

## Analysis of gene expression during embryonic development in mulberry silkworm *Bombyx mori*

Amit Singh and Karumathil P. Gopinathan

Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore 560 012, India

We have developed a method for dechoriation and devitellinization of the silkworm eggs without damage, to facilitate the analysis of gene expression during embryonic development of *Bombyx mori*. Making use of antibodies available from heterologous systems, the spatio-temporal expression patterns of *peroxidase* and *proliferating cell nuclear antigen* have been directly visualized in whole mount embryos at various stages of development without the need for generating transformed lines carrying specific reporter constructs. The *B. mori* system, previously unamenable for such studies, could thus serve as an attractive model for molecular analysis of insect development.

THE attention lavished on *Drosophila melanogaster* as the insect species *par excellence* for the genetic and molecular analysis of development, has eclipsed the earlier investigations on most other insects for a multitude of challenging biological phenomena. The mulberry silkworm, *Bombyx mori* has a genetic legacy comparable to that of *Drosophila*, but most of the efforts on the silkworm were channelled towards the improvement of the race through breeding, due to its economic importance<sup>1,2</sup>.

The extended period of embryonic development of *B. mori* (10 days as compared to 24 h in *Drosophila* and 100 h in *Manduca sexta*, another widely studied Lepidopteran insect) provides an advantage to discretely analyse without overlap, the events leading to pattern formation. The early investigations on *B. mori* were confined to the problems of diapause<sup>3</sup> and storage of embryos or towards the elucidation of morphological landmarks by electron microscopy<sup>3-5</sup> and fluorescent vital dyes<sup>6</sup>. Recently the fate mapping of *B. mori* embryo has been carried out following laser irradiation<sup>7</sup>. The investigations on gene expression during early embryonic development of silkworm have lagged behind because the dechoriation and devitellinization of embryos proved to be major hurdles. Besides, the embryonic development could not be examined due to the nonavailability of cell type-specific molecular markers as well as the lack of transgenic methodologies. In such situations the immunocytochemical approaches provide an alternative. Antibodies against gene products can be exploited as tissue- and cell-specific markers to analyse phenotypes in mutant embryos<sup>8-15</sup>. Recent studies<sup>8-17</sup> have implicated the evolutionary conservation, based on sequence similarity of developmentally important genes, in a range

of distantly related species. As a consequence, immunologic probes from heterologous sources could prove to be useful in detailed tracking of individual gene products during development. There is a distinct need, however, to adopt the existing methodologies to the *B. mori* system, where a major hurdle exists due to the presence of a thick chorion layer encasing the egg which affects the permeability of the embryo to external probes.

Here, we have improvised a protocol for the dechorionation and devitellinization of *B. mori* embryos to facilitate the analysis of spatio-temporal expression of genes during development. We have also exploited the heterologous antibodies against *Drosophila* gene products to examine the pattern of gene expression during embryonic development of silkworm and demonstrate that the system is now amenable for such analysis.

*B. mori* egg is a flattened sphere, 1.2 mm long, 0.95 mm wide and 0.63 mm thick and is encased in an envelope of chorion (Figure 1 a). The antero-posterior and dorso-ventral axes of the egg are clearly discernible from the flattened shape of the anterior side. The surface of the chorion shows hexagonal spots with no subdivisions and an anteriorly situated micropyle (Figure 1 b) which permits sperm entry.

*B. mori* follows a characteristic embryonic development pattern which essentially resembles the 'long germband' insects but with distinctive characters of its own (Figure 2). In brief, the major landmarks during embryonic development are syncytial blastoderm (12 h after egg laying), germband formation (20 h), gastrulation (25 h), generation of segmental body plan (35 h) and blastokinesis which initiates from the 4th day and lasts till 5th day. During blastokinesis, the developing larva makes a complete turnaround from its initial position and along with it the developing legs and the pseudopods get located into the interior. This revolution of embryo is an interesting feature of *B. mori* development. By the 7th day the complete functional larva is formed which hatches out only on the 10th day. Studies on the developmental expression of the genes during embryogenesis in *B. mori* could not be done previously due to problems of dechorionation and devitellinization of the eggs. The thick chorion can neither be removed mechanically during earlier stages of embryonic development as in *Drosophila*<sup>18</sup> nor its permeability be increased to stain the embryo without removal of chorion as practised in zebra fish<sup>19</sup>. Moreover, the thickness of the *B. mori* embryos reduces microscopic resolution of the staining patterns.

Eggs (embryos) were washed in distilled water to remove the glue covering them and suspended in PBT (PBS + 0.1% TritonX-100) containing 0.1% proteinase K for 15 min. They were dechorionated in 5% hypochlorite bleach for 5 min followed by the addition of

an equal volume of heptane. On dechorionation the embryos pop into the organic heptane layer preventing any damage. We tried different concentrations of the hypochlorite bleach and found that a higher concentration (5%) was necessary to dechorionate *B. mori* eggs. Pre-treatment of eggs with proteinase K further improved the effectiveness of the bleach. The presence of heptane allowed the immediate relocation of the dechorionated

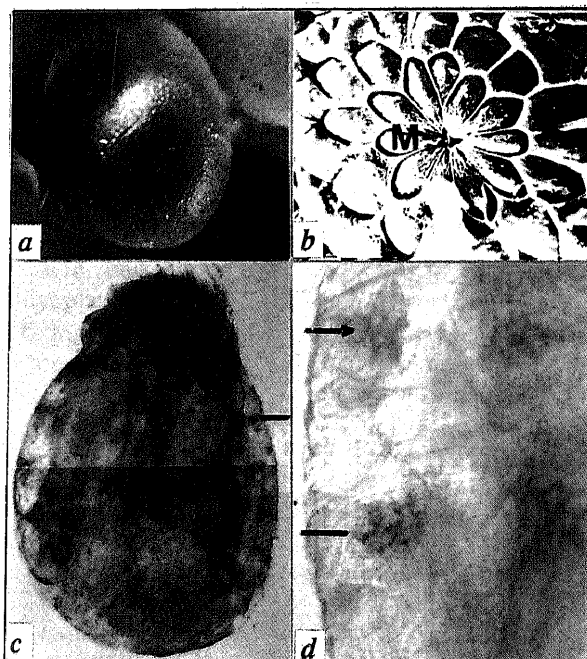


Figure 1. Whole mount embryonic staining using anti-HRP antibody. a, *Bombyx mori* egg ( $\times 15$ ), seen under stereo microscope (Olympus). b, Scanning electron micrograph (JEOL) of the egg shell surface ( $\times 1000$ ).

Eggs were collected at 30 min intervals and maintained at 25°C. The time of embryonic development is referred to as 'hours after egg laying' (AEL) and hatching occurs 10 days after oviposition. For staining, the dechorionated embryos were fixed in equal volumes of heptane and the fixative (4% paraformaldehyde in PBT, 1 mM MgSO<sub>4</sub>, 2 mM EGTA) for 40 min with continuous shaking. These embryos were flushed thrice with methanol to remove the fixative and stored in methanol at -20°C. Prior to antibody staining, the embryos were rehydrated in several changes of PBT followed by three washes of PBTA (PBT + 1% bovine serum albumin) for 2 h and incubated overnight at 4°C in primary antibody. After primary antibody reactions, the samples were washed in three changes of PBTA for 10 min each at room temperature, treated with the secondary antibody for 2 h at 4°C and the expression was monitored.

For detecting peroxidase expression (as marker for the neuronal development), the dechorionated embryos were treated with anti-horse radish peroxidase (HRP) antibody, followed by treatment with the secondary, goat anti-rabbit IgG coupled to HRP as the detection system. HRP activity was detected by staining with 0.5 mg/ml of diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub>. The stained specimen were observed under DIC optics on an Olympus microscope. c, Whole mount embryo (6th day of embryogenesis). d, Magnified view of a bundle of sensory bristles on embryonic ectoderm ( $\times 100$ ). Controls, in which the primary antibody was left out prior to the reaction with the anti-antibody, did not show colour development. For comparison of the embryonic development stage, see Figure 2.

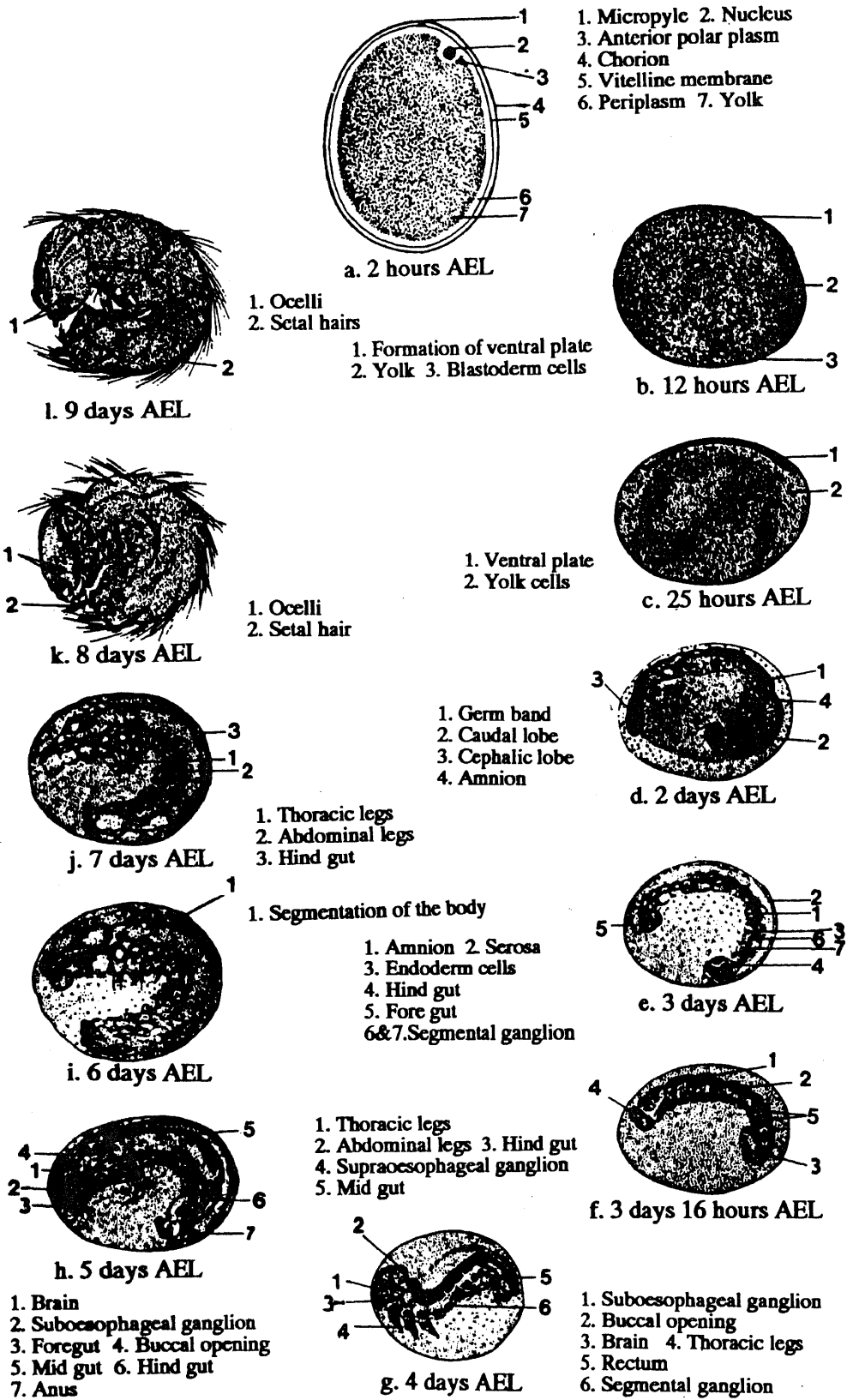


Figure 2. Schematic presentation of the embryonic development of silkworm *B. mori*. Various landmarks of the embryonic development along the temporal scale are depicted. *a*, Structure of silkworm egg. *b*, Cleavage and blastoderm formation. *c-f*, Germband formation. *g-h*, Blastokinesis. *i-l*, Completion of embryonic development.

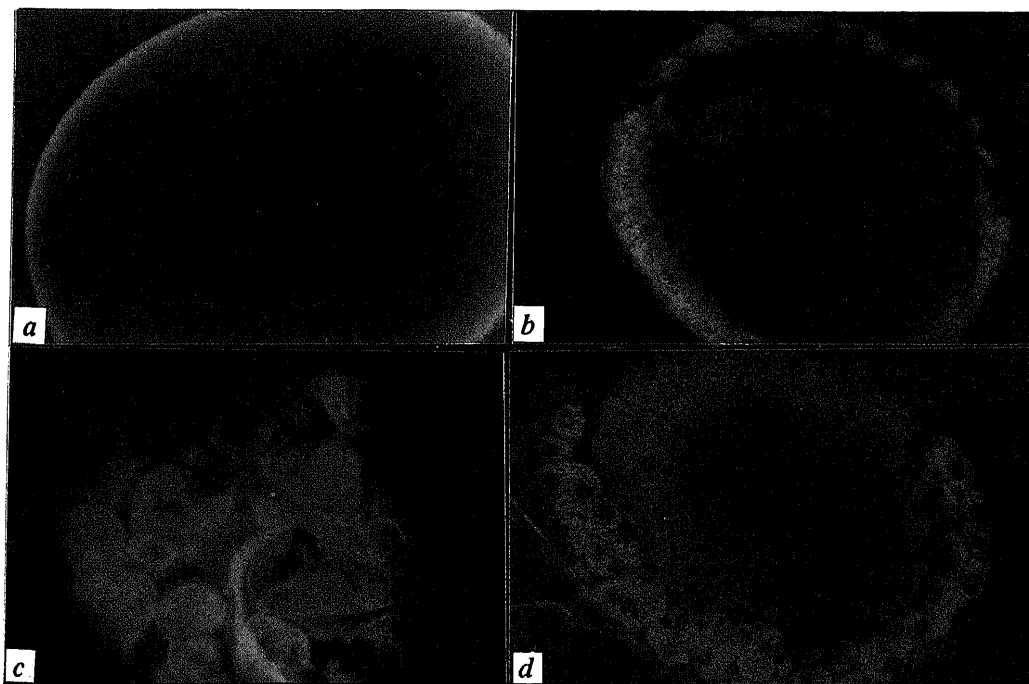
embryos to the organic layer. The relatively brief exposure of embryos to higher concentration of bleach along with an interface of heptane has allowed us to achieve chemical dechoriation successfully with higher yields and less morphological damages.

Such dechorionated embryos were devitellinized in heptane-methanol interphase<sup>18</sup> and examined for the expression of different genetic markers by making use of heterologous antibodies. The expression patterns of *peroxidase* and *proliferating cell nuclear antigen (pcna)* were examined using rabbit polyclonal antibodies raised against horse radish peroxidase (HRP) or *Drosophila* PCNA respectively. The appearance of peroxidase activity has been taken as an expression marker for nervous tissue development<sup>20</sup>. The expression of *peroxidase* was detected in basal cells of the sensory hair on the ectoderm of the silkworm embryo at the 6th day of development (Figure 1 *c,d*; arrowhead). Suitable controls were included while conducting the antibody staining reaction (where the samples were treated only with the secondary antibody with no prior treatment of primary antibodies) to rule out any possible artefacts. In fact, little information has been documented in the past on neuronal development during silkworm embryogenesis. The present approach

thus serves as a convenient means for such analyses, if various nervous system specific markers are used as probes along the temporal scale.

The approach for the detection of gene expression was extended further to study the expression of *pcna*. PCNA is involved in several vital processes of cell division and DNA replication, and its presence could be expected in all dividing cells during development. Using antibodies, *pcna* expression was detected initially at 10 h of development at the cellular blastoderm stage confined to the periphery of the embryo (Figure 3 *a*). At 20 h of development *pcna* expression further evolved in the developing germband on the ventral side of the embryos, marking all the developing segments (Figure 3 *b*). During gastrulation movements at 25 h after egg laying, it was expressed in all the developing segments of the silkworm embryos which further refined at the end of 35 h expressing in all the seven head-, three thoracic- and eleven abdominal segments of the larval body plan (Figure 3 *c,d*).

Using the methodologies established here, the *B. mori* system with its excellent genetic backup can be exploited to serve as an alternative model system to study gene expression. The extensively studied insect *Drosophila*



**Figure 3.** Embryonic expression of *proliferating cell nuclear antigen*. The dechorionated embryos were treated with rabbit polyclonal antibody to *Drosophila* PCNA and the reactions were visualized using goat anti-rabbit antibody conjugated to fluorescein isothiocyanate. The specimen was examined under an Argon Laser Confocal Microscope (Biorad MRC 1000). *a*, Expression at the syncytial blastoderm stage, optical cross section at 10 h after egg laying (AEL) ( $\times 10$ ). *b*, Developing germband with formation of the segments as shown by arrow head at 20 h AEL. *c*, Cellular movement of gastrulation at 25 h AEL. *d*, Embryo at 35 h AEL with well-developed germband. Note the expression is uniformly distributed in the entire germband with several pockets of rapidly dividing cells. Controls in which the primary antibody treatment was omitted, were always included, and they did not show staining. For comparison of the embryonic development stages, see Figure 2.

## RESEARCH COMMUNICATIONS

belongs to the phylogenetically evolved order Diptera and not necessarily represent all insect groups. The embryogenesis in *Drosophila* is rather unusual compared to other Arthropods<sup>21-24</sup>. Comparison with the more 'typical' insects may provide further insights as to which aspects of embryogenesis are widespread and which are phylogenetically restricted. Lepidopterans being separated by more than 200 million years from Diptera, should provide an important window on evolutionary innovations relative to *Drosophila*. This will offer two complementary approaches deducing both ontogenic and phylogenetic mechanisms<sup>23</sup>.

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