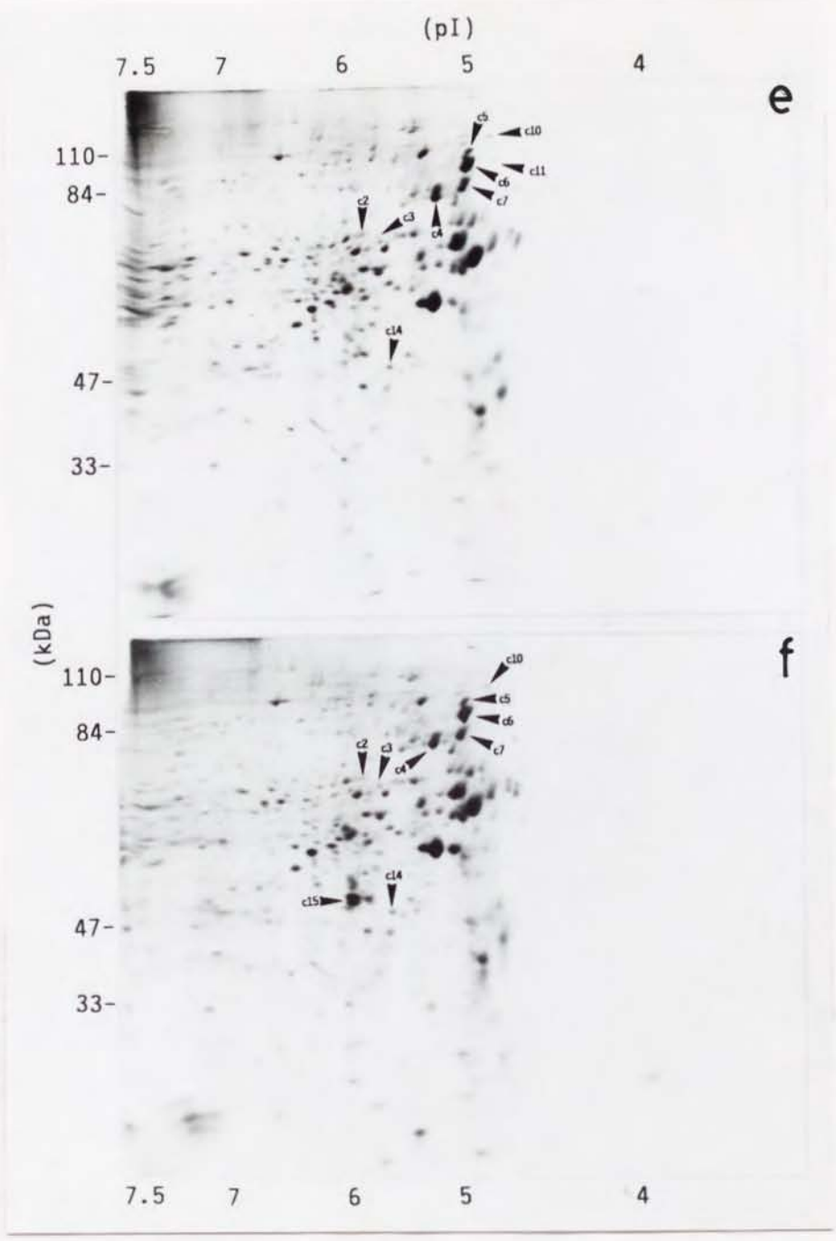


Fig. 9 (continuation).

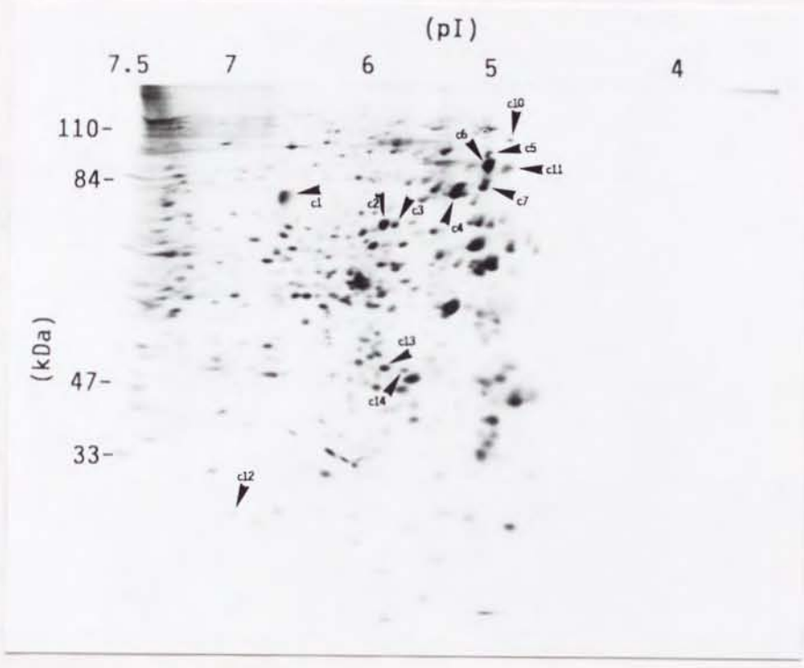


Fig. 10. Patterns of *in vivo* synthesized proteins in chilled eggs. Eggs at day 11 of chilling (started on day 2) were labeled and analyzed as described in Fig. 9. These eggs were considered to be still in a diapause phase since these did not develop normally when transferred to 25° C (see text).

be drawn on the composite chart shown in Fig. 11). This allowed the detailed survey of the patterns for their developmental fluctuation, if any, among different stages.

In the present series of fluorograms the number of total countable spots were 271, of which only 15 spots named from c1 to c15 showed developmental changes. Thus, the results exhibited very small modification from stage to stage in terms of the overall distribution of the spots and their relative intensities within a fluorography.

In the case of 6 hr of development (Fig. 9a), many spots were at a trace level, but the basic arrangement that was observed at this stage substantially remained at the later stages 12, 24, 36, 48 and 72 hr, as shown in Fig. 9b, c, d, e, and f, respectively. The number of countable spots was 264 at 6 hr, then it was 265 at 12 and 24 hr, 264 at 36 hr, 265 at 48 and 72 hr.

Many spots including a newly appeared spot (c13) became intensified at 12 hr (Fig. 9b). At 24 hr, no spot appeared newly (Fig. 9c). The spots c10, c11 and c14 were intensified and c4 appeared at 36 hr (Fig. 9d). At 48 hr, c5 and c6 appeared and other spots, c4 and c7, became strong and then at 72 hr c15 appeared (Fig. 9e and f, respectively). On the other hand, c13 disappeared at 36 hr and c8 and c9 also were not detected at 48 hr (Fig. 9d and e).

In comparison to the developing eggs, the chilled eggs on day 11 (still regarded to be in a diapause state, see MATERIALS AND METHODS) gave a slightly divergent pattern

(Fig. 10), although most part of the pattern was similar to those for the developing eggs. While c1 and c12 were newly found, c8, c9 and c15 spots were not observed. The spots c2 to c7 and c10 to c14, which changed in artificial non-diapause stages, were strong. The total number of observable spots was 268.

Summaries of the changes of in vivo *labeled products*

The positions of spots on electrophoretic gels are summarized for all the stages examined (Fig. 11). The 271 total spots were placed in this diagram. The spots that exhibited the changes according to the stage are indicated by open circles and numbered. Most of the spots undergoing changes at 36 and 48 hr of development locate in the region with pI 5 and the molecular weights more than 60 kDa. The spot c1, found in the dormant eggs during chilling, resides out of this region. The ratio of the spots whose intensities depended upon the stages were approximately 5% of the total population.

The mode of changes of the spots c1 to c15 during development of artificial non-diapause eggs are summarized in Table 2. Also illustrated here are the results of the chilled (still in a diapause state) eggs. As reference the total numbers of countable spots are drawn. The major developmental changes observable in this table are as follows: Most of the spots except c8, c9 and c15 were detected in the chilled eggs as major components. There were small changes

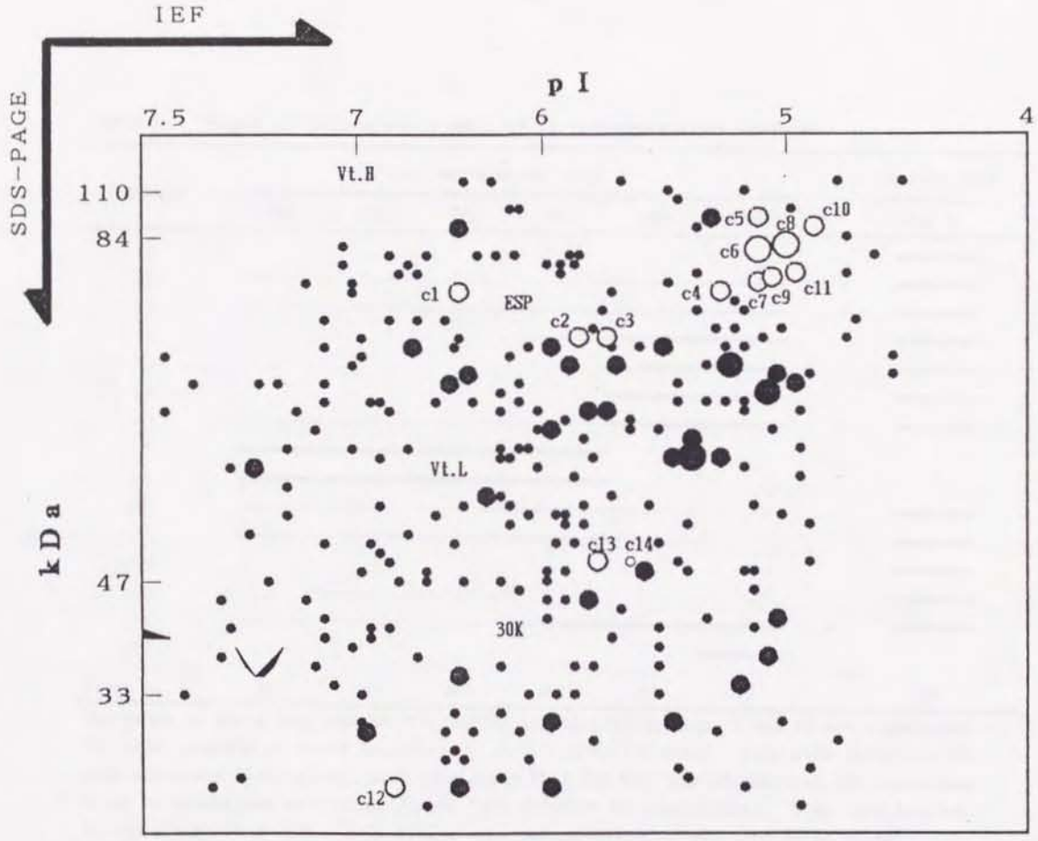


Fig. 11. Schematic representation of fluorographic patterns of *in vivo* synthesized proteins. The results shown in Figs. 9 and 10 are composed. Solid circles represent generally occurring spots whose intensities were stable. The spots drawn with open circles changed in intensity at different stages. Horizontal and vertical lines show isoelectric point and molecular weight, respectively.

Table 2. Changes of fluorographic spots of *in vivo* synthesized proteins.

Products	Artificial non-diapause eggs[a]						Chilled eggs[b]
	6hr	12hr	24hr	36hr	48hr	72hr	day 11
c1							
c2							
c3							
c4							
c5							
c6							
c7							
c8							
c9							
c10							
c11							
c12							
c13							
c14							
c15							
Total No.	264	265	265	264	265	265	268

The fates of spots that showed fluctuation (c1 to c15) on Figs. 9 and 10 are summarized. The total numbers of spots including c1 to c15 were also shown. Fine line indicates that each spots was observable. Bold line means that the spot was stronger at the concerning stage in comparison with other stages (not relative to other spots). Eggs were treated by hot HCl at 20 hr after oviposition (a), and incubated at 25℃ for 48 hr after oviposition and then chilled for 11 days (b).

in number of detectable spots at early periods of development in the artificial non-diapause eggs until 72 hr. The marked changes in the numbered spots were first seen at 12 hr. At 36 and 48 hr more extensive changes were seen. In the chilled eggs, most of the numbered spots were increased in intensity, whereas c9 and c15 disappeared.

DISCUSSION

In this chapter, the pattern of proteins labeled by the incorporation of [^{35}S]methionine *in vivo* were observed for the artificial non-diapause eggs and chilled eggs (on day 11 of chilling, the date not sufficient to evoke the normal developmental ability). The species of spots in fluorograms were in total 271. Most of these newly synthesized proteins were present in all the stages examined with only exceptional spots (c1, c10, c11, c12 and c15), which showed stage-specific mode of occurrence. These results clearly indicate that the quality of proteins detectably synthesized in the cells are constant in spite of the fact that the investigated eggs are under the extensive developmental alterations: the completion of gastrulation and the onset of obvious morphogenesis and so on. The quantitative differences in the relative rates of protein synthesis during development may be of importance; this point will be investigated in the following chapter.

The proteins, which synthesized in developing eggs and

supposed to be required for the growth of embryos, were found to be synthesized also in the chilled eggs, which must be still in a diapause state. The experiments about the synthetic activities in the dormant eggs have always been suffered from a technical difficulty: The eggs may be terminated from diapause by the microinjection, since *B. mori* embryos cease diapause when they are removed from chorions and explanted in a hanging drop of physiological saline (Takami *et al.*, 1966). It is likely that the breakage given to the chorion when the radioactive precursor was injected subsequently elevated the synthetic activity of the embryos by the injury effect. Accordingly, the pattern and/or intensity of *in vivo* incorporation into proteins may be different from those of intact eggs. Nevertheless, the present results clearly indicate the presence of distinct potency of protein synthesis in these dormant eggs. This is in agreement with the previous report that diapause eggs have active mRNAs (Saito *et al.*, 1984). Some of the spots observed here for the dormant eggs were even much more intense than those of the developing eggs. It is possible that the diapause eggs at the stage tested are equipped with an intrinsic or hidden ability of protein synthesis which permits a certain kinds of diapause specific metabolism. The spot c1 was only detected in the eggs at the dormant state as far as tested, although more detailed study will be needed to conclude whether this is a kind of diapause-specific protein or not.

In the experiments of this chapter, the proteins which

are thought to be the stress-shock proteins were scarcely detected in the artificial non-diapause eggs after the hot-HCl treatment. This is compatible with the results of RNA translation experiments described in Chapter II. At 6 hr the spots c8 and c9 appeared; these resembled the b6 spot given by the RNA translation (Fig. 7) in size and pI. However, the occurrence of the c8 and c9 was less dependent upon the stress, and remained to be synthesized at the stages far behind that at which hot-acid treatment was made. It is not likely that these are stress-shock proteins.

At any rate, the results described in the present chapter, together with those of Chapter II, indicate that stress-shock proteins that would appear at the stages shortly after oviposition in the European races (Dorel and Coulon, 1988) are scarcely synthesized in the silkworm eggs used in the present investigation. The disparity between these two samples in the occurrence of stress-shock proteins at the stage of onset of diapause was considered to be due to the respective genetical backgrounds of the European races and the Chinese/Japanese races.

The *in vivo* labeled protein patterns in the post-diapause eggs remain to be clarified. These would provide additional information on the identity of *in vivo* labeled spots to those of the *in vitro* translation products including the tentative stress-shock proteins b1 to b4.

Chapter V

Developmental changes in incorporation rate of radioactive amino acid into proteins

INTRODUCTION

The activity of protein synthesis during the early embryogenesis in the artificial non-diapause eggs has previously been approached by measuring the rates of incorporation of radioactive precursors into the acid insoluble fraction of whole eggs (Kawaguchi and Fujii, 1984; Sonobe, 1986). Also in post-diapause development, such measurements have been done referring to the increase of the polysomes content (Saito *et al.*, 1982). Despite these achievements, the activity of amino acid incorporation into proteins is to be investigated for the strain of *B. mori* used in the present study, since this type of data in general may depend upon the strain, as well as upon the experimental conditions. In the present chapter, the developmental changes of incorporation rate were pursued in the artificial non-diapause eggs, diapausing eggs and post-diapause eggs after microinjection of radioactive methionine into the eggs.

MATERIALS AND METHODS

Preparation of eggs

The eggs were obtained as described in Chapter I. For pre-diapause samples, eggs at 20 hr after oviposition were saved. The eggs treated with hot-HCl at 20 hr to prevent diapause were kept at 25° C and used at intervals. While, eggs chilled at 5° C from day 2 after oviposition until day 180 to terminate diapause were transferred to 25° C to start the post-diapause development. Alternatively, thus-activated eggs were treated with hot HCl and then transferred to 25° C. Unfertilized eggs were taken out from ovaries of the female moths before mating.

Incorporation of radioactive precursor into acid-insoluble fractions of eggs

Each egg was received 50 nl of [^{35}S]methionine by the microinjection method as described in chapter IV, and incubated at 25° C for 60 min. For one analysis 30 of injected eggs were accumulated and homogenized with 300 ul of the solution containing 5% (V/V) 2-mercaptoethanol, 2.3% (W/V) SDS, 62.5 mM Tris-HCl buffer, pH 6.8, and 1 mM PMSF. Thereafter the mixture was centrifuged at 15,000 x g for 5 min at 4° C and an 4 ul aliquot of the supernatant solution was blotted to 4 sheets of filter paper cut in a size of 1 x 1 cm. After air dried, the filter paper was fixed with 25% (W/V) trichroloacetic acid (TCA) for 15 min in an ice bath.

The filter paper was then boiled in 7% (W/V) TCA for 5 min, washed twice in ice cold 5% (W/V) TCA for 15 min, air dried and submerged in 10 ml of scintillation cocktail. Radioactivity was counted with an Aloka 1000 liquid scintillation counter.

RESULTS

Changes in incorporation of radioactive amino acid into insoluble fractions of artificial non-diapause eggs and diapausing eggs.

The radioactivity in the acid insoluble fractions after incorporation of microinjected [^{35}S]methionine was measured for the unfertilized eggs taken from the ovaries, newly deposited eggs and subsequent artificial non-diapause eggs at different stages. The results are illustrated in Fig. 12. The value of the unfertilized eggs was set at 0 hr along the abscissa. The hot-HCl treatment, done at 20 hr after oviposition to prepare the artificial non-diapause eggs, is indicated by an arrow. The incorporation increased gradually from 0 hr to 10 hr, then more rapidly until 20 hr, the time shortly before the hot-HCl treatment. After this treatment the eggs were categorized to the artificial non-diapause eggs, in which the incorporation rose, forming a peak at 36 hr after oviposition. One day after (at 48 hr after oviposition), the value decreased and remained low at 72 hr. After then the incorporation rate rose again, al-

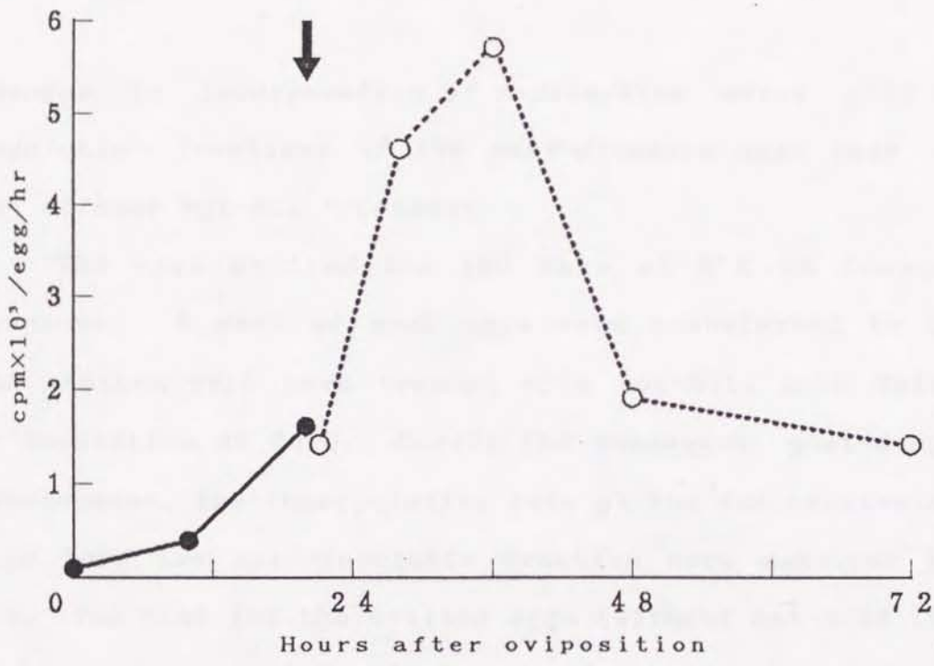


Fig. 12. Incorporation of radioactive amino acid into acid-insoluble fraction of artificial non-diapause eggs. Newly deposited eggs were kept at 25°C and treated with hot HCl at 20 hr (arrow). After then the eggs were called artificial non-diapause eggs (open circles with dotted line). At intervals, eggs were collected, injected with [^{35}S]methionine, incubated for 60 min at 25°C and extracted for TCA-insoluble fractions, which were counted for radioactivity as described in MATERIALS AND METHODS. Unfertilized eggs were used as 0 hr eggs.

though the illustration was omitted.

The same experiments were made on the eggs kept at 25° C without hot-HCl (Fig. 13, note that the ordinate is scaled-up in this figure). The incorporation became a peak at 20 to 30 hr after oviposition. However, the radioactivity decreased until 50 hr to a trace level as the eggs entered diapause and did not augment until day 9 (patterns omitted).

Changes in incorporation of radioactive amino acid into insoluble fractions of the post-diapause eggs both with and without hot-HCl treatment

The eggs chilled for 180 days at 5° C to terminate diapause. A part of such eggs were transferred to 25° C, and another part were treated with hot-HCl, both followed by incubation at 25° C. During the subsequent post-diapause development, the incorporation rate of the radioactive amino acid into the acid-insoluble fraction were measured (Fig. 14). The plot for the chilled eggs (without hot-acid treatment) was set at 0 hr. In both types of post-diapause eggs, the incorporation attained a peak at 12 hr of development. At 24 hr, the radioactivity decreased to the level of 0 hr. The level gradually rose at 48 and 72 hr. Until 48 hr, the curves of radioactivity were similar between the two types of samples. Thereafter, the eggs treated with the hot-HCl gave the higher value than those without the treatment. Although patterns were not shown, this gap lasted until 120 hr of development.

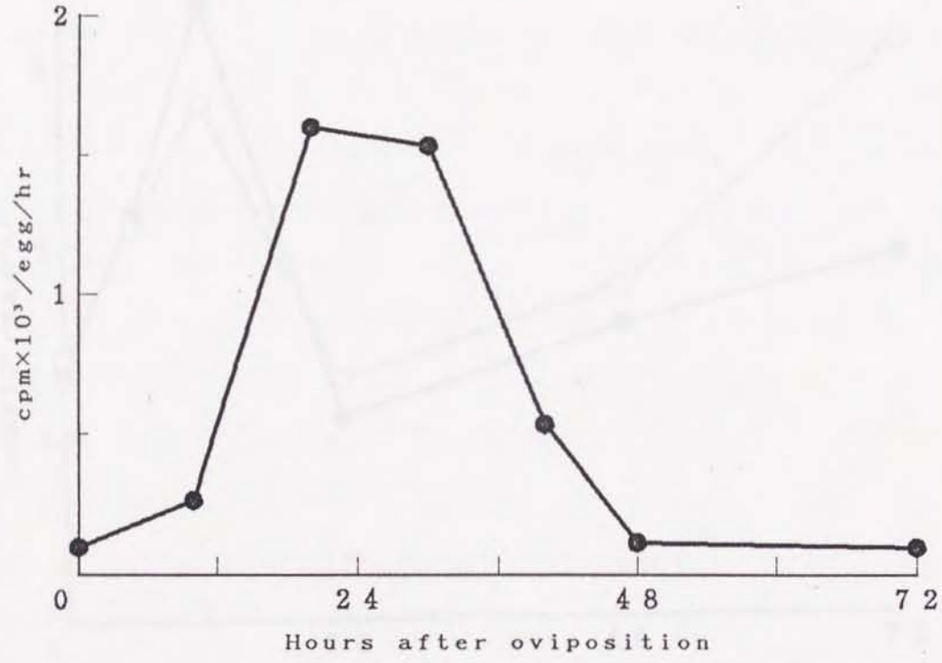


Fig. 13. Incorporation of radioactive amino acid into acid-insoluble fraction of diapausing eggs. The plots for 0 hr, 10 hr and 20 hr are copied from Fig. 12. The eggs were kept at 25°C to enter diapause. Other experimental procedures were as described in Fig. 12.

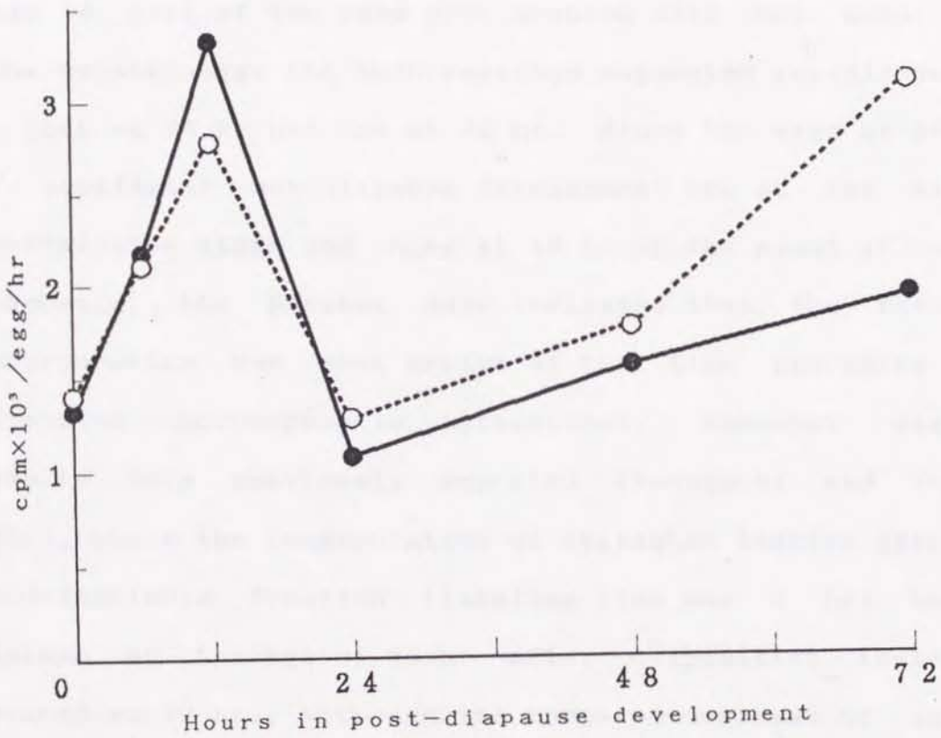


Fig. 14. Incorporation of radioactive amino acid into acid-insoluble fraction of post-diapause eggs. Eggs were incubated for 48 hr at 25°C after oviposition, chilled for 180 days and then made to initiate the post-diapause development. Solid circles represent the incorporation values for the post-diapause eggs incubated at 25°C. Open circles show the values of the similar eggs but activated by the hot-HCl treatment after chilling, followed by 25°C incubation. The procedures of experiments were similar to those described in Fig. 12.

DISCUSSION

The changes of incorporation of a radioactive precursor into egg proteins were measured. The value was low in unfertilized eggs. It rose during the pre-diapause development in the deposited, fertilized eggs until 20 hr of age, when a part of the eggs were treated with hot acid. In thus-treated eggs the incorporation augmented rapidly making a peak on 36 hr but low at 48 hr. Since the eggs at 36 hr of artificial non-diapause development are at the middle gastrulation stage and those at 48 hr at the onset of organogenesis, the present data indicated that the rate of incorporation was most active at the time preceding the extensive morphogenetic alterations. Somewhat similar results have previously reported (Kawaguchi and Fujii, 1984), where the incorporation of tritiated leucine into the acid-insoluble fraction (labeling time was 6 hr) became minimum at the age of 48 hr after oviposition (hot-acid treated at 20 hr), although the temporal decrease of incorporation was not so sharp and its time was shorter compared to the present data. In contrast, the results of similar experiments reported from another laboratory (Sonobe and Odake, 1986) gave the incorporation rate of [^{35}S]methionine (for 3 hr) which rose linearly after the hot-HCl treatment.

These discrepancies among the different investigators may be due to the different strains used and/or experimental conditions, in particular the specific activity of labeled

precursors and the duration of labeling time. In the previous experiments cited above, the labeling time was much longer than that (1 hr) applied in this experiment. Under the present conditions, the incorporation rose linearly until 30 min, leveled off until 1 hr and fell thereafter (data not shown). In future, the amino acid incorporation into proteins should be normalized by measuring the pool size of direct precursor (amino-acyl tRNA) of protein synthesis, to obtain the absolute rate of protein synthesis, although measuring the pool size requires tedious work so that the method has previously been applied for only limited species, e.g. the sea urchin embryos (Fry and Gross, 1970; Regier and Kafatos, 1977).

Nevertheless, the present investigation, together with those reported previously, confirmed that the rate of incorporation increased rapidly after the activation of eggs by hot acid.

On the other hand, the eggs which were allowed to enter diapause without hot-acid treatment displayed a decreasing pattern, leaving a temporal maximum at 20 to 30 hr of age. This result was in substantial agreement with those reported previously (Kawaguchi and Fujii, 1984; Sonobe and Odake, 1986). After the long-term chilling of hibernating eggs, the apparent ability of amino acid incorporation had already recovered as seen in the 0-hr plot of Fig. 14. The time and mode when and how this recovery occurs during the period of cold treatment remain to be

investigated.

The incorporation was measured also for the post-diapause development. In comparison between the eggs with and without the hot-HCl treatment, no difference was observed until 48 hr after the onset of the post-diapause development. The chilled eggs before the start of the development already had the activity of amino acid incorporation as discussed above. In the chilled eggs, the incubation for labeling was brought about at 25° C, and the value could be understood as an intrinsic potential of protein synthesis, since under the natural conditions these eggs would undergo limited protein synthesis at the low temperature. The value rose rapidly without a lag period in agreement with the previous report for the acid-treated post-diapause eggs (Saito *et al.*, 1982). The incorporation made a peak at 12 hr, fell thereafter and rose again from 24 hr, when the extensive organogenesis commences. The value attained another peak at 72 to 96 hr (patterns omitted). The marked fluctuation curve observed here is in contrast to the pattern seen in a previous investigation, in which the increase continued linearly until 96 hr without temporal minimum (Saito *et al.*, 1982). The discrepancy again may be ascribed to the different strains and/or experimental conditions as discussed above.

The post-diapause eggs treated by hot-HCl develop normally as do those without the treatment. Therefore, little difference in the developmental changes in amino acid

incorporation rate between the eggs with and without hot-acid treatment, as seen until 48 hr of the development, is not a matter of surprise. From this point of view, the disparity between these kinds of eggs as observed after 72 hr (the gap continued until 120 hr, patterns not shown) offers a peculiar question: Irrespective of whatever the present result mirrors some difference in absolute rate of protein synthesis or that in the pool size of methionine directly affecting the apparent rate of its incorporation into proteins (Berg and Mertes, 1970), some delayed effects of hot-acid treatment are suggested. More detailed investigation will be needed to solve the problem.

The overall results of the experiments described in this chapter confirm that marked fluctuation in rate of amino acid incorporation into whole proteins (thus probably in rate of protein synthesis) occurs at the stages investigated in the previous chapters.

GENERAL DISCUSSION

The extent of protein synthesis in the diapause eggs of *B. mori* has previously been assessed by the measurements of both the incorporation of radioactive amino acids into the acid-insoluble fraction (Kawaguchi and Fujii, 1984; Sonobe, 1986; Saito *et al.*, 1985a) and the proportion of polysomal ribosomes per total population of ribosomes (Saito *et al.*, 1982). It was concluded that the rate of protein synthesis in diapause eggs is suppressed, although these have translatable mRNAs (Saito *et al.*, 1984), suggesting the regulation or protein synthesis at the translation level is involved at the developmental switches. Soon after the onset of post-diapause development, these mRNAs will be utilized for translation in the cells, although contribution by newly transcribed mRNAs must gradually become important. On the other hand, the start of diapause and its intensification may accompany the dissociation of mRNAs from polysomes (Saito, unpublished results). Therefore, the author is interested in the problem whether mRNAs change qualitatively at the different stages of diapause and development in the eggs of *B. mori*.

The present study was mainly aimed at the analyses of the species of RNAs which could be translated in an *in vitro* protein synthesizing system. The patterns of *in vitro* translational products were compared between the stages of

pre-diapause, artificial non-diapause and post-diapause development. The post-diapause eggs were divided in two groups: those activated by chilling alone and those treated by hot HCl after chilling. RNAs from all states of eggs gave more than 130 countable spots on the 2D fluorograms, only a part of which underwent the changes in intensity. No large difference of the pattern was observed among pre-diapause eggs, chilled eggs and post-diapause eggs. These facts indicate that the extensive interchange of the pre-existing set of mRNAs does not take place in the eggs, in which physiological and morphological alteration are actively progressing. Moreover, the rate of *in vivo* incorporation of [^{35}S]methionine into the acid-insoluble fraction altered extensively, suggesting that protein synthesis is changed in terms of quantity. This situation was typical at the first 24 hr after the onset of post-diapause development; the initial activation of protein synthesis at this stage does not require the appearance of new set of mRNA species.

The present results, together with the previous knowledge of the presence of active mRNA cited above, also indicate that eggs during diapause and chilling has limited activity of transcription (and of mRNA metabolism); thus the mRNAs in the eggs after termination of diapause may contain the population present in the eggs at the beginning of diapause.

In addition to the species of translatable mRNAs, those

of the proteins synthesized *in vivo* were analyzed. In order to compare the diapausing stage (the eggs chilled for 11 days were prepared as diapause eggs) and the artificial non-diapause eggs were used for the experiments. Consequently, most of the spots were not different throughout the stages tested. These results seem to imply that a little changes in quality of proteins are required to the development. This situation is different from that in *D. melanogaster* (Summers *et al.*, 1986) and in the sea urchin (Bedard and Brandhorst, 1983), *Xenopus laevis* (Ballantine *et al.*, 1979); in these organisms, the increase of incorporation rate of radioactive amino acid is augmented in parallel with the occurrence of new protein species until the stage of gastrulation. As to the quantitative aspect, the *B. mori* eggs also exhibited an decrease in incorporation of amino acid into proteins at an early stage of gastrulation (48 hr after oviposition) and this mode of changes resembled those of the above organisms.

In the fluorographic study, chilled eggs (still had no developmental ability when transferred to 25° C) possessed the potency of incorporation to give an identical pattern to that of the developing eggs. This incorporation in the dormant eggs would be caused by the breakage of chorion. It has been argued that the diapause phase is maintained by the limited supply of oxygen (Okada, 1971; Sonobe *et al.*, 1979). Indeed, when diapause eggs are incubated in nitrogen gas and polyols (the latter are specifically ac-

cumulated in relation to diapause), apparent dormancy lasts (Okada, 1971; Kageyama and Ohnishi, 1973). The present results suggest that the diapausing eggs, in particular those during chilling, are equipped with the ability to recover protein synthesis, and the regulation mechanism could be immediately turned on, although the actual rate of protein synthesis in intact eggs would be low enough to avoid consuming the energy sources (Chino, 1957, 1978). The presence of mRNA is considered to be a strategy for rapid re-activation when the environmental conditions become adequate to initiate development. The low rate of protein synthesis in a dormant phase is explained, at least partly, by the limited access of mRNAs to the translation system. In this connection is of importance the finding that the cap site of mRNA molecules from dormant eggs are lacking in methylation (Saito *et al.*, 1985b), which is necessary for normal translation of mRNAs.

In the meantime, the post-diapause eggs activated by the hot-HCl treatment gave temporally appearing spots as revealed by the *in vitro* translation experiments. Such spots (b1, b2, b3 and b4) disappeared in the pattern at the subsequent stages tested. The spots b1 to b4 did not appear at the comparable stages after the onset of the post-diapause development that was evoked by chilling alone. On the basis of these modes of appearance, the components b1 to b4 are assumed to be stress-shock proteins like *hsp*70 of *D. melanogaster* (Buzin and Petersen, 1982; Ashburner and Bon-

ner, 1979). These components were characterized by having pI of 5 to 6 and size of about 70 kDa on the 2D gel, the values resembling those of *hsp*70 (loc. cit.).

The RNA(s) which was hybridizable with a probe containing the *D. melanogaster hsp*70 gene were also detected by the Northern blotting in the post-diapause eggs at 6 hr after the hot-HCl treatment. Such a signal was not found in the eggs at the same age without the hot-acid treatment. This situation is in parallel with that of the appearance of the b1, b2, b3 and b4 spot in the above described *in vitro* translation study. Although the band with the hybridization signal was single, and whether it constituted of plural components is unknown, it is tempting to suppose that the band contained the mRNAs for any or all of the b1, b2, b3 and b4 proteins.

In addition, a trace signal of hybridization was detected in all stages examined, suggesting that the relevant RNA has a basal expression. However, in the *in vitro* translation experiments, the corresponding appearance was not detected. It should be noted here, however, that the b1, b2 and b3 spots were detected in pre-diapause stage, indicating the presence of mRNAs at this period.

The author suggests that the tentative stress-shock proteins found in the present study may be related to the recovery from the stress that is forced to occur by the hot-HCl treatment. Also *hsp*70 has been discussed in relation to the survival at an elevated temperature (Mitchell *et*

al., 1979). In general the stress-shock proteins in developing embryos can be produced when the transcriptional ability of the embryonic genome is established; in the 2-cell stage in case of the mouse embryo (Petzoldt *et al.*, 1981). Also in the post-diapause eggs of *B. mori*, the appearance of b1 etc. may offer an indication of transcriptional ability necessary for the recovery from the stress. In the light of this inference, the fact that any of the b1, b2, b3 and b4 was not detected in the artificial non-diapause eggs after the hot-HCl treatment is rather incomprehensible. In European races, a spot induced by hot-HCl treatment has been reported (Dorel and Coulon, 1988) as discussed in Chapter II. The difference would be due to some evolutional background of the races used.

The *in vitro* translation products named b5, b6, b8, b15, b19, b20, b22, b23, b27 and b30 appeared or became intense in early periods (6 or 24 hr) in the post-diapause eggs when treated with hot HCl. These components were also seen or became strong in the post-diapause eggs without hot-HCl treatment, but only at 72 hr. Thus the time when these spots become marked was shifted by the hot-HCl treatment, suggesting that the hot-acid treatment might play a role to accelerate the expression of some mRNAs.

On the whole, the experiments described in the present article indicated very limited changes in quality of both mRNAs and proteins synthesized in different stages of *B. mori* eggs. Only a small fraction of the mRNAs and proteins

exhibited fluctuation. These include the molecules of which appearance strictly dependent upon the hot-HCl treatment, which may be a severe stress to the eggs but generally conducted to break successfully the diapause in *B. mori*. These proteins are suggested to have a role in the establishment of the normal post-diapause development after the recovery from the stress-shock.

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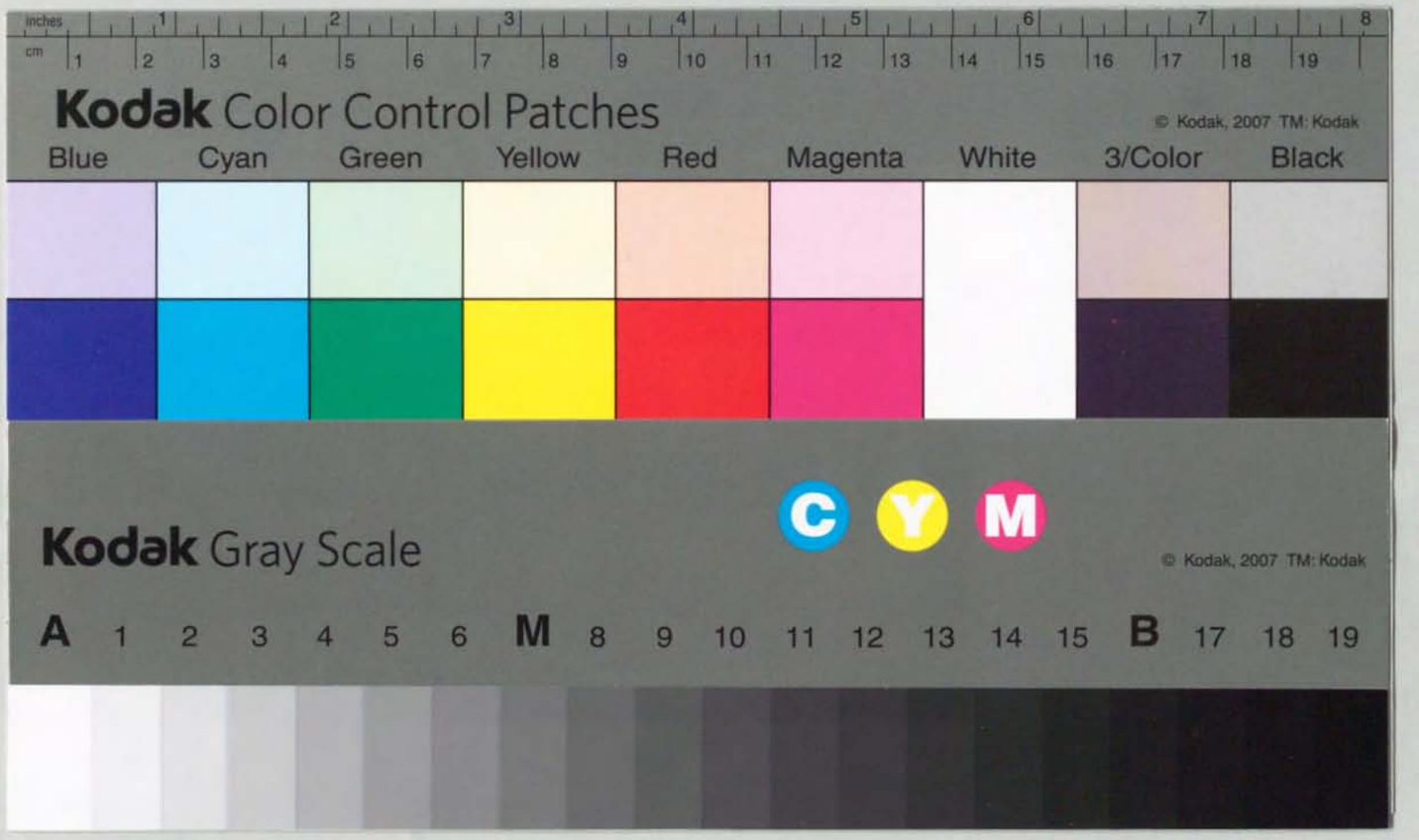
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