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Review

Genes that are involved in *Bombyx* body plan and silk gene regulation

YOSHIAKI SUZUKI*

National Institute for Basic Biology, Department of Developmental Biology, Myodaiji, Okazaki City, Japan

ABSTRACT I have summed up how silk gene regulation studies in *Bombyx mori* have been carried out. This process has brought me naturally to realize the importance of understanding the *Bombyx* body plan in comparison with the body plans of other organisms. Although their current status remains preliminary, I have tried to summarize the ongoing projects and to reveal future problems to be answered.

KEY WORDS: *Bombyx mori, body plan, silk gland, regulatory genes, tissue specific genes*

Summing up of past studies

Mendel's law was first confirmed in the animal kingdom with *Bombyx mori* (Toyama, 1906). Immediately after this report the same author described an anatomical analysis of silk gland development in *B. mori* embryos (Toyama, 1909). These pioneering studies were followed by enormous amount of silkworm genetics describing several hundred mutants on various genetic traits (see Tanaka, 1952; Tazima, 1964; Chikushi, 1972). Among these the studies on the homeotic mutants of *E* loci were superb (Sasaki, 1930; Suzuki and Ohta, 1930; Hashimoto, 1930, 1941; Itikawa, 1943, 1944, 1952; Tazima, 1964), and were pioneering works on the body plan, some of which will be referred to in later sections. Molecular biology was first introduced to the organism when the messenger RNA for silk fibroin was isolated from the posterior portion of silk gland and identified by its partial sequence analysis (Suzuki and Brown, 1972), the first chemically identified messenger RNA in eukaryotes.

Upon the isolation of fibroin mRNA the first biological question asked was whether there were any specific gene amplification events in the processes of cell differentiation (Suzuki *et al.*, 1972), since there was an example of amplification of rDNA in the oocytes of *Xenopus* and a few other organisms (Brown and Dawid, 1968). The silk fibroin gene transcribed specifically in the posterior silk gland was detected by hybridization of the pure mRNA to the genomic DNAs (Suzuki *et al.*, 1972). There was no such amplification of the gene; its concentration was constant in the producing tissue and non-producing tissues corresponding to 1-3 genes per haploid genome (Suzuki *et al.*, 1972), and later it was determined to be a single copy gene (Gage and Manning, 1976).

The next question asked was whether there were any structural changes of the gene during the differentiation processes, as in the case of immunoglobulin genes (Hozumi and Tonegawa, 1976; Bernard *et al.*, 1978). The fibroin gene was cloned (Ohshima and Suzuki, 1977) and sequenced; there was no structural difference between the genes from the producing tissue and non-producing tissue (Tsujimoto and Suzuki, 1979a,b; Suzuki and Adachi, 1984). Not even a methylation modification difference was found in the natural fibroin genes purified without using the cloning procedure from the producer tissue and non-producer tissue (Tsujimoto and Suzuki, 1984).

Later, the fibroin light chain gene expressed coordinately with the fibroin (heavy chain) gene was cloned and characterized (Yamaguchi *et al.*, 1989; Hui *et al.*, 1990b).

Knowing the constancy of the fibroin gene during silk gland differentiation, our attention was forwarded to the analysis of elements and factors involved in transcription regulation. Transcription studies of the fibroin gene were initiated by measuring the mRNA expression pattern (Suzuki and Suzuki, 1974; Suzuki and Giza, 1976; Maekawa and Suzuki, 1980) and followed by the development and use of cell-free transcription systems (Tsuda and Suzuki, 1981, 1983; Tsujimoto *et al.*, 1981; Hirose *et al.*, 1982, 1984, 1985; Tsujimoto and Suzuki, 1984; Tsuda *et al.*, 1986; Suzuki *et al.*, 1986, 1990; Hirose and Suzuki, 1988; Obara and Suzuki, 1988; Takiya and Suzuki, 1989, 1993; Hui and Suzuki, 1989; Takiya *et al.*, 1990) and a transfection cell system (Tokunaga *et al.*, 1984). It should be emphasized that the cell-free extracts from the silk gland (Tsuda and Suzuki, 1981) were the first example of tissue extracts which can transcribe genes faithfully reflecting appropriate regulations (Suzuki *et al.*, 1986, 1990); even a differential transcription of the fibroin gene and the sericin-1 gene has been accomplished in these extracts (Suzuki *et al.*, 1990).

The sericin (Okamoto *et al.*, 1982; later renamed as sericin-1) and sericin-2 (Michaille *et al.*, 1990) genes transcribed specifically in the middle portion of the silk gland were cloned and sequenced. Transcription studies of the sericin-1 gene were carried out also in the cell-free systems (Obara and Suzuki, 1988; Hui and Suzuki, 1989; Matsuno *et al.*, 1989, 1990; Suzuki *et al.*, 1990).

***Address for reprints**: National Institute for Basic Biology, Department of Developmental Biology, Myodaiji, Okazaki City 444, Japan. FAX: 81-5647566.

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These studies in the cell-free systems indicated approximate locations of *cis*-acting elements on both the fibroin and sericin-1 promoters. The DNase I footprint and gel shift assays with the wild type as well as mutant oligonucleotides indicated precise locations and important nucleotides of *cis*-acting elements in the promoters and the presence of *trans*-acting factors in the silk gland extracts (Suzuki and Suzuki, 1988, 1991a,b; Matsuno *et al.*, 1989, 1990; Hui *et al.*, 1990a,b; Takiya and Suzuki, 1993).

Fibroin factor 1, which is presumed to function on the enhancer I of fibroin gene under the co-existence of fibroin factor 2, was purified and characterized (Suzuki *et al.*, 1991a,b; Ohno, unpublished). Another transcription factor, SGF-1, which is presumed to function on both proximal promoter regions of the fibroin and sericin-1 genes (Hui *et al.*, 1990a; Matsuno *et al.*, 1990), has been purified and sequenced (Mach, unpublished data). Another factor, OBF-1, which binds to octamer-like sequences in the enhancer II of the fibroin gene (Takiya *et al.*, 1990), has also been partially purified (Takiya, unpublished). Currently we plan to clone these factors. Recently, the SGF-1 was identified as a *fork head* homolog (Mach and Takiya, unpublished data). SGF-3 binds to the sericin-1 gene at the SC region accommodating a sequence for an octamer-binding protein (Matsuno *et al.*, 1989, 1990; Hui *et al.*, 1990a) and is presumed to be a key factor to stimulate transcription of the gene (Matsuno *et al.*, 1990). Its cDNA was cloned from the middle silk gland, sequenced, and named POU-M1 (Fukuta *et al.*, 1993) because of its accommodation of a POU domain identical with that of *Drosophila Cf1-a* (Johnson and Hirsh, 1990). A genomic DNA fragment encompassing the whole coding region of POU-M1 as well as the flanking regions was cloned and sequenced (Xu *et al.*, 1994a). Using the cell-free transcription system from the middle silk gland, several positive and negative *cis*-acting elements of the *POU-M1* promoter have been determined (Xu *et al.*, 1994a). POU-M1 protein binds to the PB region of the promoter and suppresses its transcription indicating a negative autoregulation of the gene.

Based on these studies in the past we are currently trying to understand 1) the *Bombyx* body plan for its specificity and generality and 2) the mechanism determining the labial segment identity so that the silk gland can be induced in the labial segment and differentiated to result in the tissue-specific transcription of the fibroin and sericin-1 genes.

Current and future problems

Bombyx body plan genes

The more we understand the regulation hierarchy of the silk gene transcription, the more we realize the importance of knowing the body plan in *Bombyx,* under which a specific part of the body, the silk gland, is determined for its fate and specialized. Among many of the important candidate genes for body plan we have begun characterizing homologs of *caudal* (Xu *et al.*, 1994b), *engrailed, invected* (Hui *et al.*, 1992), *Wnt-1* (Amanai *et al.*, in preparation), *Antennapedia* (Hui *et al.*, 1992; Nagata *et al.*, in preparation), *Deformed, Sex combs reduced* (Kobubo *et al.*, in preparation), *Cf1-a* (Fukuta *et al.*, 1993; Xu *et al.*, 1994a), *fork head* (Takiya, unpublished), *Ultrabithorax, abdominal-A,* and *Abdominal-B* (Ueno *et al.*, 1992).

Clones containing whole open reading frames of *Bombyx caudal (Bm cad)* were isolated from a cDNA library of *Bombyx* embryos (Xu *et al.*, 1994b). We are interested in studying the nature of the *Bm cad* for two reasons; (1) there are still no reports on the maternal genes at the top of hierarchy in insects other than the long germ band type *(Drosophila)*, and (2) there is no direct molecular identification on the concentration gradient spanning anteroposterior axis in insects other than *Drosophila* (Gehring, 1973; Nüsslein-Volhard, 1979; Mlodzik *et al.*, 1985; Mlodzik and Gehring, 1987; Driever and Nüsslein-Volhard, 1988). Northern hybridization with a *Bm cad* probe revealed the presence of single maternal transcript of 2.3 kb. A stronger signal of the transcripts was detected from the unfertilized eggs to the embryos 36 h after deposition. The transcripts decreased rapidly by 48 h and a weak signal was maintained until hatching. *In situ* hybridization experiments revealed that *Bm cad* transcripts were firstly accumulated in the nurse cells and transferred into the oocyte (Xu *et al.*, 1994b). The *Bm cad* mRNA and protein form concentration gradients spanning anteroposterior axis during the gastrulation stage (Xu *et al.*, 1994b), while the mRNA and protein of *Drosophila cad* reveal the corresponding expression profile during the syncytial blastoderm stage (Mlodzik *et al.*, 1985; Mlodzik and Gehring, 1987); a clear difference in the body plans of *Drosophila* and *Bombyx*. What kind of a cascade modification would be produced under this difference?

From a morphological analysis, the *Bombyx* embryos seem to belong to either intermediate or short germ band type; a stage-6 (36 h) embryo reveals a non-segmented morphology at the presumed head, gnathocephalon, and thorax regions and an ambiguous growing morphology at the future abdominal region (Takami and Kitazawa, 1960; Amanai, unpublished). By stage 16 (48 h) the embryo is very much elongated by growth and segmented from head to tail. This point should become clearer when we use homologs of *engrailed* and *Wnt-1* as markers to analyze the segmentation processes (Kornberg *et al.*, 1985; Heuvel *et al.*, 1989; Patel, 1993). Since we already had *Bombyx engrailed (Bm en)* cDNA from the middle silk gland (Hui *et al.*, 1992), we cloned *Wnt-1* homolog from a cDNA library of *Bombyx* embryos (Amanai *et al.*, in preparation). Upon *in situ* hybridization of a *Bm en* probe, the first stripe appeared at 24 h in the anterior region, and 3 stripes followed slightly after 24 h in the anterior and middle regions of the embryo. About 8 stripes were detected in the anterior half of the embryo at 36 h, leaving the posterior half with no signals, and about 17 stripes were observed in the entire region of the elongated embryo at 48 h. Later, most of these stripes disappeared from the anterior and middle regions, leaving a few stripes in the posterior region (Amanai *et al.*, in preparation). The appearance of *Bombyx* WNT-1 protein in stripes together with a wider band in the growing region of the embryo preceded the expression of the *Bm en* (Amanai *et al.*, in preparation). Based on these observations and the morphological appearance, we conclude that the *Bombyx* embryo is clearly different from the long germ band type like *Drosophila* and belongs to either the intermediate or short germ band type (Patel, 1993); further studies are necessary to decide which type.

Several *Bombyx* homologs of the homeobox genes that specify the segment identities have been cloned and studied. *Bombyx Deformed* and *Sex combs reduced* are expressed in the mandibular and maxillary segments and the labial segment, respectively (Kokubo *et al.*, in preparation), which will be referred to again in the following section in relation with silk gland development. *Bombyx Antennapedia (Bm Antp)* was first found to be expressed strongly in the larval middle silk gland (Hui *et al.*, 1992; Hui and Suzuki, 1994; Nagata *et al.*, submitted), which will also be described in the following section. Recently we found that the *Nc* mutation (Itikawa, 1943, 1944, 1952) was caused by a partial deletion of *Bm Antp* (Nagata *et al.*, submitted). The *Nc*/*Nc* embryos reveal a partial

fusion of the prothorax and the mesothorax (Itikawa, 1952; Nagata *et al.*, submitted), transformation of legs in the prothorax into antennae, and an abnormal morphology at the gnathocephalon region (Nagata *et al.*, submitted). The last abnormality is associated with a dwarf development of the silk gland from the labial segment.

The *E* loci in *B. mori* contain homeotic genes specifying the identities of the larval abdominal segments (Hashimoto, 1941; Itikawa, 1943; 1944, 1952; Tazima, 1964). This homeotic gene complex possibly consists of *Bombyx Ultrabithorax (Bm Ubx), abdominal-A (Bm abd-A), and Abdominal-B (Bm Abd-B)* (Ueno *et al.*, 1992), and is thought to be located on the 0.0 locus of the sixth chromosome linkage group, and over 30 types of mutations were found. All of these mutations are dominant and induce ectopic expression of the markings or the legs in the abdominal segments. Most of the mutations in the *E* complex are lethal in the homozygous condition. Recently, we found (Ueno *et al.*, 1992) that the *Bm abd-A* gene is deleted in the E^{Ca} chromosome (Itikawa, 1943, 1952), and *Bm abd-A* and *Bm Ubx* genes are deleted in the E^{N} chromosome (Itikawa, 1943, 1952). Morphologically, E^{Ca}/E^{Ca} embryos lack all of the abdominal legs which should be formed in A3-A6 segments of the wild type embryos (Itikawa, 1943, 1952). Now we interpret that this is caused by the deletion of *Bm abd-A* gene in the E^{Ca}/E^{Ca} embryos transforming the A3-A6 segments into A1 type segments. Additional deletion of the *Bm Ubx* gene in the E^{N}/E^{N} embryos causes transformation of all the abdominal segments into metathorax type segments with thorax type legs except the A8 segment which reveals intermediate type legs (Ueno *et al.*, 1992). The distance between the *Nc* locus which probably belongs to *Bombyx Antennapedia* complex (Nagata *et al.*, submitted) and one of the genes in the *E* loci (*Bombyx Bithorax* complex; Ueno *et al.*, 1992) was estimated to be about 1.4 cM (Itikawa, 1952).

One of the gross morphological differences between *Drosophila* and *Bombyx* embryos is the lack of leg formation in *Drosophila* embryos. It is probably not a lack of gene cascade responsible for the leg formation in *Drosophila* because *Drosophila* adults have an ability to form the thorax legs. In this sense it is quite interesting to study the mechanism of leg formation in *Bombyx* embryos. We have begun searching for the target genes of *Bm abd-A*, some of which are supposed to be regulatory and/or structural genes constructing the abdominal legs (Ueno, unpublished).

Genes involved in silk gene regulation

In *Bombyx* embryos the salivary gland (Tanaka, 1928) and the silk gland (Toyama, 1909; Nunome, 1937) develop in the mandibular segment and the labial segment, respectively, while in *Drosophila* only the salivary gland develops in the labial segment. What would cause this difference? To answer this question and decipher the regulation network, especially in the *Bombyx* labial segment, we have begun the following studies.

In *Drosophila* embryos the *Scr* gene is expressed in the labial segment (Martinez-Arias *et al.*, 1987), the salivary gland formation is disturbed in *Scr* mutants, and ectopic salivary glands are induced by a forced expression of introduced *Scr* gene (Panzer *et al.*, 1992). Therefore, the *Scr* is essential for salivary gland induction and belongs to an upper part of the cascade required for salivary gland formation. The *in situ* hybridization signals of *Bombyx Deformed (Bm Dfd)* are detected in the mandibular and maxillary segments at around 36-48 h of development. The *Bombyx Sex combs reduced (Bm Scr)* expression appears in the ectoderm of the labial segment slightly later than the *Bm Dfd* expression (H. Kokubo *et al.*, in preparation). Interestingly, at stage 19, when the silk gland starts to invaginate (Toyama, 1909; Nunome, 1937; Takami and Kitazawa, 1960), the *Bm Scr* expression is suppressed at the invagination site leaving the expression in other regions of the labial segment.

Since the *POU-M1* was thought to be a homolog of *Drosophila Cf1-a*, it has remained enigmatic that the *POU-M1* is expressed in the silk gland and controls the sericin-1 gene transcription (Fukuta *et al.*, 1993). The expression of the *POU-M1* in *Bombyx* embryos was studied by *in situ* hybridization (P-X. Xu, unpublished). To our surprise the *POU-M1* was expressed first at stage 18-19 specifically at the site of the silk gland invagination in the labial segment, and the expression region was expanded inwardly along with the silk gland development. It was only at stage 22 when the *POU-M1* signal was observed in the neural cells as expected for the *Cf1-a* homolog. It would be interesting to find out whether the *POU-M1* has diverged to play dual or more functions specifically in *Bombyx* or whether other functions of the *Cf1-a* remain to be detected in *Drosophila*. The disappearance of the *Bm Scr* in the invagination site seems to be complemented by the strong expression of the *POU-M1*. Does the *Bm Scr* control the *POU-M1* or is the *Bm Scr* controlled by the *POU-M1*?

The elements that can serve as binding motifs for homeo- or POU-domain containing proteins were detected in the silk gene promoters (Hui and Suzuki, 1990; Hui *et al.*, 1990a,b; Matsuno *et al.*, 1990), and actually *Bm en*, *Bm in*, *Bm Antp* (Hui *et al.*, 1992) and *POU-M1* (Fukuta *et al.*, 1993) cDNAs were cloned from middle silk gland libraries. Analysis of *Bm Antp* expression in the silk glands of the wild type and the *Nc*/+ larvae resulted in the discovery that the *Nc* mutation is caused by a partial deletion of *Bm Antp* at around the homeobox region (Nagata *et al.*, submitted). Now we know that the *POU-M1* is expressed at the exact time when the silk gland begins its development and continues to be expressed throughout silk gland development, and the silk gland development is severely disturbed by the *Bm Antp* mutation. *In situ* hybridization experiments with a *Bm Antp* probe indicated that a stronger expression centers around the thorax region but a weaker expression extends into the labial segment as well as the abdominal segments at stage 18 (X. Xu, unpublished). The expression in the labial segment is restricted to the posterior half by stage 19, and is no longer detectable at stage 20. These results also suggest that the *Bm Antp* plays important roles in silk gland development.

By the time that more transcription factors of the silk gene regulation are cloned and the regulation cascades were deciphered, we will clearly understand how the labial segment is specified to form and develop the silk gland and how the tissue-specific expression of the silk genes is controlled.

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