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

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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 (Unshima 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 sercin-1) and sercin-2 (Michaille et al., 1990) genes transcribed specifically in the middle portion of the silk gland were cloned and sequenced. Transcription studies of the sercin-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).
These studies in the cell-free systems indicated approximate locations of cis-acting elements on both the fibroin and sercin-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 sercin-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 sercin-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 Cft-1-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., 1994). 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 sercin-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), en (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 (Kokubo et al., in preparation), Cft-1-a (Fukuta et al., 1993; Xu et al., 1994a), fork head (Takiya, unpublished), Ultrathorax, abdominal-A, and Abdominal-B (Ueno et al., 1992).

Clones containing whole open reading frames of Bombyx caudal (Bmcad) were isolated from a cDNA library of Bombyx embryos (Xu et al., 1994b). We are interested in studying the nature of the Bmcad for two reasons; (1) there are still no report 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; Modzik et al., 1985; Modzik and Gehring, 1987; Driever and Nüsslein-Volhard, 1988). Northern hybridization with a Bmcad 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 Bmcad transcripts were firstly accumulated in the nurse cells and transferred into the oocyte (Xu et al., 1994b). The Bmcad 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 (Modzik et al., 1985; Modzik 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, 1980; 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 gnathocphalon 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 Ultradithorax (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 homoygous condition. Recently, we found (Ueno et al., 1992) that the Bm Abd-A gene is deleted in the Elocus chromosome (Itikawa, 1943, 1952), and Bm Abd-A and Bm Ubx genes are deleted in the E' locus chromosome (Itikawa, 1943, 1952). Morphologically, ECa/Eca 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 ECa/Eca embryos transforming the A3-A6 segments into A1 type segments. Additional deletion of the Bm Ubx gene in the E' locus embryos causes transformation of all of 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 Scrmutants, and ectopic salivary glands are induced by a forced expression of introduced *Sogene* (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 first expressed 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 Bm 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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**References**


