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[REVIEW]

Bombyxin: An Insect Brain Peptide that Belongs to the Insulin Family

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ABSTRACT—Bombyxin is a 5 kDa secretory brain peptide that belongs to the insulin family. Bombyxin of the silkmoth *Bombyx mori* can induce adult development when injected into brain-removed dormant pupae of the saturniid moth *Samia cynthia ricini* by activating the prothoracic glands to synthesize and release ecdysone. *Bombyx* bombyxin has been shown to lower the concentration of the major haemolymph sugar, trehalose, and to elevate the trehalase activity in the midgut and muscles in *Bombyx*, but the doses required to be effective are higher than the amounts in the feeding larvae. The exact physiological function of bombyxin in *Bombyx* itself is therefore still obscure, but its insulin-like structure suggests it has important roles. Bombyxin comprises a mixture of highly heterogeneous molecular forms whose amino acid sequences have 40% identity with human insulin. The *Bombyx* bombyxin gene encodes a precursor consisting of the signal peptide, B chain, C peptide, and A chain, in that order from the N terminus. So far, 32 bombyxin genes have been identified in *Bombyx*, and they are classified into 7 families, A to G, according to their sequence similarity. The bombyxin genes have no introns and cluster in unique distribution patterns. The gene arrangement in the cluster has been classified into three categories: gene pairs, gene triplets, and single genes. Nucleotide sequence analysis indicates that equal and unequal crossings-over and duplications may have generated these unique distribution patterns. The *Bombyx* bombyxin genes are expressed predominantly in the brain and at low levels in a number of other tissues. Genes of all 7 families are expressed in four pairs of the medial neurosecretory cells of the brain. Detailed examination indicated that only a limited number of genes in the A, B and C family members are expressed and that their expression shows a gene-arrangement-dependent pattern.

INTRODUCTION

Insect molting and metamorphosis have remained interesting and important subjects throughout the history of developmental biology and endocrinology. In 1922, Kopeć found that a humoral factor from the gypsy moth *Lymantria dispar* was responsible for the induction of larval to pupal molting (Kopeć, 1922). This finding was the first demonstration of the presence of hormone in invertebrates. He named that hormone the brain hormone. His finding was unfortunately long ignored, until 1940 when Wigglesworth threw light on the brain hormone by confirming Kopećs discovery (Wigglesworth, 1940). Shortly after, Fukuda demonstrated that the prothoracic glands in the thorax secrete a hormone essential for insect development (Fukuda, 1944). The hormone secreted from the prothoracic gland is now known as ecdysone, a hormone directly responsible for the development of peripheral tissues. Fukuda's work on the silkmoth *Bombyx mori* was one of the outstanding scientific achievements in Japan. His work was immediately confirmed by Williams (1947). Williams then proved that the brain hormone, now known as the prothoracicotropic hormone (PTTH) stimulates the prothoracic glands to secrete ecdysone. By those pioneering works, an important concept, known as the classical scheme, was established (for review, Bollenbacher and Granger, 1985).

In the early 20th century, Japan's economy was highly dependent on silk production so a large amount of research was done on *Bombyx* (for review, Okada, 1994). The abundance of the silkmoth larvae was a great advantage for biologists at that time. In the course of the purification of PTTH, a peptide was identified in an extract from *Bombyx* brains (Ichikawa and Ishizaki, 1963) and, after many years, the pep-

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tide was purified almost homogeneously from millions of *Bombyx* heads (Suzuki *et al*., 1982). The peptide has a molecular weight of 5 kDa and the ability to stimulate the prothoracic glands of the saturniid moth *Samia cynthia ricini* to synthesize and release ecdysone. Soon after, the 5 kDa peptide was however revealed to be inactive in *Bombyx* at a physiological dose, but instead a 30 kDa peptide is active in *Bombyx* itself (Kawakami *et al*., 1990). Thus, the 5 kDa peptide has not been regarded as a "pure" PTTH. Unexpectedly, the 5 kDa peptide was shown to be homologous to insulin, a peptide hormone that plays crucial roles in the energy metabolism and growth of vertebrates (Nagasawa *et al*., 1984, 1986). Although the existence of insulin-like peptides in invertebrates was predicted in 1960s through biological and/or immunological assays, little was known about their structure (for review, Kramer, 1985). The elucidation of the structure of the 5 kDa peptide was therefore the first demonstration of the presence of insulin or insulin-related peptides in invertebrates at the molecular level. The silkmoth insulin-related peptide is now called bombyxin and has been proved to exist widely in insects. In this review, molecular aspects of bombyxin, especially the structure and expression of its gene, are summarized.

STRUCTURE OF BOMBYXIN AND THE BOMBYXIN GENE

Bombyxin comprises highly heterogeneous molecular forms. Five molecular forms, I, II, III, IV and V, have so far been identified from *Bombyx* heads (Nagasawa *et al*., 1984, 1986, 1988; Jhoti *et al*., 1987; Maruyama *et al*., 1988). The primary structures have been determined completely for bombyxins II and IV and partially for bombyxins I, III and V. They are heterodimers of the A and B chains whose amino acid sequences show about 50% and 30% identity to the A and B chains of human insulin (Fig. 1). The A and B chains of bombyxin are connected by two inter- and one intra-chain disulfide bonds in exactly the same manner as insulin. Bombyxins II and IV have been chemically synthesized and proved to have the same prothoracicotropic activity in *Samia* as natural bombyxins (Nagasawa *et al*., 1988; Maruyama *et al*., 1990). Molecular modeling for the three-dimensional structure showed that bombyxin resembles insulin in adopting a globular-like core structure (Jhoti *et al*., 1987). Solution structure analysis of bombyxin by nuclear magnetic resonance further demonstrated that the overall main-chain fold of bombyxin is similar to those of insulin in solution, insulin in the crystalline T-state, and relaxin in the crystalline form (Nagata *et al*., 1995). In fact, a hybrid molecule consisting of the A chain of *Bombyx* bombyxin and the B chain of human insulin stimulates 2-deoxyglucose uptake and DNA synthesis in CHO cells. Bombyxin resembles relaxin in having a pyroglutamic acid residue at the B chain N terminus (Nagasawa *et al*., 1986).

Thirty-two genes that encode bombyxin have been cloned from the *Bombyx* genome (Iwami *et al*., 1989, 1990; Kawakami *et al*., 1989; Kondo *et al*., 1996; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997, 1998). These genes have been classified into 7 families, A, B, C, D, E, F and G, according to their sequence similarity. The family B members can be further divided into three subfamilies BI, BII and BIII (Kondo *et al*., 1996). The family A bombyxin consists of 10 gene copies, the family B 12 gene copies, the family C 6 gene copies. The bombyxin fami-

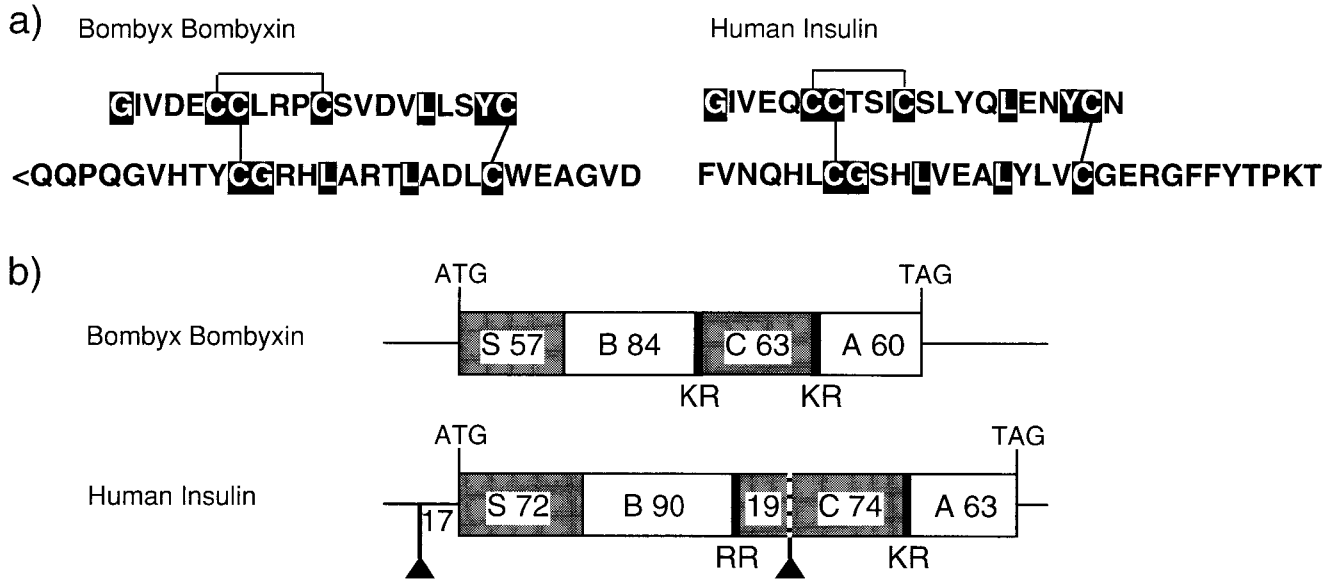

Fig. 1. a) Amino acid sequences of bombyxin II, one of the molecular forms of *Bombyx* bombyxin (Nagasawa *et al*., 1986; Maruyama *et al*., 1988), and human insulin (Bell *et al*., 1980). The identical residues between the two peptides are boxed. Disulfide bonds are indicated by bars. <Q, pyroglutamate residue. b) Schematic representation of the genes encoding prepropeptides for bombyxin (bombyxin A1) and human insulin. ATG and TAG represent the translation initiation site and termination site, respectively. Open boxes designate the domains that form mature molecules and hatched boxes those which are excised after translation. Dibasic amino acid residues, KR and RR, indicate the post-translational processing sites. S, signal peptide; B, B chain; C, C peptide; A, A chain. Numbers in boxes indicate the nucleotide number in the respective coding segments. Solid triangles indicate the positions of introns. Modified from Iwami *et al*., 1989.

lies D to G stand as single genes. The amino acid sequences deduced from the bombyxin genes show that bombyxin II is the product of genes A6 and/or A7 (Kondo *et al.*, 1996) and bombyxin IV is that of gene E1 (Tsuzuki *et al.*, 1997). On the other hand, bombyxins III and V do not coincide with any bombyxins deduced from the genes, indicating that more bombyxin genes remain undetected. The deduced amino acid sequences of the bombyxin genes show 41% to 56% identity with each of the other families and 28% to 35% identity with human preproinsulin, as shown in Table 1. Preprobombyxins within the same family have at least 73% identical sequences with each other. Compared with the limited structural variation of vertebrate insulins (Steiner *et al.*, 1985), *Bombyx* bombyxin has a large diversity in structure. In vertebrate insulins, there may be little room for mutational divergence to occur because of the low copy number of their genes (see Discussion in Kondo *et al.*, 1996). It is reported that even point mutations resulting in abnormal human insulins can cause diabetes mellitus (Steiner *et al.*, 1985).

Table 1. Overall sequence identity among the prepropeptides of *Bombyx* bombyxins and human insulin

			Bombyxin							Human
			A1	B1	C1	D1	E1	F1	G1	Insulin
Bombyxin	A1	(89)	100							
	B1	(73)	55	100						
	C1	(91)	51	51	100					
	D1		55	51	52	100				
	E1		48	49	47	47	100			
	F1		48	47	51	53	48	100		
	G1		50	54	47	56	41	50	100	
Human Insulin			35	33	34	33	34	31	28	100

Amino acid sequence identity was calculated using the computer program Maximum Matching in DNASIS (Hitachi Software). Numbers in parentheses indicate the minimum sequence identity of preprobombyxins within the same family. Amino acid sequences are from A1, Iwami *et al.*, 1989; B1, Kawakami *et al.*, 1989; C1, Iwami *et al.*, 1990; D1, Kondo *et al.*, 1996; E1, Tsuzuki *et al.*, 1997; F1, Yoshida *et al.*, 1997; G1, Yoshida *et al.*, 1998; human insulin, Bell *et al.*, 1980.

The amino acid sequences of the protein products deduced from the genes are listed in Fig. 2. Except for C3 to C6, all the products have the same basic structure as that of preproinsulin (Kondo *et al.*, 1996). They consist of the signal peptide, B chain, C peptide, and A chain, in that order from the N terminus. Bombyxin genes C3 to C6 have an in-frame stop codon so as to encode only the signal peptide and an N terminal portion of the B chain. The bombyxin A10 gene also has an in-frame stop codon at about the center of the region coding for the A chain (Kondo *et al.*, 1996). These genes are thus presumably pseudogenes.

The amino acid sequences of preprobombyxins show a high similarity in the A and B chains throughout the 7 families, and the level of conservation is remarkably high within each family (Fig. 2). On the other hand, the conservation of amino acid sequences is relatively low in the C peptide and even lower in the signal peptide, as has been shown for preproinsulins (Steiner *et al.*, 1985). The sequences of the C

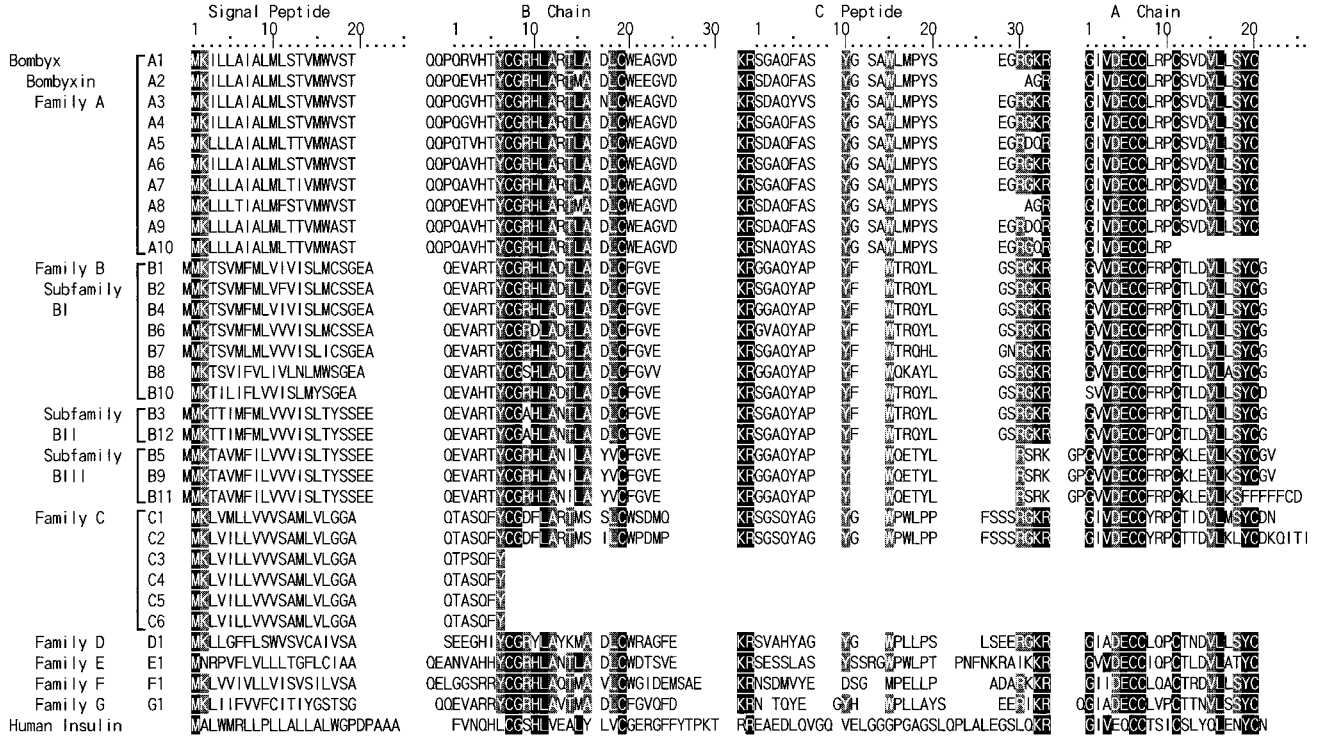

Fig. 2. Amino acid sequences of *Bombyx* preprobombyxins deduced from nucleotide sequences of their genes and human preproinsulin. Solid boxes represent identical residues among all the insulin family prepropeptides listed and hatched boxes those among preprobombyxins. Amino acid residues are numbered from the N terminus of each domain of insulin. Amino acid sequences are from Bell *et al.*, 1980; Iwami *et al.*, 1989, 1990; Kawakami *et al.*, 1989; Kondo *et al.*, 1996; Tsuzuki *et al.*, 1997; Yoshida *et al.*, 1997, 1998.

peptide and those of the signal peptide within each family show a remarkably high level of conservation between families. High similarities are found among bombyxin members in cysteine and hydrophobic residues responsible for the hydrophobic core formation of bombyxin and insulin (Jhoti *et al*., 1987). The surface patch formed by the central part of the B chain is of critical importance for recognition by the bombyxin receptor (Nagata *et al*., 1995). Intriguingly, the amino acid sequences of the surface patch are conserved or conservatively substituted among bombyxin members. Also conserved is glycine at position 8 of the B chain. In insulin, the glycine residue maintains the conformation through its contribution to the main-chain turn. The role of the N terminus of the bombyxin II A chain was investigated using bombyxin analogues with modifications (Büllesbach, 1999). Modification of the A chain dramatically reduced the affinity to the bombyxin receptor, suggesting that bombyxin exhibits a mammalian insulin-like structural sensitivity for the A chain modifications. The paired basic amino acid residues are also conserved among preprobombyxins, which suggests that the mature bombyxins are generated through excision of the C peptide, exactly the same as insulin is (Chan *et al*., 1981).

Interestingly, the signal peptide sequences of the subfamily BII members are more similar to those of the subfamily BIII members than to those of the subfamily BI members, whereas, in contrast, the C peptide and A chain sequences of the subfamily BII members are more similar to those of the subfamily BI members than to those of the subfamily BIII members (Fig. 2). This tendency is also pronounced in the nucleotide sequences of the three subfamily members. A subfamily BII prototype gene therefore must be generated by a crossing-over in the B chain region between prototype genes belonging to the subfamilies BI and BIII (see also Fig. 4).

PHYSIOLOGICAL FUNCTION OF BOMBYXIN

The *Bombyx* brain contains two types of molecules that stimulate the synthesis and release of ecdysone, i. e. bombyxin and PTTH (Ishizaki *et al*., 1983). *Bombyx* bombyxin can induce adult development when injected into brain-removed dormant pupae of *Samia*. The amount of bombyxin that induces adult development is estimated to be roughly 0.1–0.4 ng (Nagasawa *et al*., 1984). When *Bombyx* bombyxin was injected into brain-removed *Bombyx* pupae, even at amounts than 1,000 times, it did not elicit adult development, whereas only 0.1 ng of the *Bombyx* PTTH was effective in the brain-removed *Bombyx* pupal assay. In addition, the dose required for attaining maximum activation by bombyxin was as high as 1,600 times of that of PTTH when assayed using *in vitro* culture of the *Bombyx* pupal prothoracic glands (Kiriishi *et al*., 1992). Therefore, it was concluded that *Bombyx* bombyxin is not a “pure” *Bombyx* PTTH active in *Bombyx* itself (Kawakami *et al*., 1990). The insulin-related structure of bombyxin both in its primary and tertiary forms, however, strongly suggests that bombyxin plays important roles in sugar metabolisms and growth of insects.

Bombyx haemolymph contains a large amount of trehalose at a concentration of about 10 mM (Oda *et al*., 1997). Bombyxin II was shown to have the hypotrehalosemic activity that reduces the concentration of trehalose, when injected to the neck-ligated larvae (Mizoguchi, 1994; Satake *et al*., 1997). Although bombyxin II shows the activity in a dose-dependent manner, the dose required to elicit a half maximal effect is 10 ng, which is higher than the amount in the feeding *Bombyx* larvae. It is not known whether bombyxin molecules other than bombyxin II also have a hypotrehalosemic activity. The concentration of trehalose in the haemolymph is controlled by the relative rates of trehalose synthesis in the fat body and trehalose uptake by the tissues. Bombyxin II was demonstrated to cause elevated trehalase activity in the *Bombyx* midgut and muscles, which suggests that bombyxin induces hypotrehalosemia by promoting the hydrolysis of trehalose to glucose and thereby facilitating its transport into tissue cells (Mizoguchi, 1994; Satake *et al*., 1997). Bombyxin II was also shown to reduce the glycogen content in the fat body by elevating the percentage of the active form of glycogen phosphorylase (Satake *et al*., 1997). In the adult moths, bombyxin II, however, does not affect the concentration of haemolymph trehalose nor the activity of trehalase in the muscles, which indicates the occurrence of a change in the action of bombyxin during metamorphosis (Satake *et al*., 1997).

Developmental changes in the titer of bombyxin in *Bombyx* haemolymph were investigated using a monoclonal antibody against bombyxin II by radioimmunoassay (Saegusa *et al*., 1992) and time-resolved fluoroimmunoassay (Satake *et al*., 1999). It was shown that after eclosion the haemolymph titers of bombyxin were low in both males and females, increased steeply in males to a very high level and that this titer was maintained for several hours, whereas the titer increment in females was small and transient. The difference in the change of bombyxin titer between males and females suggests that bombyxin is responsible for the regulation of physiological changes underlying sexually different behavior of the adult moth (Satake *et al*., 1999).

In the nematode *Caenorhabditis elegans*, the *daf*-2 gene, which regulates longevity and diapause, was identified as encoding an insulin receptor-like molecule (Kimura *et al*., 1997). This indicates that the ligand of DAF-2 may be an insulin-like peptide and act in metabolic and diapause control in the nematode. It is recalled that bombyxin was discovered through its induction of adult development when injected into dormant pupae whose development had been arrested by the removal of the brain. Further, *Bombyx* bombyxin was reported to bind specific receptors on the ovarian cells (Fullbright *et al*., 1997) and to induce meiosis in the ovarian cells (Orikasa *et al*., 1993) and morphological changes in a cell line derived from the ovarian tissue (Tanaka *et al*., 1995). The control of reproductive maturation of the germ line was also shown to require DAF-2 activity in the nematode (Hsin and Kenyon, 1999). In addition, the haemolymph titer of bombyxin attains a peak value during pupal-adult development in which tissue remodeling and productive maturation are in maximum

progress (Saegusa *et al*., 1992). It is therefore highly probable that bombyxin plays critical roles in metamorphosis and reproduction through its ability to metabolize energy and growth and/or developmental controls. Further study is necessary for the elucidation of the real physiological function(s) of bombyxin.

STRUCTURE, ORGANIZATION, AND EVOLUTION OF BOMBYXIN GENE

All insulin genes except the murine insulin-I gene have two introns, one in the 5'-untranslated region and the other in the C peptide region. The murine insulin-I gene has only one intron in the 5'-untranslated region and is inferred to be a functional processed gene due to its unique structure in respect to the processed gene (Soares *et al*., 1985). The typical hallmarks of processed genes are (1) the lack of introns, (2) the presence of a poly(A) tract 3' to the transcriptional terminator, and (3) the presence of direct repeats bounding the transcribed region (Vanin, 1985). All bombyxin genes lack introns both in the 5'-untranslated region and in the C peptide region (Iwami, 1990, 1995; Iwami *et al*., 1989, 1990; Kondo *et al*., 1996; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997, 1998). In addition, not a few bombyxin genes have boundary short repeats and the remnants of a poly(A) tract immediately preceding the downstream repeat, though the repeats and remnants are not complete in some cases (Iwami *et al*, 1989; Iwami, 1990; Tsuzuki *et al*., 1997). The structural features of bombyxin genes thus indicate that bombyxin genes are functional processed genes.

The bombyxin genes in the *Bombyx* genome show a unique spatial organization (Kondo *et al*., 1996; Yoshida *et al*., 1997, 1998). Thirty-one out of the 32 bombyxin genes are clustered in the genome as shown in Fig. 3. Segment A in Fig. 3 contains 25 bombyxin genes in a 50 kb range and segment B 6 genes in a 15 kb range. The topological relationship of segment A to segment B is unknown. The arrangement of the bombyxin genes in the clusters is classified into three categories: (1) gene pairs–two genes that belong to different families are opposed to form a gene pair with opposite transcriptional orientations. Most of such gene pairs are composed of the family A and B members. Nine sets of bombyxin gene-pairs can be found in the *Bombyx* genome. The distance between the two genes of the pairs ranges from 0.5 to 2.3 kb. (2) Gene triplets–all triplets are composed of the family B, C and A genes, in this order from 5' to 3', and the three genes are apposed. The transcriptional direction of the family C and A genes is opposite to that of the family B gene. All the family C genes in the triplets C3, C4, C5 and C6 are pseudogenes. Four triplet sets are found in segment A. (3) Single genes–the bombyxin D1 gene is present singly, not forming either a pair or a triplet. The bombyxin E1 gene, which is not included in either segment A or B, is also present singly (Tsuzuki *et al*., 1997). However, the possibility cannot be excluded that the two genes form pairs or triplets with yet undetected bombyxin genes.

Nucleotide sequence comparison suggests that the gene pairs form the basic unit of bombyxin genes in the genome and that the gene triplets are generated by an unequal crossing-over between the two gene pairs (Kondo *et al*., 1996). By nucleotide sequence analysis of the genes and their spacers together with restriction site analysis of the surrounding regions, the present form of the tandemly arranged bombyxin gene pairs was shown to be generated by gene duplication of a putative original pair (Kawakami *et al*., 1989; Kondo *et al*., 1996). Further, the present form of the four tandemly arranged bombyxin gene triplets should be generated by two duplications. An ancestral bombyxin gene triplet duplicated to form two closely situated gene triplets, as triplets B11/C5/A9 and B12/C6/A10, and the resulting double-triplet sets duplicated again to form the present triplet-sets on segment A in Fig. 3 (Kondo *et al*., 1996). It has been further demonstrated that a crossing-over between a gene triplet and a gene pair occurred which resulted in the diversification of the gene triplets, as shown in step (8) of Fig. 4 (Yoshida and Iwami, unpublished results). Thus, the very high degree of diversification in structure and genomic organization of bombyxin genes results from equal and unequal crossings-over as well as gene and gene-set duplications. A model for generation of the diversity is as follows (Fig. 4) (Kondo *et al*., 1996): (1) An ancestor bombyxin gene diverged into genes ancestral to at least 7 families, A to G, by gene duplication followed by mutations. (2) The family B gene diverged further into two genes ancestral to subfamilies BI and BIII by point mutations. (3) Two gene pairs, BIII/C and BI/A, were generated and the other family genes, not il-

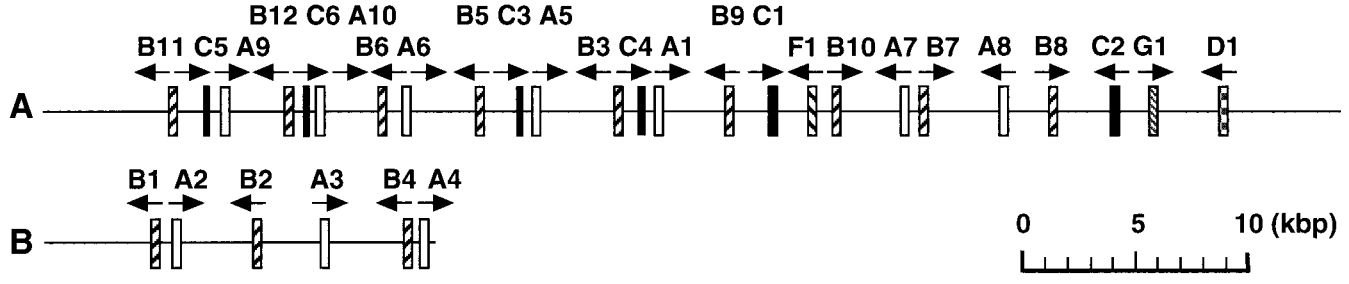

Fig. 3. Schematic representation of the two segments, A and B, of the *Bombyx* genomic DNA showing the organization of 31 bombyxin genes. Segment A carries 25 bombyxin genes in a 50 kbp range and segment B 6 bombyxin genes in a 15 kbp range. Boxes on the maps represent the bombyxin genes. Arrows over the genes indicate their transcriptional directions. The bombyxin E1 gene is not included in either segment A or B. Combined from Kondo *et al*., 1996; Yoshida *et al*., 1997, 1998.

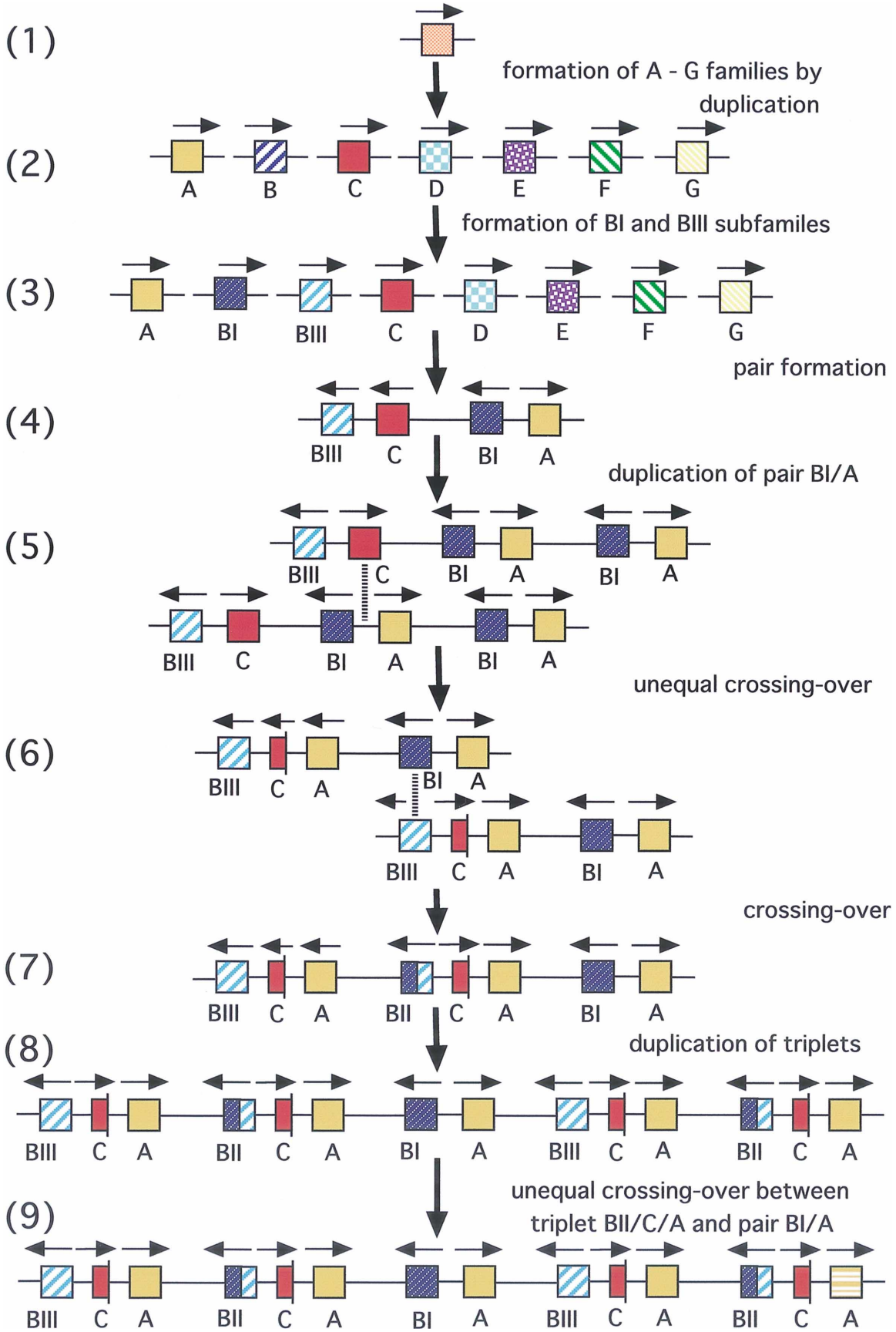

Fig. 4. A schema for the molecular evolutionary history of the bombyxin genes in the *Bombyx* genome. Boxes represent the bombyxin genes, and arrows over the genes indicate the transcriptional directions. Modified from Kondo *et al.*, 1996.

lustrated in the figure, were duplicated and diverged. (4) The gene pair BI/A was duplicated to form a tandem array of two gene pairs. (5) Subsequently, an unequal crossing-over between gene pairs BIII/C and BI/A generated a gene triplet BIII/C/A. (6) Then, a crossing-over between gene pair BI/A and gene triplet BIII/C/A generated a tandem array of the two triplets, one of which contains a new subfamily BII. (7) Gene duplication of the triplet sets BIII/C/A and BII/C/A generated four gene triplets. (8) Lastly, an unequal crossing-over between gene triplet BII/C/A and gene pair B/A generated the present form of gene triplet B3/C/A1, as seen in the *Bombyx* genome. Point mutations have occurred throughout these process to diversify the structure of bombyxin genes (see Discussion in Kondo *et al*., 1996).

EXPRESSION OF THE BOMBYXIN GENE

The bombyxin gene is expressed predominantly in the brain and at low levels in a number of other tissues (Iwami *et al*., 1996b), in contrast to the insulin gene which is expressed in the gastroenteric organs and is almost silent in the brain. The brain is the only tissue that expresses the bombyxin gene throughout *Bombyx* development from the embryonic to adult stages when analyzed by northern hybridization (Adachi *et al*., 1989; Iwami, 1990). Reverse transcription-PCR analysis, however, demonstrated the presence of bombyxin family A and B mRNAs in all larval tissues examined although the levels of expression were very low (Iwami *et al*., 1996b). The bombyxin mRNA expression was detected in ganglia, epidermis, testis, ovary, fat body, silk gland, Malpighian tubule, midgut, and hindgut of the fifth instar larvae, which indicates the ubiquitous expression of bombyxin mRNA in the larvae. In the *Bombyx* brain, genes of all 7 families are expressed in four pairs of the medial neurosecretory cells as shown in Fig. 5 (Iwami, 1990, 1995; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997, 1998). The concentration of bombyxin mRNA in the bombyxin-producing cells (BPCs) is remarkably high; for example, the concentration of the family A mRNA is about

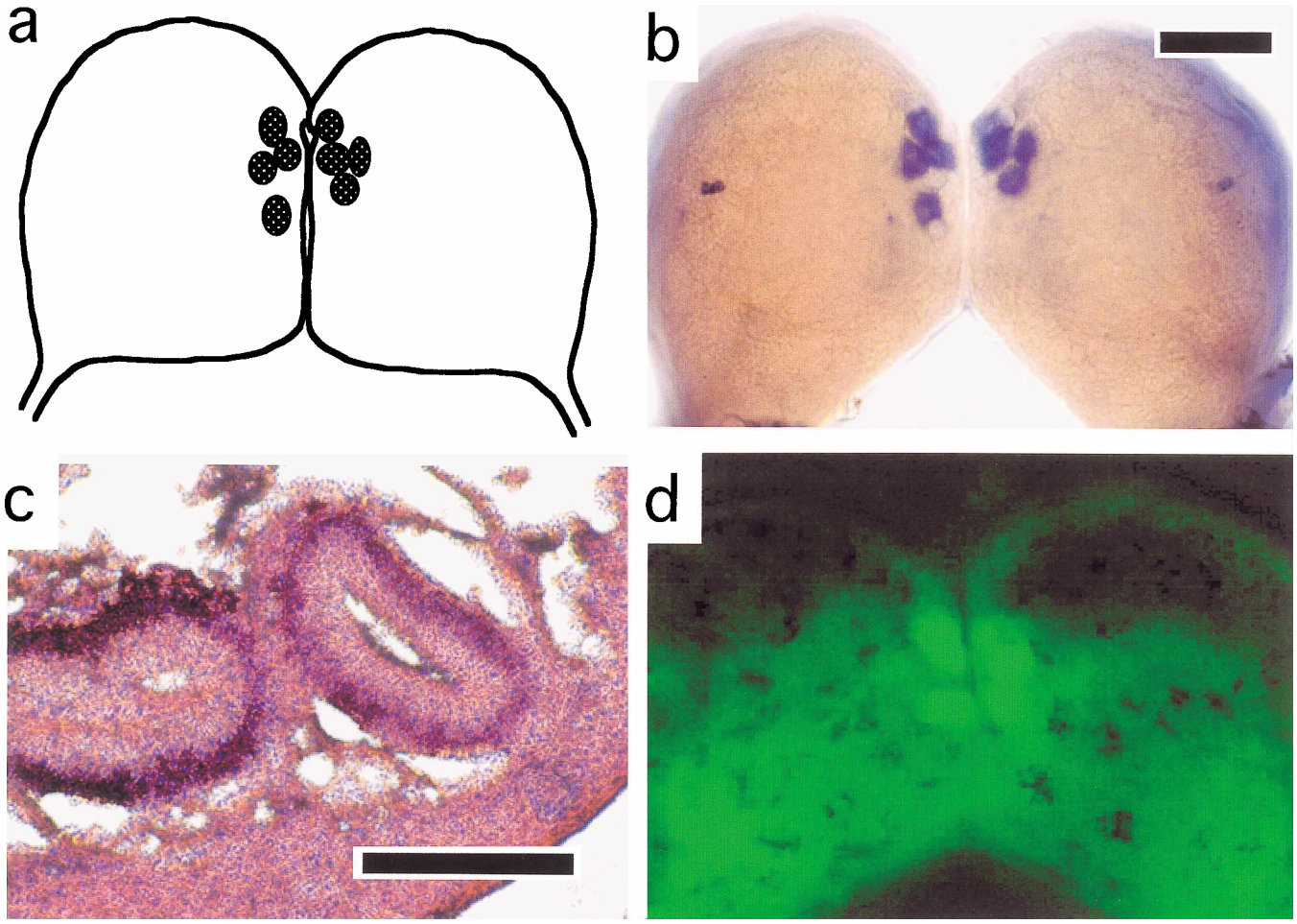

Fig. 5. a). Schematic representation of the bombyxin-producing cells, BPCs, of the *Bombyx* brain. b) Whole-mount *in situ* hybridization of a *Bombyx* fifth-instar larval brain. The bombyxin family-A mRNAs were detected only in four pairs of BPCs (shown as dark blue). Bar, 100 µm. c) Section *in situ* hybridization of a *Bombyx* fifth-instar ovary. The bombyxin B1 DNA fragment was used as a probe. The bombyxin mRNA-producing cells are shown as dark blue. Bar, 100 µm. d) Fluorescence microphotograph of a Bombyx fifth-instar brain electroporated with the bombyxin/GFP reporter, pC4/B3::EGFP. The GFP fluorescence is visible as bright green in five cells, two in the left hemisphere and three in the right. Modified from Moto *et al*., 1999.

2.8×10^9 molecules/μg of total RNA (Adachi *et al*., 1989). The amounts of the family A, B, and C mRNAs decrease gradually during larval to pupal development (Iwami, 1990). Immunohistochemical analysis using anti-bombyxin I monoclonal antibody also demonstrated the same spatial specificity (Mizoguchi *et al*., 1987). The immunohistochemistry revealed that the axons for the BPCs innervate the corpora allata and that their terminals are preferentially localized on the surface region of the corpora allata. Thus, bombyxin is presumed to be liberated into haemolymph from the corpora allata. Developmental fluctuation of the bombyxin content in the *Bombyx* brain was also investigated by immunohistochemistry using the monoclonal antibody (Mizoguchi *et al*., 1990). Interestingly, the four bombyxin cells in a brain hemisphere behaved immunohistochemically as two groups, each of which comprised two cells. On many occasions, two cells stained heavily while the other two stained lightly. At the mRNA level, there exists no such differential expression pattern. Therefore, the differential immunostaining might represent a pulsatile secretion of bombyxin (Mizoguchi *et al*., 1990) as demonstrated in PTTH (Gilbert *et al*., 1981).

The spatial specificity is also shown in the *Bombyx* larval ovary where two or three cell layers on the outer surface of each ovariole are found as shown in Fig. 5c (Iwami *et al*., 1996b). The cells of the inner surface of an ovariole and the cell layers beneath the ovarial capsule also express the bombyxin mRNA. It is noteworthy that the presence of bombyxin molecules has been demonstrated in the developing embryos shortly after oviposition but before appearance of neurosecretory cells in the brain (Fugo *et al*., 1987). It is thus probable that bombyxin in the ovariole is transferred to the embryo. The presence of a large number of bombyxin gene copies in the *Bombyx* genome might meet the demand for a large quantity of bombyxins for growth, development, and reproduction at a specific stage such as pupal to adult development.

In order to analyze the BPC-specific expression of the bombyxin gene, a gene-transfer technique was developed that enables introduction of a reporter gene into the brain by electroporation (Moto *et al*., 1999). By combination of this technique with the green fluorescent protein (GFP) reporter assay under UV-microscopy, we developed an assay system of the cell-specific expression of bombyxin gene. The reporter gene, which consists of a bombyxin gene spacer between B3 and C4 and the GFP coding region, was introduced into *Bombyx* brains by electroporation (Fig. 5d). The observation of fluorescence exclusively in the BPCs indicates that the reporter is under the control of the bombyxin gene promoter in a BPC-specific manner and that the spacer between B3 and C4 contains such a promoter. The expression pattern of the reporter has also been observed when the *Bombyx* pupal brains were used for the recipient tissue (Moto *et al*., 1999). The exact promoter site is now under investigation.

A recent study showed that a limited number of bombyxin genes are expressed in the brain just after pupation (Ino *et al*., unpublished results). Thirteen out of the 28 genes belonging to the family A, B and C are expressed while the other 15 genes are silent or almost silent. The 13 bombyxin genes expressed show a gene-arrangement-dependent manner that can be classified into two categories: (1) gene-pair-specific expression of the family A and C genes–the family A and C genes expressed are located exclusively in the gene pairs but not in the gene triplets. The family A and C genes belonging to the gene triplets are silent or almost silent. Thus, the family A and C genes show a gene-pair-specific expression. (2) gene-triplet-specific expression of the family B genes–the family B genes belonging to the gene triplets are expressed. In the gene pairs, some family B genes are expressed, whereas the other family B genes are not expressed. The mechanism of these unique expression patterns of bombyxin genes remains to be elucidated.

CONCLUDING REMARKS

The study of bombyxin is an odyssey from classic experimental morphology to modern molecular biology (see also Ishizaki, 1986). The study of bombyxin began in the 1950s when H. Ishizaki, the author's supervisor at Nagoya University, set up his research with M. Ichikawa at Kyoto University. Ishizaki tried to purify the so-called brain hormone, because it controls insect postembryonic development. The hormone assay was fully dependent on the methods of classical experimental morphology and the purification techniques were primitive because of the lack of modern molecular techniques such as peptide analysis. The purification was therefore very difficult work. After a 30-year struggle, Ishizaki purified bombyxin and PTTH from *Bombyx* heads in collaboration with biochemists of the University of Tokyo, S. Tamura, A. Suzuki, H. Nagasawa, and H. Kataoka. Shortly after the hormone purification, the author and his colleagues succeeded in cloning the genes encoding bombyxin and PTTH.

In parallel with bombyxin study in *Bombyx*, the author and his colleagues isolated 10 bombyxin genes in other Lepidopteran species. In *Samia*, six bombyxin genes have been cloned and proven to be expressed in the medial neurosecretory cells in the *Samia* brain (Kimura-Kawakami *et al*., 1992; Yagi *et al*., 1995; Inoue *et al*., unpublished results). They have a domain organization and nucleotide sequence very similar to the *Bombyx* bombyxin gene. The 6 genes isolated have been classified into two families, A and B, by nucleotide sequence similarity. They form a cluster in the genome, and two genes belonging to different families are localized close to each other with opposite transcriptional orientations (Kimura-Kawakami *et al*., 1992), resembling the *Bombyx* bombyxin gene. From the hornworm *Agrius convoluvuli*, three cDNA clones that encode *Agrius* bombyxins have been isolated and classified into two families, A and B (Iwami *et al*., 1996a). Southern hybridization analysis demonstrated the presence of multiple gene copies of the *Agrius* bombyxin gene in the genome. The *Agrius* bombyxin genes also have the insulin-like domain structure and an expression site that is localized to four pairs of the medial neurosecretory cells in the brain as the *Bombyx* bombyxin genes are (Iwami *et al*., 1996a). An antibody against *Bombyx* bombyxin detected the immunore-

active molecules in the oligochaete *Eisenia foetida* (Sauber *et al*., 1990), the locust *Locusta migratoria* (Zachary *et al*., 1988), the wax moth *Galleria mellonella* (Žitňan *et al*., 1990), the hornworm Manduca sexta (Dai *et al*., 1994), and the fruit fly Drosophila melanogaster (Žitňan *et al*., 1993). Those studies demonstrate that bombyxin and/or bombyxin-related peptides exist ubiquitously in insects. The study of bombyxin has thus expanded the insulin world.

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